Ana Clara Corujas Redondo

FAMILIAL AMYLOIDOTIC POLYNEUROPATHY: STUDY OF STRUCTURAL DETERMINANTS IN AMYLOIDOGENESIS

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Supervisor: Professor Maria João Saraiva

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Aos meus pais,
por continuarem a gostar de tomar conta de mim.

De acordo com o disposto no nº 2 do Artigo 8º do Decreto-lei nº 388/70, foram utilizados nesta dissertação os resultados dos artigos publicados abaixo indicados.

No cumprimento do disposto no referido Decreto-lei, esclarece-se serem da nossa responsabilidade a execução das experiências que permitiram a obtenção dos resultados apresentados (excepto quando referido em contrário), assim como a sua interpretação e discussão.

Redondo, C., Damas, A. M., Saraiva, M. J. (2000) Designing transthyretin mutants affecting tetrameric structure: implications in amyloidogenicity. *Biochem J* **348** (15): 167-172.

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CONTENTS

Summary

The amyloidoses are a group of protein misfolding diseases characterised by the accumulation of insoluble fibrillar protein in several organs and tissues leading to cell death and tissue degeneration and to date, 18 different proteins and polypeptides have been identified in disease-associated amyloid deposits. An aminoacid substitution in the amyloid protein is associated with some forms of amyloidosis. To date, more than 85 different mutations have been reported in humans in association with transthyretin (TTR) amyloid deposition. The molecular mechanism behind the transformation of a soluble native TTR tetramer into insoluble aggregates leading to the development of Familial Amyloidotic Polyneuropathy (FAP), a lethal neuropathic disease, has been under intensive research in the last two decades.

The main objectives of this work were to contribute to the understanding of this mechanism, characterising amyloidogenic determinants in FAP. We first tried to elucidate the nature of the subunits that compose TTR amyloid fibrils, searching for key areas of the molecule strategic for tetrameric destabilisation to occur. Compelling evidences point to the involvement of monomers as subunits of TTR fibrillogenesis, though there are still some researchers that oppose this idea proposing a dimer or a multiple of dimers as the amyloid fibril building block. We have produced three "stabilised mutants" by introducing extra cysteines replacing specific residues thought to be located in crucial areas for stabilising the tetrameric structure, and we performed several biochemical studies to test their resistance to dissociation and susceptibility to *in vitro* amyloid formation. Under acidic pH, mutants Ser117Cys and Glu92Cys were both dimeric in nature and the double mutant Val30Cys/Leu55Cys dissociated into stable monomers. All three mutants, occurring as dimeric or monomeric species, were unable to polymerise into amyloid even at pH 3.2, due to disulphide bonding. When the amyloid formation assay was repeated in the presence of β-mercaptoethanol it was observed that upon disruption of the disulphide bridges on these stable dimers or monomers, amyloid fibrils could be formed. These evidences clearly suggest that monomers rather than dimers are the repeating structural subunits composing amyloid fibrils, in *in vitro* amyloid induced by acidification, and further support that partially unfolding or destabilisation of monomers is requested for fibrillogenesis. A rearrangement of strands C and D might be part of the modifications that occur in the native tetrameric TTR structure, which lead to dissociation into partially unfolded or unstable monomers involved in the formation of amyloid fibrils. We also present evidence for the importance of the AB loop and the contacts between strands G and H for the stabilisation of the tetrameric TTR structure. Our results with two "destabilised mutants", Asp18Asn and Leu110Ala showed that substitutions affecting key residues in those areas favour the appearance of altered surfaces that contribute strongly to aggregation.

The investigation of altered conformations that might represent intermediate structures in TTR fibrillogenesis was also approached in our work, aiming at identifying cryptic regions specific of determined steps of the amyloidogenic cascade. We tried to destabilise the TTR tetrameric structure, based on the known crystallographic data of the structure of Leu55Pro, a highly aggressive TTR mutant. We produced a mutant with a substitution at position 78, bearing in mind that the original residue, in WT TTR, is hydrogen bonded to residue 18 from the AB loop and that in the crystal structure of Leu55Pro, one of the conformational changes involved in the opening of new surfaces involved in aggregation, is the destabilisation of the AB loop. Tyr78Phe was isolated by HPLC as a tetrameric soluble form though under acidic pH was shown to be prone to form amyloid. Furthermore, this soluble tetrameric form of TTR was recognised by a monoclonal antibody (Mab 15), previously reported to react only with highly amyloidogenic mutants lacking the tetrameric fold and with amyloid fibrils. We suggest that the tetrameric structure of Tyr78Phe is altered due to the loosening of the AB loop,

leading to a structure that might represent an early intermediate in the fibrillogenesis pathway, exposing a cryptic epitope that is common to amyloid fibrils.

To further investigate TTR conformational intermediates with exposed cryptic epitopes that might reveal amyloidogenic surfaces, we performed a Gal4-based two-hybrid screening for TTR interactions with a 20-mer-aptamer library. Using as *bait*s the highly aggressive mutant Leu55Pro TTR and WT TTR, we first saw that transformed yeast cells were capable of expressing both soluble and aggregated forms of TTR and that a polyclonal antibody and Mab 15 could be used as markers for soluble and aggregated forms of the protein, respectively. We screened the library of putative interacting aptamers aiming at identifying regions in the TTR molecule being important for binding during polymerisation and/or others, exposed before polymerisation in intermediate forms, and still conserved in fibrils. Under the conditions tested, seven different types of interacting aptamers were obtained and organised into groups according to the different immuno-reactivity patterns between [Abs-TTR *bait*s]-interaction and [Abs-complex *bait*-aptamer]-interaction. We performed BIAcore competition studies and kinetic determinations for the affinity of interactions between Abs and five selected aptamers to several TTR forms (WT and mutant). Using these aptamers shown to be specific for certain TTR forms, we believe to have identified putative cryptic regions, some specific for determined TTR structures that at some point will suffer further rearrangements, and others which are conserved in the final fibrillogenic structure. The five peptides studied in this work were able to recognise and bind to three different types of epitopes conserved or lost along the amyloidogenic process: (i) one epitope expressed on WT TTR that is lost in the conversion of native into fibrillar structures; (ii) one cryptic epitope specifically expressed on amyloid fibrils and (iii) cryptic epitopes expressed on TTR amyloidogenic intermediates and on TTR amyloid fibrils, involving three different regions of the molecule. These evidences allow us to propose three candidate regions for amyloidogenic cryptic epitopes. One of these epitopes is probably located in an area involved in TTR destabilisation previously reported to be recognised by Mab 15, the C strand-CD loop area, as suggested by the results with aptamers 40W, C26 and possibly 29B. We also found two new epitopes, one specifically exposed in fibrils, and another undergoing conformational changes between TTR soluble forms and fibrils, revealed by aptamers C45 and 67, respectively. Our results support the idea that a native tetramer gradually suffers amyloidogenic changes, evolving from a partially modified structure (soluble Leu55Pro TTR) to a highly modified tetrameric arrangement (soluble Tyr78Phe TTR) culminating in its dissociation into disarranged monomers, which finally associate into fibrils.

We explored further the specificity of aptamer C45 to the fibrillar form of Leu55Pro TTR by testing its ability to inhibit *in vitro* amyloid formation, upon incubation with a fluorescent marker for amyloid, thioflavine T (ThT). When the aptamer was mixed with Leu55Pro TTR, fibril formation occurred. However, after 5 days of incubation at 37^0C, the ThT fluorescence was reduced by about a factor of 2, until the 22^{nd} day of the experiment, providing indication that C45 was interfering with fibrillogenesis and that the decrease in ThT fluorescence required this aptamer. C45 did not prevent β-sheet formation or fibril initiation, but it might bind to the growing fibril and affect the elongation process. More effective inhibitors, for example, shorter peptide sequences, based on C45, could function as amyloid disrupting agents.

Altogether, the results reported in this thesis brought new insights into the amyloidogenic process and might contribute in therapeutic intervention designed to act specifically against the formation or evolution of early intermediate amyloidogenic structures in localised steps of this pathogenic phenomenon.

Resumo

As amiloidoses são um grupo de doenças que envolvem o desarranjo estrutural de proteínas e que se caracterizam pela acumulação de proteína fibrilar insolúvel em diversos órgãos e tecidos conduzindo à morte celular e à degeneração dos tecido e até à data, foram identificados 18 polipeptídeos e proteínas diferentes em depósitos de amilóide associados a patologias. Uma substituição aminoacídica na proteína amloidogénica está associada com algumas formas de amiloidoses. Até à data, mais de 85 mutações diferentes foram relatadas nos seres humanos associadas com a deposição de transtirretina (TTR) sob a forma de amilóide. O mecanismo molecular que está por trás da transformação de um tetrâmero de TTR nativo e solúvel nos agregados insolúveis que conduzem ao desenvolvimento da Polineuropatia Amiloidótica Familiar (PAF), uma doença neuropática letal, tem estado sob pesquisa intensiva nas últimas duas décadas.

Os objectivos principais deste trabalho visavam contribuir para a compreensão deste mecanismo, caracterizando determinantes amiloidogénicos na PAF. Tentou-se primeiramente elucidar a natureza das sub-unidades que compõem as fibras de amilóide de TTR, procurando áreas chaves na molécula, estratégicas para que a destabilização tetramérica ocorra. Fortes evidências apontam para o envolvimento de monómeros como as subunidades na fibrilogénese da TTR, embora existam ainda alguns investigadores que se opõem a esta ideia, propondo um dimer o ou um múltiplo de dímeros como a subunidade da fibra de amilóide. Produziram-se três "mutantes estáveis" introduzindo cisteínas extra em substituição de resíduos específicos, possivelmente situados em áreas cruciais na estabilização da estrutura tetramérica da TTR. Procedeu-se à execução de diversos estudos bioquímicos para testar a resistência desses mutantes á dissociação bem como a sua susceptibilidade para formar amilóide *in vitro*. Sob pH ácido, os mutantes Ser117Cys e Glu92Cys eram naturalmente diméricos e o duplo-mutante Val30Cys/Leu55Cys dissociava em monómeros estáveis. Todos os três mutantes, ocorrendo como espécies diméricas ou monoméricas, foram incapazes de polimerizar sob a forma de amilóide mesmo a pH 3.2, devido às ligações dissulfureto. Quando o ensaio para formação de amilóide foi repetido na presença de β-mercaptoetanol observou-se que após o rompimento das pontes dissulfureto tanto os dímeros quanto os monómeros estáveis eram capazes de formar fibras. Estas evidências sugerem claramente que as subunidades constituintes das fibras de amilóide induzidas *in vitro* por acidificação, são os monómeros e não os dímeros, apoiando que o desarranjo parcial ou a destabilização dos monómeros é necessário/a para a fibrilogénese. O re-arranjo das "strands" C e D pode fazer parte das modificações que ocorrem na estrutura tetrameric nativa da TTR e que conduzem à dissociação em monómeros parcialmente desarranjados ou instáveis, posteriormente envolvidos na formação de fibras de amilóide. Nós apresentamos também evidência para a importância do "loop" AB e dos contactos entre as "strands" G e H para a estabilização da estrutura tetramérica da TTR. Os nossos resultados com os dois "mutantes instáveis" Asp18Asn e Leu110Ala mostraram que as substituições que afectam estes resíduos-chave nas áreas que os circundam, favorecem o surgimento das superfícies alteradas que contribuem fortemente para a agregação.

A investigação de conformações alteradas que podem representar estruturas intermediárias na fibrilogénese da TTR foi também alvo de interesse no nosso trabalho, visando a identificação de regiões crípticas específicas para determinadas etapas na cascata amiloidogénica. Tentámos destabilizar a estrutura tetramérica da TTR, baseando-nos em dados cristalográficos conhecidos para a estrutura da Leu55Pro, um mutante de TTR altamente agressivo. Produziu-se um mutante com uma substituição na posição 78, tendo em mente dois factos: que o resíduo original na TTR normal (WT), está ligado por uma ligação de hidrogénio ao resíduo 18 do "loop" AB; e que na estrutura cristalográfica da Leu55Pro, uma das alterações conformacionais envolvidas na exposição de novas superfícies envolvidas na

agregação, é a destabilização do "loop" AB. O mutante Tyr78Phe foi isolado por HPLC como uma forma tetramérica solúvel embora a pH ácido tenha demonstrado propensão para formar amilóide. Além disso, esta forma mutante de TTR tetramérica e solúvel, foi reconhecida por um anticorpo monoclonal (Mab 15), relatado previamente como sendo capaz de reconhecer somente mutantes altamente amyloidogénicos sem conformação tetramérica e fibras de amilóide. Estes factos levaram-nos a sugerir que a estrutura tetramérica do mutante Tyr78Phe está alterada devido ao afrouxar do "loop" AB, conduzindo a uma estrutura que pode representar um intermediário inicial no processo da fibrilogénese, expondo um epítopo críptico que é comum às fibras de amilóide.

Prosseguindo na investigação de intermediários conformacionais de TTR expondo epítopos crípticos que pudessem revelar superfícies amiloidogénicas, procedeu-se a uma selecção baseada num sistema de "Two-hybrid"-Gal4 para interacções de TTR com uma biblioteca de aptâmeros com 20 aminoácidos de comprimento. Usando como *"baits"* um mutante de TTR altamente agressivo (Leu55Pro) e a TTR WT, observámos inicialmente que as células transformadas de levedura eram capazes de expressar as formas de TTR solúvel e agregada e que dois anticorpos (Abs) (um policlonal e o Mab 15) poderiam ser usados como marcadores para as formas solúvel e agregada da proteína, respectivamente. A partir da biblioteca de aptâmeros aleatórios, seleccionaram-se alguns que interagindo com diferentes formas de TTR nos permitiriam identificar as regiões na molécula que provavelmente estão envolvidas no contacto durante a polimerização e/ou outras, expostas antes da polimerização em formas intermédias, podendo vir a conservar-se nas fibras. Nas condições testadas, obtiveram-se sete tipos diferentes de aptâmeros interactuantes e organizámo-los em grupos de acordo com os distintos padrões de imuno-reactividade obtidos entre: interacção de Abs-TTR e interacção Abs-complexo (TTR-aptâmero). Realizaram-se estudos de competição em BIAcore e determinações de cinéticas para a afinidade das interacções entre Abs/aptâmeros e entre diversas formas de TTR (WT e mutante). Usando cinco aptâmeros seleccionados, específicos para determinadas formas de TTR, poderemos ter identificado algumas das regiões crípticas, umas especificamente expostas em determinadas estruturas de TTR que em algum ponto sofrerão re-arranjos adicionais, e outras que se conservam na estrutura fibrilogénica final.

Os péptidos estudados neste trabalho reconhecem e ligam-se a três tipos diferentes de epítopos conservados ou transientes ao longo do processo amiloidogénico: (i) um epítopo expresso na TTR WT e que é perdido na conversão de estrutura nativa em estrutura fibrilar; (ii) um epítopo críptico expresso especificamente em fibras de amilóide, e (iii) epítopos crípticos expressos em intermediários amiloidogénicos de TTR e nas fibras de amilóide, envolvendo três regiões diferentes da molécula. Estas evidências permitem a sugestão de três regiões candidatas a epítopos crípticos amiloidogénicos. Um destes epítopos situa-se provavelmente numa área envolvida na destabilização da TTR, reconhecida pelo Mab 15 (como relatado previamente), ou seja, a área da "strand" C-CD "loop", como sugerido pelos resultados obtidos com os aptâmeros 40W, C26 e possivelmente o 29B. Detectámos também dois epítopos novos, um exposto especificamente em fibras, e outro que parece sofrer alterações conformacionais na transição de formas solúveis para fibrilares, revelados pelos aptâmeros C45 e 67, respectivamente. Os nossos resultados apoiam a ideia de que um tetrâmero nativo sofre gradualmente mudanças amiloidogénicas, evoluindo de uma estrutura parcialmente modificada (TTR Leu55Pro solúvel) para um arranjo tetramérico altamente modificado (TTR Tyr78Phe solúvel) culminando na sua dissociação em monómeros desarranjados, que por fim se associam em fibras.

Explorou-se a especificidade do aptâmero C45 para a forma fibrilar da TTR Leu55Pro, testando a sua capacidade de inibir a formação de amilóide *in vitro*, procedendo-se a incubações com um marcador fluorescente para amilóide, a tioflavina T (ThT). Quando o aptâmero foi misturado com a TTR Leu55Pro, ocorreu formação de

fibras. No entanto, após 5 dias de incubação a 37^{0}C, a fluorescência da ThT foi reduzido aproximadamente num factor de 2, até ao vigésimo segundo dia de experiência, fornecendo a indicação de que o C45 interfere com a fibrilogénese e que a diminuição na fluorescência da ThT requeria a presença deste aptâmero. O C45 não impediu a formação de folhas pregueadas-β ou a iniciação das fibras, mas poderá ter-se ligado à fibra crescente afectando o processo de alongamento. Inibidores mais eficazes, como por exemplo, sequências peptídicas mais curtas, baseadas no C45, poderão funcionar como agentes disruptores de amilóide.

No seu conjunto, os resultados relatados nesta tese constituem novas pistas para a compreensão do processo amiloidogénico e podem contribuir na intervenção terapêutica projectada para agir especificamente contra a formação ou evolução de estruturas amiloidogénicas iniciais intermediárias, que ocorrem em etapas localizadas deste fenómeno patogénico.

Résumé

Les amyloidoses sont un groupe des maladies impliquant le dévoilement d'une protéine, caractérisées par l'accumulation de la protéine fibrillaire insoluble dans plusieurs organes et des tissus, et qui mène à la mort de cellules et à la dégénération tissulaire. Jusqu'ici, 18 protéines et polypeptides différents ont été identifiés dans les dépôts amyloïdes maladie-associés. Une substitution de l'aminoacid (aa) dans la protéine amyloïde est associée à quelques formes d'amyloidoses. Plus de 85 mutations différentes ont été enregistrées chez l'homme en association avec le dépôt amyloïde de la transthyrétine (TTR). Le mécanisme moléculaire derrière la transformation d'un tétramère soluble de TTR sauvage dans les agrégats insolubles menant au développement de la Polyneuropathie Amyloidotique familialede (PAF), une maladie neuropathique mortelle, a été sous la recherche intensive dans les deux dernières décennies.

Les objectifs principaux de ce travail étaient de contribuer à la compréhension de ce mécanisme, caractérisant des causes déterminantes amyloidogéniques dans la PAF. Nous avons commencé pour élucider la nature des sous-unités qui composent les fibrilles amyloïdes de TTR, recherchant les zones principales de la molécule stratégique pour que la déstabilisation tetrameric se produise. De plusieurs évidences point à la participation des monomères comme sous-unités de fibrillogenése de TTR, bien qu'il reste quelques chercheurs qui s'opposent à cette idée proposant un dimère ou un multiple des dimères comme module amyloïde de la fibrille. Nous avons produit trois « mutants stables » en présentant des cystéines supplémentaires en substitution de résidus possiblement situés dans des zones cruciales pour stabiliser la structure tetramérique, et nous avons réalisé plusieurs études biochimiques pour tester leur résistance à la dissociation et à la susceptibilité à la formation amyloïde *in vitro*. Sous le pH acide, les mutants Ser117Cys et Glu92Cys étaient dimères dans sa nature et le double mutant Val30Cys/Leu55Cys dissociait dans des monomères stables. Chacun des trois mutants, se produisant en tant qu'espèce dimère ou monomérique, ne pouvait pas polymériser dans amyloïde même à pH 3.2, du à l'existence des liaisons bisulfure. Quand le test de formation d'amyloïde a été répété en présence du β-mercaptoéthanol on a observé que lors de l'interruption des ponts bisulfure sur ces dimères ou monomères stables, des fibrilles amyloïdes pourraient être formées. Ces évidences suggèrent clairement que les monomères plutôt que les dimères sont les sous-unités structurales de répétition composant les fibrilles amyloïdes, dans l'amyloïde *in vitro* induite par l'acidification, et davantage support que le dévoilement partiel ou la déstabilisation des monomères est demandé pour la fibrillogenése. Une remise en ordre des « strands » C et D pourrait faire partie des modifications qui se produisent dans la structure tetramérique de la TTR sauvage, qui mènent à la dissociation dans les monomères partiellement dévoilés ou instables impliqués dans la formation des fibrilles amyloïdes. Nous présentons également l'évidence pour l'importance du « loop » AB et des contacts entre les « strands » G et H pour la stabilisation de la structure tetramérique de la TTR. Nos résultats avec deux « mutants instables », Asp18Asn et Leu110Ala ont prouvé que les substitutions affectant les résidus principaux dans ces zones favorisent l'occurrence des surfaces modifiées qui contribuent fortement à l'agrégation.

La recherche sur les conformations modifiées qui pourraient représenter des structures intermédiaires dans la fibrillogenèse de TTR a été également approchée dans notre travail, visant identifiant des épitopes cachés spécifiques des étapes déterminées dans la cascade amyloidogénique. Nous avons essayé de déstabiliser la structure tetramérique de la TTR, basée sur les données cristallographiques connues de la structure de Leu55Pro, un mutant fortement agressif de TTR. Nous avons produit un mutant avec une substitution dans la position 78, considérant que le résidu initial, dans la TTR sauvage, est joint pour une liaison d'hydrogène au résidu 18 du « loop » AB, et que dans la structure cristallographique de Leu55Pro, un changement de

conformation impliqué dans l'ouverture d'une nouvelle surface impliquer dans l'agrégation, est la déstabilisation du « loop » AB. Le mutant Tyr78Phe a été isolé par HPLC comme une forme soluble tetramérique, cependant sous le pH acide s'est avéré sujet pour former l'amyloïde. En outre, cette forme tetramérique soluble de TTR a été identifiée par un anticorps monoclonal (Mab 15), précédemment enregistré pour réagir seulement avec les mutants fortement amyloidogeniques manquant du pli tetramérique et avec les fibrilles amyloïdes. Nous proposons que la structure tetramérique de Tyr78Phe soit modifiée en raison du desserrement du « loop » AB, menant à une structure qui pourrait représenter une première intermédiaire dans la voie de fibrillogenése, exposant un épitope caché qui est commun aux fibrilles amyloïdes.

Pour étudier plus loin les intermédiaires de conformation de TTR avec des épitopes cachés exposés qui pourraient indiquer les surfaces amyloidogéniques, on a effectué un criblage utilisant le système de « two-hybrid » basé sur Gal4, pour identifier des interactions de TTR avec une bibliothèque de 20-« mer »-aptamers. En utilisant comme « baits » le mutant fortement agressif Leu55Pro TTR et la TTR WT, nous avons vu la première fois que les cellules transformées de levure étaient capables d'exprimer la forme soluble et les formes agrégées de TTR et qu'un anticorps polyclonal et Mab 15 pourraient être utilisés comme repères pour la forme soluble et agrégée de la protéine, respectivement. Nous avons effectué un criblage sur la bibliothèque des aptamers que pourrait interagit, visant identifiant des régions dans la molécule de TTR étant importante pour l'interaction pendant la polymérisation et/ou d'autres, exposées avant polymérisation sous les formes intermédiaires, et toujours conservé dans les fibrilles. Dans les conditions testées, sept types différents d'aptamers ont été obtenus et organisés en groupes selon les différentes configurations d'immunoréactivité entre l'interaction [anticorps-TTR « baits »] et l'interaction [anticorps-compléxe TTR « bait »-aptamer]. Nous avons ensuite exécuté des études de concurrence de BIAcore et des déterminations cinétiques pour l'affinité des interactions entre anticorps/aptamers et les plusieurs formes de TTR (WT et mutants). L'utilisation de ces aptamers spécifiques pour certaines formes de TTR, a permis l'identification des putatives régions cachées, quelques spécifiques pour structures déterminées de TTR qui à un certain point souffriront d'autres remises en ordre, et d'autres ce qui sont conservées dans la structure fibrillogenic finale. Les cinq peptides étudiés dans ce travail pouvaient reconnaître et lier à trois types différents d'épitopes, conservés ou a détruit au long du processus amyloidogénique: (i) un épitope exprimé sur la TTR WT qui est détruit dans la conversion de la structure native dans les structures fibrillaires; (ii) un épitope caché spécifiquement exprimé sur les fibrilles amyloïdes et (iii) épitopes cachés exprimés sur les intermédiaires amyloidogéniques de TTR et sur les fibrilles amyloïdes de TTR, impliquant trois régions différentes de la molécule. Ces évidences nous permettent de proposer trois régions de candidat pour les épitopes cachés amyloidogéniques. Un de ces épitopes est probablement situé dans une zone impliquée dans la déstabilisation de la TTR précédemment enregistrée pour être reconnu par Mab 15, la zone « strand » C-« loop » CD, comme suggéré par les résultats avec les aptamers 40W, C26 et probablement 29B. Nous avons également trouvé deux nouveaux épitopes, un spécifiquement exposé dans les fibrilles, et an autre qui souffre des changements de conformation entre les formes solubles de TTR et les fibrilles, indiqué par les aptamers C45 et 67, respectivement. Nos résultats supportent l'idée qu'un tétramère natif souffre graduellement des changements amyloidogéniques, évoluant d'une structure partiellement modifiée (TTR Leu55Pro soluble) à un arrangement tetramérique fortement modifié (TTR Tyr78Phe soluble) aboutissant à sa dissociation dans des monomères désorganisés, qui s'associent finalement dans des fibrilles.

La spécificité de l'aptamer C45 à la forme fibrillaire de Leu55Pro TTR a été exploré en testant sa capacité d'empêcher la formation d'amyloïde *in vitro*, lors de l'incubation avec un repère fluorescent pour l'amyloïde, la thioflavine T (ThT). Quand l'aptamer a été mélangé à la TTR Leu55Pro, la formation de fibrilles s'est produite.

Cependant, après 5 jours d'incubation à 37^0C, la fluorescence de la ThT a été réduite par un facteur de 2, jusqu'au vingtième jour de l'expérience, fournissant l'indication que le C45 gênait la fibrillogenése et que la diminution de la fluorescence de ThT a exigé la présence de c'est aptamer. C45 n'a pas empêché la formation de beta-feuilles ou le déclenchement des fibrilles, mais il pourrait lier à la fibrille croissante et affecter le processus d'élongation. Des ordres plus courts de peptide, basés sur la structure du C45, pourraient fonctionner comme agents de perturbation d'amyloïde.

Tout à fait, l'ensemble des résultats décrits dans cette thèse représente un apport nouveau pour la compréhension du processus amyloidogénique et pourraient contribuer dans l'interposition thérapeutique conçue pour agir spécifiquement contre la formation ou l'évolution des premières structures amyloidogéniques intermédiaires, dans des étapes localisées de ce phénomène pathogène.

PART I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. Transthyretin (TTR)

TTR was first isolated from human plasma in 1956 by Schultze and co-workers, and was originally named prealbumin due to its mobility in electrophoresis of whole plasma at pH 8.6 (Ingbar, 1958), running in front of serum albumin. Highly conserved in evolution, TTR has a well-defined double-role acting as a transport protein for thyroxine (Woeber and Ingbar, 1968) and vitamin A (Goodman, 1987). The transport of vitamin A (retinol) is accomplished by the formation of a complex with retinol-binding protein (RBP). TTR was first identified in the cerebrospinal fluid (CSF) (Kabat *et al.*, 1942) and only later in plasma (Seibert and Nelson, 1942). In humans, TTR expression occurs mainly in the liver, choroid plexus of the brain and in the eye.

1.1. TTR gene structure and regulation

The gene encoding human TTR is a single copy gene located in the long arm of chromosome 18 (Whitehead *et al.*, 1984). Assigned to region 18q11.2-q12.1, the TTR gene spans 7.6 kb and contains four exons with approximately 200 bp each and 3 introns: A, B and C (Tsuzuki *et al.*, 1985). Exon 1 encodes a signal peptide (20 aminoacid residues) and the first 3 aminoacids of the mature protein; exon 2 encodes for aminoacid residues 4-47; exon 3 for aminoacid residues 48-92 and exon 4 for aminoacid residues 93-127. Introns B and C contain two *Alu* sequences with opposite polarity suggesting a hairpin formation in the precursor mRNA. Introns A and C contain two open reading frames (ORF) of unknown significance and it was suggested that they might encode for gene expression regulatory proteins (Tsuzuki *et al.*, 1985).

Upstream the transcription initiation site, consensus sequences include a TATA box at position -30, a GC rich region of about 20 bp, a CAAT box at position -101 and, further up, two overlapping sequences homologous to glucocorticoid-responsive elements at positions -224 and -212. The $(CG)_n$ dinucleotide is the only known repeated motif in the entire TTR gene, including regulatory regions. A polyadenylation signal (AATAAA) was identified 123 bp downstream from the coding sequence (Sasaki *et al.*, 1985).

Most studies on TTR gene regulation have been performed using the mouse and rat genes, due to the high homology among DNA sequences of the coding region and regulatory sequences of the mouse, rat and human TTR gene (82% and 90%

homology of the mouse and rat genes to human gene, respectively) (Costa *et al.*, 1986).

Also, studies on regulation and expression of mouse TTR gene were performed in human hepatoma cells (HepG2) (Costa *et al.*, 1986; Costa *et al.*, 1989) leading to the identification of two major regulatory regions in the mouse TTR gene: a promoter proximal region, at approximately -150 bp relatively to the cap site, and a distal enhancer sequence, at -1.86 to -1.96 kb (Costa *et al.*, 1986); these findings led to the identification of regulatory sequences in the human gene by comparative analysis (Figure 1).

The promoter region of the human gene contains elements for hepatocyte-specific expression, including binding sites for HNF-1, C/EBP, HNF-3 and HNF-4. Comparative analysis of human and mouse sequences showed that the binding sites for HNF-3 and HNF-4 are well conserved in the human TTR gene, but not those for C/EBP (Sakaki *et al.*, 1989). Two other liver-specific nuclear protein potential binding sites were identified in the human gene at positions -216 ~ -221 and -199 ~ -204. The motif (TGG/AA/CCC/T) is common to factors Tf-LF1, Tf-LF2 and LF-A1.

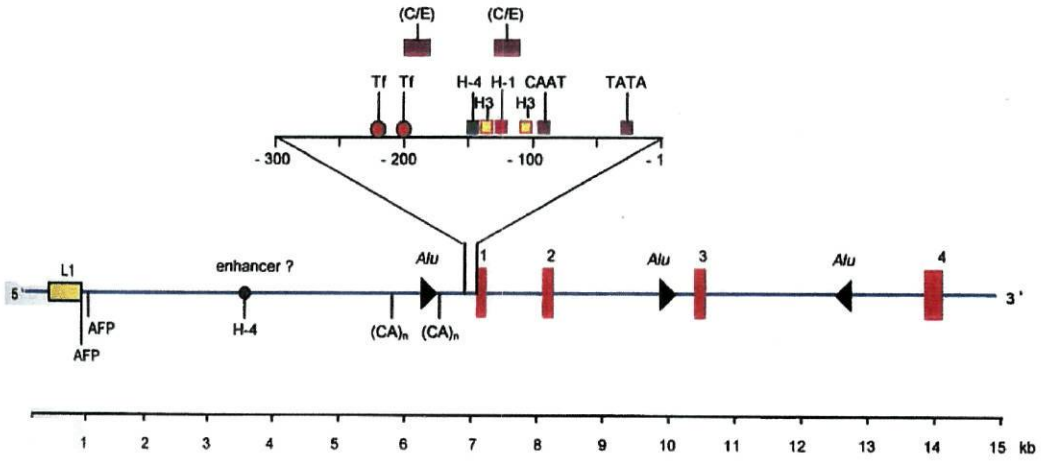


Figure 1. Schematic representation of the structure of the human TTR gene and localisation of the respective regulation regions. The exons (red boxes) are numbered from 1 to 4. *Alu*, L1 and $(CA)_n$: respective repetitive sequences. TATA: TATA box; CAAT: CAAT box. H1, C/E, H3, H4: binding sites of hepatocyte nuclear factors HNF-1, C/EBF, HNF-3 and HNF-4, respectively. Tf: common motif to Tf-Lf-1, Tf-LF-2, LF-A1. Enhancer: a region highly homologous to a tissue-specific enhancer of the mouse TTR gene. AFP: binding site for AFP-1 factor (from Sakaki *et al.*, 1989).

These DNA segments contain the binding sites for several liver specific nuclear proteins and their presence is sufficient for normal hepatic expression in transgenic animals, but it was necessary to introduce 3 kb of upstream sequences for expression in the choroid plexus (Yan *et al.*, 1990). These results suggested that choroid plexus genes and liver genes are regulated differently, possibly by using different cis and/or trans-acting factors (Costa *et al.*, 1990). This differential gene regulation by liver and choroid plexus had already been suggested, since TTR mRNA levels in liver decrease during the acute-phase response, and protein mal-nutrition (Wade *et al.*, 1988) but not in the choroid plexus (Dickson and Schreiber, 1986). Transgenic mice for the human TTR gene showed that a sequence of 600 bp upstream contained the cis-elements sufficient for liver and yolk sac expression, whereas sequences lying at 6 kb upstream are required for developmental, tissue specific and quantitatively normal expression (Yan *et al.*, 1990; Nagata *et al.*, 1995).

1.2. TTR structure

TTR is a tetrameric protein, composed of four identical subunits, each formed by 127 aminoacid residues, the sequence of which was determined by Kanda *et al.* (1974). The molecular mass of each subunit, based on the aminoacid composition, is 13,745 kDa and therefore, the mass of the tetramer is 54,980 kDa.

The three dimensional structure of TTR was determined by crystallographic and X-ray diffraction analysis by Blake and Swan (1971). The dominant secondary structural element of the TTR monomer is the β-pleated sheet: about 45% of the aminoacid residues are organised into eight β-strands, labelled A through H, interacting in an anti-parallel manner, with the exception of strands A and G. These β-strands are 6-9 aminoacids long with the exception of strand D that is formed by 3 aminoacids only, and are organised into two β-sheets, composed of strands DAGH and CBEF of each monomer, respectively. The single α-helical segment in the TTR monomer is formed by residues 75-83 located at the end of the β-strand E (Figure 2). The remaining residues, not organised in strands, constitute seven loops linking the eight β-strands together and varying in length and character, plus a ten-residue N-terminal and a five-residue C-terminal segments. The five first nine residues of the polypeptide chain are considered to be disordered, as well as the last five.

The tertiary structure of the TTR subunit is largely determined by the association of the two β-sheets DAGH and CBEF. The dominant interaction in the formation of the dimer is the hydrogen bonding between the respective strands F and H of the two β-sheets in each monomer (Figure 2), extending the two four-stranded sheets in the monomers to eight-stranded structures in the dimers. The strand arrangement becomes DAGHH'G'A'D' and CBEFF'E'B'C', where strands A-H belong to one monomer and strands A'-H' represent the equivalent strands of the other monomer.

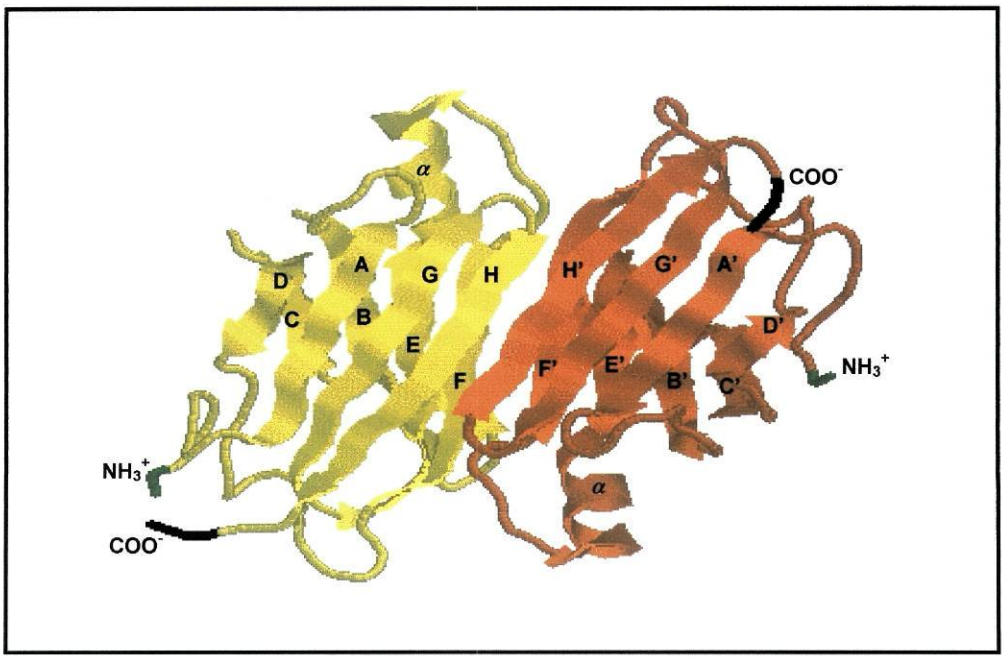


Figure 2. Diagram representation of a TTR dimer (adapted from De la Paz *et al.*, 1992).

Two dimers assemble to form the tetramer with their inner eight-stranded sheets DAGHH'G'A'D' face-to-face, about the molecular z axis, forming a cylinder with a central hydrophobic channel, where two binding sites for thyroxine (T_4) exist. The dimer-dimer contacts are derived from residues located on two loops at the edge of the sheets: the GH loop (residues 112-115) and the AB loop (residues 17-23).

The TTR quaternary structure is characterised by exhibiting a compact arrangement of the monomers (Figure 3), stabilised by hydrogen bonding and hydrophobic interactions, which accounts for the unusual stability of the TTR molecule (Raz and Goodman, 1969). The central hydrophobic channel running through the

molecule, delimited by 16 β-strands and two semi-cylindrical surface depressions, is complementary to the structure of the DNA double-helix, and forms a binding pocket for the thyroid hormone thyroxine. Two monomers of the TTR tetramer contribute to the formation of the TTR:RBP complex, mainly through the A strands and the α-helices.

TTR has been shown to be a highly conserved protein. TTR sequences from mammals show 87% sequence identity between species; chicken and lizard TTR show 75 and 66% sequence similarity with the human protein, respectively (Duan *et al.*, 1995). One of the differences between the structure of chicken TTR and mammal TTR is the existence of a loop involving aminoacid residues 75-83, instead of the α-helix previously described (Sunde *et al.*, 1996). Most of the substitutions are located on the outer surface of the protein leaving the regions corresponding to the central channel almost completely conserved.

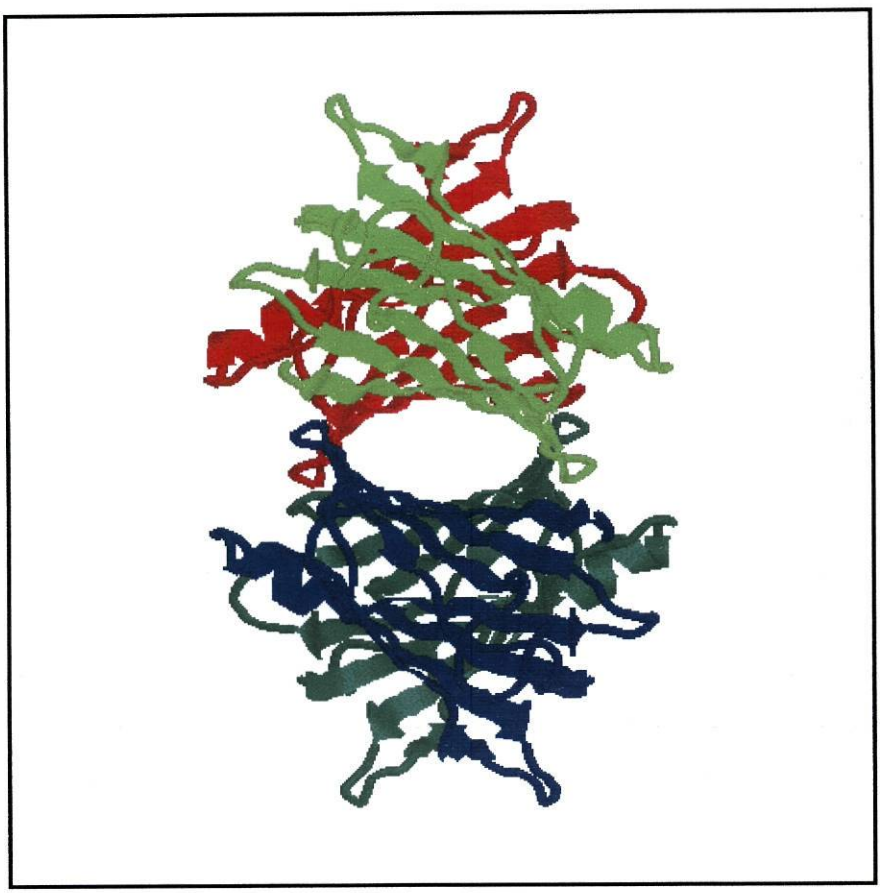


Figure 3. Diagram representation of human TTR tetramer showing the thyroxine-binding channel.
Protein data bank (1TTA)

The hydrophobic core, the monomer-monomer and dimer-dimer interaction sites, and the hormone-binding channel are the most conserved regions of TTR. The RBP-binding site is little conserved in comparison to the hormone-binding channel. A reason for this is the possibility for coupled evolution of RBP, which is only 42% identical or conserved in the complex-forming region, in contrast to the constant structure of the thyroid hormone molecules. The surface-exposed region displays the lowest evolutionary conservation (Eneqvist and Sauer-Eriksson, 2001).

In all the crystallographic structures available for TTR in the literature, at present, the tetrameric form of the protein could be observed, indicating that crystallisation occurred from the protein in a tetrameric conformation or rather that the packing interactions led to an association of dimers or monomers where the tetramer was present (Damas and Saraiva, 2000).

1.3. Physiological role of TTR

TTR main biological function is the plasma transport of the thyroid hormone thyroxine (Fergunson *et al.*, 1975) and of retinol (vitamin A alcohol), the latter *via* the formation of a protein complex with retinol-binding protein (RBP) (Kanai *et al.*, 1968).

1.3.1. Transport of T_4

Thyroid hormones are very important in basal metabolism regulation, oxygen consumption, in controlling cellular growth and differentiation, and are particularly essential for the normal development of mammalian brain (Stein *et al.*, 1991). T_4 can be taken up from the free hormone pool, and this has been regarded as the principal mechanism of uptake *in vivo* (Mendel, 1989), whilst protein mediated uptake by co-internalisation with TTR, or enhanced dissociation of the hormone carrier protein complex due to a transient interaction with the cell surface, have been proposed as alternative mechanisms of uptake (Pardridge, 1987).

In human plasma, TTR is one of the three proteins responsible for the distribution of thyroxine throughout the body, the others being thyroxine-binding globulin (TBG) and albumin. TBG is the protein with the highest affinity for T_4 ($K_a=1x10^{10}$ M^{-1}) and is responsible for the transport of 70% of plasma T_4, despite its much lower concentration in serum than the other T_4 transport proteins. TTR affinity for

T_4 is $7x10^7$ M^{-1}, and albumin presents the lowest affinity of the three binding proteins with a $K_a=7x10^5$ M^{-1} (Robins, 1991). TTR also binds triiodothyroxine (T_3), the biologically active hormone, but with much lower affinity.

Plasma TTR carries only about 15% of total thyroxine, suggesting that its role in the transport of this hormone is not essential. But this does not seem to be the case in the central nervous system, in which, 80% of T_4 is bound to TTR (Herbert *et al.*, 1986). TTR is synthesised at high rate by the epithelial cells of the choroid plexus and is present in high concentration in the cerebrospinal fluid (Dickson *et al.*, 1986; Palha *et al.*, 2000a).

TTR has two structurally identical binding sites for T_4 (Blake *et al.*, 1978), each located between two of the four subunits. In the centre of the channel, the aminoacid residues Leu^{110}, Ser^{115} and Ser^{117} originate a constriction that separates the two binding sites. These binding sites display very different affinities, $1.05x10^8$ M^{-1} and $9.55x10^5$ M^{-1}, respectively, therefore implying negative cooperativity in T_4 binding to TTR (Andrea *et al.*, 1980). In each hormone-binding site there are six pockets capable of binding an iodine substitute (de La Paz *et al.*, 1992); when T_4 binds, four of these pockets become occupied by the iodines of the hormone molecule. The thyroxine-binding channel is characterised by three chemically distinct regions: a charged region at the entrance of the channel, a hydrophilic central region and a hydrophobic patch. This hydrophilic-hydrophobic-ionic character of the binding site matches the chemistry of the hormones.

Several authors reported that the T_4 binding sites are capable of accommodating other molecules like 3',5'-dibromo-2',4,4',6-tetrahydroxyaurone, milrinone, retinoic and flufenamic acids, and 3,3'-diiodo-L-thyronine, as well as PCB metabolites (reviewed by Damas and Saraiva, 2000).

Thyroid hormones circulate mainly bound to plasma proteins; their free fraction is less than 0.1% of the total circulating hormone. The equilibrium between bound/free hormone fractions is sometimes affected by changes in the plasma-binding proteins concentration and affinity. In the majority of cases these variations do not have dangerous physiological consequences since the free hormone levels usually remain unaffected.

The effect of TTR in T_4 transport across the plasma membrane is still a controversial issue. It was initially suggested that given the high lipophilicity of the thyroid hormones, they could easily cross the double lipid layer plasma membrane, by passive diffusion (Lein and Dowben, 1961). However, the demonstration of plasma

membrane binding sites for T_4 in several organs and cells (Krenning and Docter, 1986; Davis, 1991), suggested a membrane receptor mediated transport.

Several evidences point to the validity of the free-hormone transport theory, according to which hormones enter into the tissues exclusively through its free fraction after dissociating from the protein-hormone complexes. Mendel and colleagues (1988) observed that T_4 tissue influx constant rate is very high, as would be expected for an uptake that occurs exclusively through the hormone free fraction; also, the rate of T_4 tissue uptake never exceeds the rate of spontaneous T_4 dissociation from its binding proteins (Mendel, 1989). Furthermore, in albumin deficient rats (Mendel *et al.*, 1989) and in total or partial TBG deficient humans (Bartalena, 1993), thyroid hormone transfer into the tissues is normal. With respect to TTR, displacement studies of T_4 bound to TTR, using a synthetic flavonoid, suggested that TTR is not essential for liver and kidney T_4 uptake (Mendel *et al.*, 1992).

As mentioned, another view on T_4 transport supports the involvement of plasma-binding proteins as mediators of hormone transfer into the tissues. Pardridge (1981; 1987) claimed that the hepatic rate of uptake of thyroid hormones exceeded their dissociation from the plasma-binding proteins and, therefore, the hormone protein bound fraction would represent an important fraction of hormone transferred into the tissues.

The function of TTR in transporting T_4 to the brain still raises controversy. One of the mechanisms for T_4 to reach the brain is the involvement of TTR synthesised by the choroid plexus and secreted to the CSF or simply by diffusion through the lipid plasma membrane of epithelial and endothelial cells, of choroid plexus and cerebral capillars, respectively.

Several evidences suggest an essential role for TTR in T_4 transport into and within the brain: TTR is the major T_4 binding protein in CSF (Hagen and Solberg, 1974) and the regulation of its synthesis in the choroid plexus is independent from that of the liver (Wade *et al.*, 1988). It was shown that rat choroid plexus explants, *in vitro*, accumulate thyroid hormones from the surrounding media, and that when the equilibrium is reached, the thyroid hormone intracellular/extracellular concentration ratio decreases when TTR media content is increased (Dickson *et al.*, 1987). Furthermore, it was shown that after intravenous radioactive T_4 injection, the radioactivity first appears in the choroid plexus, followed later by the brain (Dickson *et al.*, 1987; Schreiber *et al.*, 1990). It was then proposed that T_4 is transported from the blood into the choroid plexus where it binds to TTR before being secreted into the CSF.

Equally plausible and given its high lipophilicity, thyroid hormones should be able to cross the blood-brain barrier outside the choroid plexus without the intervention of a plasma transport protein. Studies where ^{125}I- T_4 covalently bound to TTR was intravenously injected in rats showed that the serum T_4 bound to TTR fraction is not transported into the CSF (Chanoine *et al.*, 1992). Also, Palha *et al.* (1994), conducted studies in TTR knock-out mice (*ttr*-) showing that TTR was not absolutely required for T_4 to reach the brain and later reported that the whole brain total T_4 levels were decreased in these animals (Palha *et al.*, 1997). Schreiber and co-workers (1997) proposed that given the redundancy of T_4-binding proteins (namely, TBG, albumin and TTR), the absence of one protein would be compensated by the others. Though this concept may apply for serum its appliance for the blood-choroid plexus-CSF barrier of TTR-null mice, was argued by Palha *et al.* (2000) indicating that CSF TTR is not required for normal maintenance of the thyroid hormone in brain. Their results support the free hormone hypothesis for T_4 uptake, proposing that under normal physiological conditions, TTR has a storage role for T_4 in blood, choroid plexus and CSF. In the absence of TTR as a reservoir in the choroid plexus and CSF, T_4 exits from the CSF to the cerebral and, ultimately, the general circulation might be accelerated.

Some TTR mutants, involving aminoacid replacements in the T_4 binding channel have been shown to present an altered affinity for the hormone (Refetoff *et al.*, 1996; Almeida *et al.*, 1997). Other mutations, not directly involved in T_4 binding, have also been described to bind the hormone in a distinct manner, indicating that slight modifications in the TTR molecule can affect thyroid hormone binding (Rosen *et al.*, 1993; Almeida *et al.*, 1996).

1.3.2. Transport of retinol

Vitamin A compounds are essential for vision, reproduction functions and for the maintenance of differentiated epithelia. The transport of retinol from retinoid stores in the liver to target tissues is accomplished exclusively through retinol-binding-protein (RBP) that protects retinol from oxidation, makes it soluble and protects the tissues from the toxic action of the compound. Inside the cells, intracellular binding proteins with high affinity for retinol, such as cellular-retinol-binding-protein (CRBP) I and II would then be responsible for shuttling retinol to various enzymes for storage or metabolism (Sundaram *et al.*, 1998).

TTR associates with RBP, contributing thus to the transport of retinol (Kanai *et al.*, 1968) and RBP circulates in plasma as a 1:1 molar protein complex with TTR. This association is thought to protect RBP from glomerular filtration and renal catabolism (Raz *et al.*, 1970) assuring the delivery of retinol to the target cells and increasing RBP's half-life by about threefold. The complexed RBP mobilises retinol from its sources in the liver and delivers it to tissues throughout the body. Sivaprasadarao *et al.* (1988) suggested that TTR played an important role in retinol distribution to the tissues, by controlling the levels of free RBP.

The TTR tetramer is capable of binding two molecules of RBP, though four potential binding sites for this molecule were shown to be present (van Jaarsveld *et al.*, 1973; Kopelman *et al.*, 1976). Monaco and co-workers (1995) showed that the RBP molecules establish molecular interactions with the same TTR dimer *in vitro*, and each also makes contacts with one of the remaining monomers, thus blocking the other two potential binding sites in the tetramer. The dissociation constants for the two RBP molecules were found to be $1.9\pm1.0\times10^{-7}$ M for the first RBP molecule and 3.5×10^{-5} M for the second. In spite of these *in vitro* determinations, under physiological conditions, only one RBP molecule binds to the TTR tetramer due to the limiting physiological concentrations of RBP in plasma (2 mM), about half the concentration of TTR. The binding affinity of the interaction between these two proteins is approximately 1×10^{-7} M.

The X-ray structure of the complex human TTR:chicken RBP was first reported by Monaco and co-workers (1995) and the structure of the human TTR:RBP complex was published by Naylor and Newcomer (1999) (Figure 4). Human RBP has a C-terminal 8 aminoacids longer than chicken RBP; its interaction with human TTR revealed that this longer sequence, which is characteristic of all mammalian RBPs, is involved in the protein-protein recognition interface.

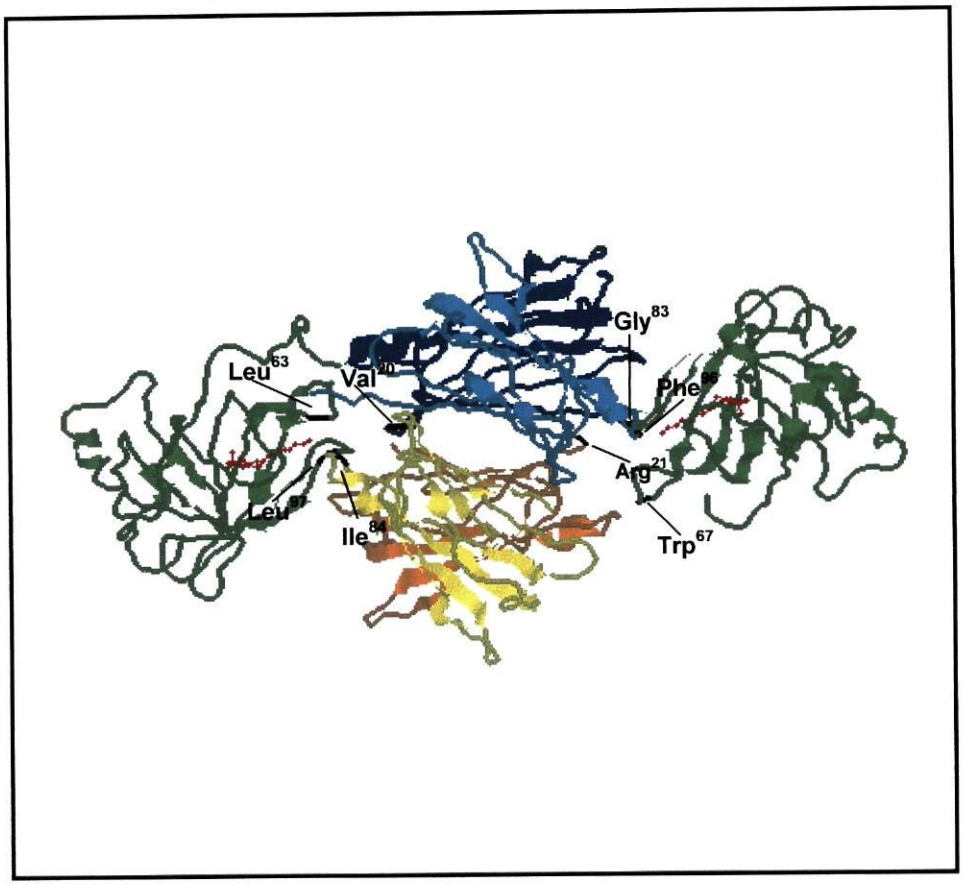


Figure 4. Structure of human Retinol-binding protein with its carrier protein Transthyretin.
The TTR tetramer can be seen in the centre (monomers represented in blue, cyan, yellow and orange) interacting with the carboxyl terminal of two RBP molecules (represented in green) conjugated with retinol (in red). The main areas of contact between both proteins correspond to residues highlighted in black (20, 21, 83, 84 from TTR and 63, 67, 96, 97 form RBP). Protein data bank (1QAB).
Adapted from Naylor and Newcomer (1999)

The RBP molecule interacts with the Ile^{84} from two different chains of TTR and the retinol hydroxyl group establishes a hydrogen bond with the carbonyl group of the Gly^{83} of TTR; this residue also interacts with Leu^{35} of RBP and is very important in the transition of holo to apo form (with or without retinol, respectively). This transition involves a conformational change on the loop that extends from aminoacid 34 to 37, in particular Leu^{35} and Phe^{36} (Zanotti *et al.*, 1993). Other important contacts between TTR and RBP include residues Arg^{21} and Val^{20} contributed by two TTR monomers and Trp^{67}, Phe^{96}, Leu^{63} and Leu^{97} from RBP.

The interaction of retinol with an aminoacid of the TTR molecule explains why the formation of the TTR-RBP complex stabilises the binding of retinol to RBP and

why, after delivery of retinol to cells and tissues, RBP shows a reduced affinity for TTR. The importance of the aminoacid residue at position 84 has been physiologically demonstrated by the existence of two molecular variants of TTR in which the isoleucine is replaced by serine (TTR Iso84Ser) and asparagine (TTR Iso84Asn), where carriers of these variants have been reported to have lowered plasma RBP levels, possibly representing an altered affinity of RBP for these mutant TTRs (Waits *et al.*, 1995).

The transmembrane transport of retinol has been quite controversial and three possible mechanisms have been suggested for its cellular uptake. Some authors (Fex and Johannesson, 1988; Noy and Xu, 1990) assumed that retinol would be transported into the target cells by simple diffusion through the lipid bilayer. Despite some evidences that could support this theory, this mechanism would imply that following dissociation from RBP, retinol would bind to several other non-specific lipid-binding proteins being distributed to all tissues; together with the fact that no appreciable levels of retinol were found to be associated with these proteins, this non-specific mechanism, should it occur, is a wasteful process, as this nutrient would be presented to tissues where it might have little or no physiological role. In TTR knockout mice, hepatic retinol levels are raised when compared to WT animals and levels of retinol in the testis, kidney, spleen and eyecups are normal (Wei *et al.*, 1995). This suggests that TTR does not affect the uptake nor the storage of dietary retinol and supports the possibility that tissue uptake of plasma retinol is more effective when the retinol:RBP complex is not bound to TTR. However, this non-retinol deficient status can also be due to the fact that as the plasma levels of RBP in these mice are very low, retinol transport and internalisation occurs through binding to chylomicrons.

Other authors have reported the presence of specific cell surface receptors for RBP on several cell types and two different mechanisms were proposed. A receptor-mediated transport being extremely specific, offers a distinct physiological advantage to the organism by not only directing the delivery of retinol to the tissues that require vitamin A to function but also preventing cellular damage by free retinol.

One of these mechanisms suggests that RBP charged with retinol specifically interacts with its receptor on the plasma membrane and this interaction results in the endocytosis of the retinol-RBP complex, followed by the subsequent release of retinol in the lysosomes. Gjoen *et al.* (1987) and Senoo *et al.* (1990) conducted studies *in vivo*, from which they suggested that hepatocytes might take up retinol from plasma not only through RBP, but also via the RBP-TTR complex.

The second mechanism, also involving a receptor, proposes a role for CRBP as an acceptor for the retinol molecule, after a receptor mediated transport process across the plasma membrane, where the resultant apo-RBP remains outside. The apo-RBP appears to lose its affinity to the receptor and TTR and due to its low molecular weight, is filtered in the kidneys and excreted in the urine. Sivaprasadarao *et al.* (1994) showed that TTR inhibited RBP binding to its receptor, suggesting that the RBP-TTR complex may function as a reservoir for RBP, releasing more RBP as it binds to the receptor. This fact was found to be in agreement with the next finding that the binding sites of RBP-TTR interaction overlapped with the binding sites for the interaction RBP-cellular receptor (Sivaprasadarao and Findlay, 1994).

1.3.3. Other TTR functions

Together with its two best-known functions, TTR has been shown to interact with a variety of compounds, though the significance of these interactions is, in most cases, unexplained or poorly understood. These minor TTR interactors include noradrenaline oxidation products (Boomsma *et al.*, 1991), pterins (Ernstrom *et al.*, 1995), chicken lutein, hemin and hemoglobin (Martone and Herbert, 1993), polyhalogenated biphenyl compounds (Cheek *et al.*, 1999), retinoic acid - an inhibitor of T_4 binding to TTR (Smith *et al.*, 1994), apolipoprotein ApoAI (Sousa *et al.*, 1999) and others.

Some studies have also implicated TTR in immunological processes by interfering with the secretion of interleukin I (Borish *et al.*, 1992) and in the formation of stable complexes with amyloid-β peptide, preventing Aβ accumulation in cell culture experiments (Mazur-Kolecka *et al.*, 1995).

1.4. TTR metabolism

TTR, as most plasma proteins, is synthesised in the liver (Gitlin and Gitlin, 1975), in hepatocytes, and secreted into the serum. Normal serum TTR concentration in humans varies between 20-35 mg/dl. The two main places of extra hepatic TTR synthesis are the choroid plexus of the brain and the eye. The choroid plexus epithelium of cerebral ventricles synthesises and secretes large quantities of TTR into the CSF, where TTR levels range from 2-4 mg/dl (Soprano *et al.*, 1985; Herbert *et al.*,

1986). This high concentration of TTR in CFS first suggested that TTR would preferentially be transported through the blood-brain barrier and/or that TTR synthesis would occur in the central nervous system (Weisner and Roethig, 1983). In the eye, the retinal pigment epithelium is the specific site of TTR synthesis (Dwork *et al.*, 1990). Both epitheliums have been considered structurally and functionally homologous, and the ability of both to produce TTR further supported this hypothesis.

Other sites of extra hepatic TTR expression have been demonstrated in several studies and include: pancreatic islets (Jacobson, 1979), pineal gland (Martone *et al.*, 1993), stomach, heart, skeletal muscle and spleen (Soprano *et al.*, 1985).

TTR is expressed very early in embryonic development as shown in several studies performed in humans, where TTR mRNA was detected in hepatocytes and choroid plexus epithelial cells as early as the eighth week, and in pancreatic islets from mid-term pregnancy (Jacobson *et al.*, 1989). In humans, TTR is first expressed in the *tela choroidea*, the precursor of the choroid plexus, followed by expression in the liver (Harms *et al.*, 1991; Richardson *et al.*, 1994). Also, in mice, TTR mRNA was identified from the tenth day of gestation in hepatocytes, *tela choroidea* and visceral yolk sac (Murakami *et al.*, 1987).

TTR synthesis in the brain seems to have occurred first in evolution, being detected in reptiles (Achen *et al.*, 1993), whereas TTR synthesis by the liver only begins in placental mammals, diprodont marsupials and birds (Richardson *et al.*, 1993, 1994; Schreiber *et al.*, 1993). These and other studies could support the hypothesis that the primordial role of TTR is the transport of T_4 in the brain. Targeted TTR null-mice present depressed levels of retinol and T_4, but are otherwise phenotypically normal. According to Palha *et al.* (2000), the absence of TTR does not produce measurable features of hypothyroidism in these animals, including the brain parenchyma. Therefore, in the absence of TTR, compensatory mechanisms for T_4 and retinol metabolism might operate and can potentially be revealed under stressful conditions. Though a similar situation was never described in humans, carriers of different TTR mutations are euthyroid and do not reflect major symptoms associated with retinol deficiency, even when the mutations alter affinity for T_4 and/or RBP.

Several kinetic studies on TTR metabolism and turnover have been carried out in different species. In humans (Vahlquist *et al.*, 1973), the biological half-life determined for TTR was of 2-3 days and the total body turnover (synthetic rate) obtained was of 250-300 $mg/m^2/day$.

Using TTR labelled with tyramine cellobiose and the trapped ligand method, Makover *et al.* (1988) were able to assess the sites of TTR degradation in the rat. The main organ found for TTR catabolism was the liver, responsible for 35-40% of total TTR degradation, followed by muscle (10-15%), skin (11%) and kidney (6%). The mean fractional turnover of plasma TTR was 0.15/h, and that of the total body TTR was 0.04/h. In other to investigate the degradation of choroid plexus-derived TTR, the same authors conducted another study where it was found that the major sites of labelled TTR degradation, injected in CSF or in plasma, were approximately the same. The estimated turnover of CSF TTR was approximately 0.33/h. No specific transfer of plasma TTR to the nervous system or degradation of plasma TTR in the nervous system was observed.

The normal physiology of TTR is not fully characterised and in particular, its cellular uptake is poorly understood. Early studies had been suggestive of a receptor-mediated internalisation of TTR, in both human hepatoma cells (HepG2) (Divino *et al.*, 1990) and in chicken oocytes (Vieira *et al.*, 1995). Because megalin (a multiligand endocytic receptor) has been implicated in the renal re-uptake of plasma proteins carrying lipophilic compounds, Sousa *et al.* (2000) investigated the possibility that this receptor might play a role in renal uptake of TTR. These authors performed binding/uptake assays using immortalised rat yolk sac cells with high expression levels of megalin and radiolabelled TTR, free as well as in complex with T_4 or RBP. They observed that the cells rapidly took up TTR, and this uptake was strongly inhibited by a polyclonal megalin antibody and by the receptor-associated protein (RAP), a chaperone like protein inhibiting ligand binding to megalin. They concluded from this work that TTR is a novel megalin ligand with potential importance in T_4 transepithelial transport reinforcing the concept that megalin is a general endocytic receptor for protein in the proximal tubule.

Recently, Sousa and Saraiva (2001) explored TTR uptake using hepatomas and primary hepatocytes and showed direct evidence for internalisation of TTR by a specific receptor. Given previous evidence that in the kidney, megalin, a member of low-density lipoprotein receptor family (LDLr), internalises TTR and that a fraction of TTR is associated with high-density lipoproteins (HDL) (Sousa *et al.*, 1999), led the authors to investigate whether TTR and lipoproteins could share related degradation pathways. It was observed that RAP, a multifunctional ligand binding all members of the LDLr family antagonising their ligand binding activity, inhibits TTR uptake. This inhibition was observed in different cell lines and in primary hepatocytes, re-enforcing

the fact that the ~90 kDa TTR-receptor complex observed by cross-linking on hepatoma cells was also RAP-sensitive. In the case of cells from liver origin, however, none of the candidate members of the LDLr family were involved in TTR uptake, leading the authors to conclude that TTR uptake is occurring via another yet unidentified RAP-sensitive receptor.

1.5. Molecular variants of TTR

More than 80 TTR variants have been described so far. All of them result from single base substitutions translated into single aminoacid substitutions in the polypeptide chains (the deletion of an aminoacid was only reported in one case). Most of these variants are related to familial amyloidoses. However, a few of these variants occur in the normal, healthy population and do not have any pathogenic consequences.

Most of the mutated TTR variants are associated with the deposition of amyloid fibrils in several tissues, causing predominantly polyneuropathy and/or cardiomyopathy, characteristic of two types of hereditary amyloidoses: Familial Amyloidotic Polyneuropathy (FAP) or Familial Cardiac Polyneuropathy (FAC), respectively. A major question remains unsolved: why is TTR an amyloidogenic protein with clinically heterogeneous pathogenic consequences? TTR has a high content of β-pleated sheet structures, which is characteristic of amyloid, therefore, a potential amyloidogenic molecule. A particular modification in the structure of the protein, introduced by a single aminoacid substitution can lead to the formation of a new critical structural domain crucial for amyloid formation. Distinct amyloidogenic substitutions could share this amyloidogenic conformation and worldwide efforts have been directed towards the establishment of the pathogenic mechanisms underlying amyloidogenesis.

2. Amyloid related disorders

The term amyloid was introduced for the first time in 1854 by Virshow when using iodine to stain cerebral *corpora amylacea* that had an abnormal macroscopic appearance. Upon blue staining, he concluded that the substance was cellulose and named it amyloid. However, in 1859, Friedreich and Kekule demonstrated both the presence of protein in a "mass" of amyloid and the apparent absence of carbohydrate.

Attentions were shifted to the study of amyloid first as a protein and later as a class of proteins, with a propensity to undergo changes in conformation that result in fibril formation (Cohen, 1986).

Amyloidoses are a group of diseases characterised by the deposition of abnormal fibrillar proteins; these proteins assemble forming amyloid fibrils that accumulate in the extracellular space, leading to organ dysfunction. Although presenting a very diverse biochemical composition, all types of amyloid fibrils share a number of common features.

Amyloid deposits show an apple-green birefringence under polarised light, in Congo red stained tissue samples, and present a typical electron microscopic appearance of linear, non-branching fibrils of variable length, that appear to be composed of several parallel filaments, occasionally twisting around each other (Cohen and Calkins, 1959; Shirama and Cohen, 1967). Several protein/peptides have been identified as precursors of these amyloid fibrils, but the mechanisms by which these soluble proteins self-assemble into a fibrillar structure are largely unknown.

2.1. Amyloid fibrils

The unique tissue staining properties of amyloid, such as Thioflavine and its changes in fluorescence emission on binding to amyloid, and particularly Congo red and its red/green birefringence in polarised light, indicated that amyloid was not an amorphous protein deposit, but one that possessed an organised substructure. Amyloid deposits of diverse origins in humans and animals exhibit a similar fibrillar submicroscopic structure: bundles of straight, rigid fibrils ranging in width from 60 to 130 Å (average 75 to 100Å) and variable length (Figure 5).

Another feature of amyloid fibrils is their high insolubility under physiological conditions, suggesting that this characteristic would prevent their complete proteolytic degradation *in vivo*. This resistance to proteolysis, however, is not absolute. Clinical as well as experimental data indicate clearly that amyloid deposits might regress when the precursor pool from which the deposits are drawn is depleted.

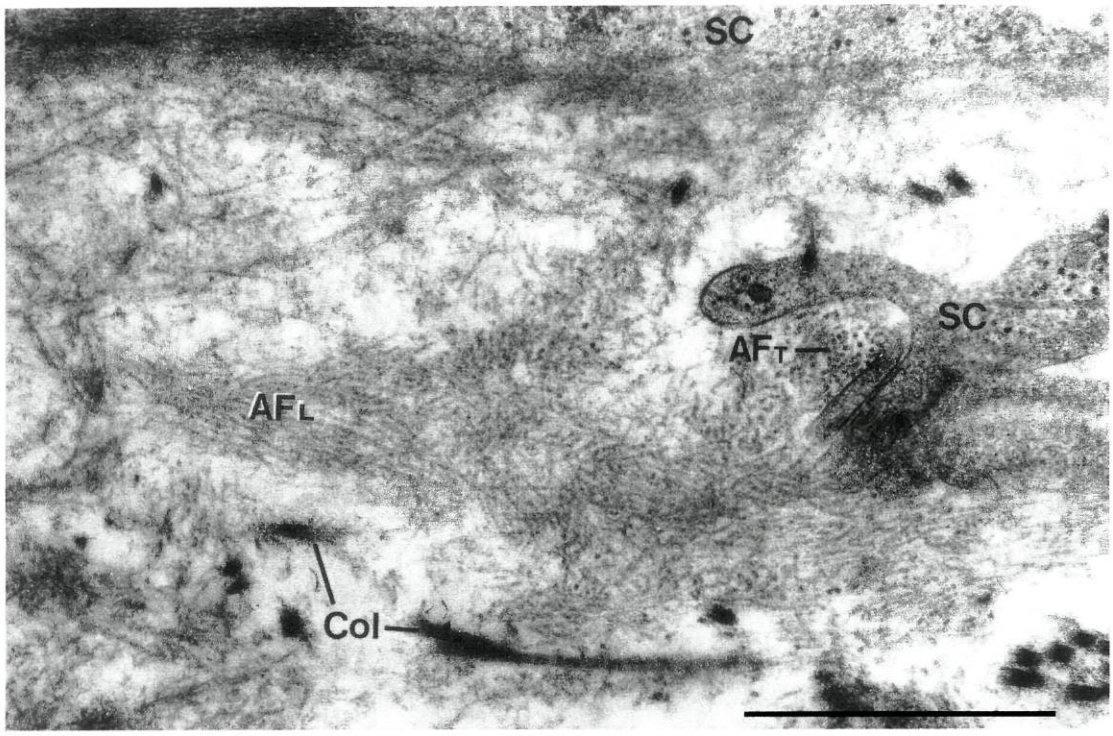


Figure 5. Electron microscopy demonstration of amyloid fibrils in the extracellular space of FAP tissue in the vicinity of a Schwann cell. Amyloid is identified as assemblies of fibrils, which were sectioned either longitudinally (AF_L) or transversely (AF_T). SC, processes of Schwann cells; Col, collagen fibrils. X 41,800. Bar = 1 μm. (Inoue *et al.*, 1998)

The biochemical composition of amyloid fibrils is variable and several proteins have been described as amyloid precursors; these proteins usually exist as normal, soluble, circulating precursors that, by still unknown mechanisms, precipitate and form amyloid fibrils. Using different amyloidogenic peptides or proteins as examples, such as Aβ (Alzheimer's disease), islet amyloid polypeptide (IAPP or amylin) or transthyretin, the *in vitro* assembly of fibrils appears to follow kinetics that are consistent with a probabilistic nucleation process and where the time course of quantitative fibril accumulation is sigmoid in shape (reviewed by Kisilevsky, 2000).

2.2. Amyloid-associated molecules

The process of polymerisation of the main amyloid protein may involve a number of diverse molecules that appear associated with amyloid deposits. These molecules inhibit or enhance the process, as stated in the examples next presented.

Common components present in all types of amyloid fibrils are serum amyloid P component (Coe *et al.*, 1981; Prelli *et al.*, 1985), sulphonated glycosaminoglycans (GAGs) (Snow *et al.*, 1987), apolipoproteins E (Wisniewski and Frangione, 1992) and J (Choi-Miura *et al.*, 1992), membrane basement components such as fibronectin, laminin and collagen type IV (Husby *et al.*, 1994), complement proteins (Husby and Natvig, 1972) and metal ions (Clements *et al.*, 1996).

Serum amyloid P component (SAP) is a decameric plasma glycoprotein that binds in a calcium dependent manner to all types of amyloid fibrils. This molecule is highly resistant to proteolytic degradation in the presence of the ion, and therefore, is thought to contribute to the resistance of the amyloid deposits to enzymatic digestion.

Another usual component of amyloid deposits is included in the group of sulphated glycosaminoglycans (GAGs), notably, heparan sulphate and dermatan sulphate (Snow *et al.*, 1987; Nelson *et al.*, 1991). It is possible that GAGs may be ligands to which SAP binds (Husby *et al.*, 1994). It was also described that heparan sulphate influences the secondary conformation of Aβ, increasing its β-sheet structure considerably and affecting the linear growth and lateral aggregation of Aβ fibrils.

Despite the fact that SAP and heparan sulphate proteoglycans are always present in tissues with amyloid fibrils, amyloid fibrils can be formed *in vitro* in their absence from several natural polypeptides, including insulin, glucagon, calcitonin, immunoglobulin light chains, β2-microglobulin and transthyretin (Sipe, 1994).

Many other substances and molecules have been shown to associate with amyloid deposits and to influence *in vitro* amyloid formation, though some times, contradictions appear in the literature. Several factors will certainly be responsible for the selectivity of amyloid deposition, observed not only in localised amyloidoses, but also in some cases of systemic amyloidoses. Tissue specific enzymes, such as proteases as well as other factors such as receptors and other binding sites may be responsible for different patterns of selective, tissue specific amyloid deposition (Sipe and Cohen, 2000). In particular, the reason why different TTR variants deposit in different organs in TTR-related amyloidoses, is not known, but it can be hypothesised that different variants may interact distinctively with these putative factors.

2.3. Amyloid diseases

Amyloid diseases can be divided into two categories, depending on the distribution of the amyloid deposits (Table I): localised amyloidoses, in which amyloid is confined to one tissue or organ, in the close vicinity of the precursor producer cells, and systemic amyloidoses, in which a more generalised deposition occurs. In the latter, the amyloid proteins are usually derived from the circulating precursors that are either present in excess, abnormal, or both.

Table I: Amyloidoses in humans

Amyloid protein	Precursor	Systemic (S) or Localised (L)	Syndrome and/or Involved Tissues
Aβ	Aβ protein precursor (AβPP)	L	Alzheimer's disease; aging Familial (prototype Dutch)
$A\beta_2M$	β_2-microglobulin	S	Associated with chronic haemodialysis
AA	(Apo) serum AA	S	Secondary, reactive
AANF	Atrial natriuretic factor	L	Isolated atrial amyloid
AApoAI	Apolipoprotein AI	S	Familial
ACalc	(Pro)calcitonin	L	In medullary carcinomas of the thyroid
ACys	Cystatin C	S	Familial (prototype Icelandic)
AFib	Fibrinogen α-chain	S	Familial
AGel	Gelsolin	S	Familial (prototype FInnish)
AH	Immunoglobulin heavy chain	S,L	Primary Myeloma-associated
AIAPP	Islet amyloid peptide	L	Islets of Langerhans Diabetes type II, Insulinomas
AIns	Insulin	L	Iatrogenic
AL	Immunoglobulin light chain	S,L	Primary Myeloma-associated
ALac*	Lactoferrin	L	Cornea
ALys	Lysozyme	S	Familial
APro	Prolactin	L	Aging pituitary Prolactinomas
$APrP^{SC}$	Prion protein	L	Spongiform encephalopathies
ATTR	Transthyretin	S	Familial (prototype Portuguese, Japanese, Swedish); Senile systemic

** Preliminary*

Adapted from Westermark et al (1999)

2.3.1. Localised Amyloidoses

Among the localised amyloidoses, the more common are atrial amyloidosis (AANF), Alzheimer's disease (AD) and islet amyloid polypeptide derived amyloidosis (AIAPP). Not so much spread are the prion protein diseases (APrP) (Prusiner, 1994) and amyloidoses related to calcitonin (Acalc) (Sipe and Cohen, 2000), prolactin (Apro) (Westermark *et al.*, 1997), insulin (Ains) (Hellman *et al.*, 1990) and lactoferrin (Alac) (Klintworth *et al.*, 1997).

AANF is a condition that occurs with some frequency in aged individuals, associated with congestive heart failure and chronic rheumatic disease. In this type of localised amyloidoses, amyloid protein is mainly composed of atrial natriuretic factor (ANF), a 28 aminoacid C-terminal segment of a prepropolypeptide, and deposits are mainly found in the heart, along the sarcolema, in the walls of small vessels (Westermark, 1994).

Alzheimer's disease (AD) is a neurodegenerative disorder causing dementia late in life. The majority of Alzheimer's cases are sporadic, but familiar cases, with an autosomal dominant pattern of inheritance (familial AD-FAD), have also been described (Schellenberg, 1995). Three main features are characteristic of AD (Ghiso *et al.*, 1994): (i) Senile or neuritic plaques, which are spherical lesions formed by a central amyloid core, dystrophic neurites, and activated astrocytes and microglial cells; (ii) Presence of intracellular neurofibrillary tangles in neurons which are composed of paired helical filaments, made of abnormal phosphorilated *tau* proteins; (iii) Cerebrovascular amyloidosis characterised by amyloid deposits in small and medium vessels of the leptomeninges and cerebral cortex. The major component of amyloid fibrils is a peptide, the amyloid β-peptide (Aβ), derived from a large transmembrane glycoprotein precursor, designated as amyloid β protein precursor (AβPP), that presents several forms arising from alternative splicing. The AβPP is a ~ 190 kDa protein, with a large extracellular domain, a membrane spanning region and a short cytoplasmic tail (Kang *et al.*, 1987). The Aβ sequence encompasses parts of both the transmembrane and the extracellular domains, thus requiring proteolytic cleavage of AβPP, at both its N- and C-terminals, to be released. Selkoe *et al.* (1994) proposed several functional roles for AβPP or its soluble derivatives, namely mediating cell adhesion and neuronal attachment, promoting cellular growth and neuronal activity, and some forms might also be involved in the repair process of vascular injury and wound healing.

Islet amyloidosis occurs in the majority of individuals with non-insulin dependent diabetes, in insulinoma endocrine tumours and also in more than 50% individuals over 60 years of age, although, in the latter, affecting just a few islets. The amyloid deposits contain islet amyloid polypeptide, a 37 aminoacid residue long (also known as amylin), synthesised by pancreatic islet β cells as a prepropolypeptide (Westermark, 1992; 1994).

2.3.2. Systemic amyloidoses

Systemic amyloidoses comprise a large group of amyloid diseases with very different amyloid protein precursors and can be grouped in non-hereditary and hereditary amyloidoses.

2.3.2.1. Non-hereditary amyloidoses

In humans, the most frequent forms of non-hereditary systemic amyloidoses are amyloid A associated amyloidosis (AA) and light and heavy immunoglobulin chains associated amyloidosis (AL and AH, respectively).

Amyloid A, secondary or reactive amyloidosis, is a complication of common chronic infections, inflammatory disorders such as familial Mediterranean fever (FMF) - an autosomal recessive disorder. Amyloid deposits are mainly found in the liver, spleen and kidney and the main constituent of the amyloid fibrils is protein AA, a 76-residue N-terminal fragment derived by proteolysis of serum amyloid A (SAA), an acute-phase reactant protein (Husebekk *et al.*, 1985). The physiological function of SAA remains unclear; it is thought to modulate the role of HDL in reverse cholesterol transport and appears to be involved in the recruitment of leukocytes (Sipe, 1994; Xu *et al.*, 1995). AA is also the major fibril protein found in spontaneous and experimentally induced amyloidosis in animals (Sipe, 1994; Husby *et al.*, 1994).

AL and AH are uncommon diseases that occur more frequently in men and show an increasing incidence rate with advancing age. They are generally associated with overproduction of monoclonal light/heavy chains as a result of plasma cell dyscrasias, with systemic deposition of amyloid. The organ distribution is highly

variable, and in some cases localised deposits in the lung and skin are observed (Kyle, 1991).

In AL, the amyloid fibrils consist of either the whole molecule or fragments of monoclonal immunoglobulin light chains (5-23 kDa). The fragments are derived from the N-terminal region and consist of the variable region with or without a portion of the constant region. Among the amyloidogenic light chains, there is a prevalence of the λ over κ class and some λ isotypes also predominate (Solomon *et al.*, 1994a; Osaki *et al.*, 1994). In AH, the amyloid precursor was identified as immunoglobulin heavy chain fragments, corresponding mainly to the variable region (Solomon *et al.*, 1994b).

β_2-Microglobulin (β_2M) amyloidosis is frequent among patients in long-term dialysis. The amyloid fibrils are composed of monomers, dimers and polymeric forms of intact β_2M, but fragmentation has also been described (Gejyo *et al.*, 1985). β_2M is a member of the immunoglobulin super-family, with a high β-sheet content, that is present in the surface of mammalian cells as a component of class I HLA antigens.

2.3.2.2. Hereditary amyloidoses

Hereditary amyloidoses are a group of late onset, autosomal inherited diseases. The most common forms of hereditary amyloidoses are related to six main proteins: TTR, apolipoprotein AI, lysozyme, gelsolin, cystatin C and fibrinogen.

Transthyretin-related amyloidoses are the most prevalent type of hereditary systemic amyloidoses and include familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomyopathy (FAC) and systemic senile amyloidosis (SSA). SSA is not an inherited condition and affects about 25% of the world population over 80 years of age (Cornwell *et al.*, 1988), in which normal (non-mutated) TTR forms amyloid deposits in the heart.

FAP is clinically heterogeneous regarding organ involvement and age of onset. This phenotypic diversity is associated not only with the genetic heterogeneity of the amyloid precursor (TTR, gelsolin or ApoAI), but also with other unknown factors. For these reasons, FAP has been considered in the past divided into four clinical types: FAP type I, also called Portuguese or Andrade type, corresponds, basically, to the clinical picture described by Andrade (1952), and is characterised by a generalised polyneuropathy, beginning distally in the lower limbs and accompanying autonomic

features; FAP type II, or Indiana type, characterised by an upper limb entrapment neuropathy followed by a generalised polyneuropathy; FAP type III or Iowa type, characterised by peripheral neuropathy, peptic ulcers and nephrotic syndrome and FAP type IV of Finnish type, showing cranial neuropathy, followed by a mild generalised polyneuropathy as the major clinical manifestations. This classification is no longer in use, due to the difficulties in characterising the syndrome, the overlapping of symptoms and the phenotypic diversity displayed. The classification now used is based on the protein/variant involved: TTR variants are the main components of amyloid fibrils in FAP types I and II, whereas in type III amyloid deposits are composed of fragments of apolipoprotein AI and in FAP type IV, the amyloid deposits are composed of protein fragments related to gelsolin.

In apoAI amyloidosis, amyloid fibrils are characterised by the deposition of N-terminal fragments of variable length of the mutated protein. Several amyloidogenic variants of apolipoprotein AI (apoAI), the 28 kDa major apolipoprotein component of HDL, have been reported. The majority of the amyloidogenic apoAI variants carry an extra +1 charge with respect to normal apoAI and have their mutation in the N-terminal region. The clinical phenotype of this disease includes peripheral neuropathy, renal and cardiac amyloidosis. Some of the variants described, however, do not produce change in charge from neutral to positive, resulting in a unique clinical presentation of cutaneous amyloid deposition and restrictive cardiomyopathy (Asl *et al.*, 1999).

In gelsolin-related amyloidosis (Agel), amyloid deposition of a gelsolin fragment occurs in the cornea, cranial nerves and various internal organs (Meretoja, 1969). The amyloidogenic fragment is homologous to aminoacid residues 173-243 of gelsolin, with mutations occurring at aminoacid at position 187 (Maury, 1991; De la Chapelle *et al.*, 1992). The mutations result in aminoacid substitutions with a charge change in the gelsolin molecule, postulated to alter the susceptibility for proteases thereby rendering the molecule amyloidogenic. Of the two forms of gelsolin, secretory and cytoplasmic, the secretory plasma form is the likely source of amyloid (Kiuru, 1998). Gelsolin is a calcium-dependent actin-modulating protein that nucleates actin filament growth (Paunio *et al.*, 1997).

Hereditary cerebral haemorrhage with amyloidosis, Icelandic type (HCHWA-Icelandic), is an autosomal dominant disease characterised by the deposition of amyloid in arterial walls, with particular incidence in the cerebral cortex and

leptomeninges. The protein depositing as amyloid corresponds to cystatin C, without ten amino terminal residues, and with a glutamine for leucine substitution at position 68 (Ghiso *et al.*, 1986).

Hereditary non-neuropathic systemic amyloidosis was associated with lysozyme variants by Pepys *et al.* (1993). This is a rare autosomal dominant disease in which amyloid deposition in the viscera is usually fatal by the fifth decade. The mutations occur in highly conserved aminoacid residues, namely Thr56Ile and His67Asp. Lysozyme is a ubiquitous bacterolytic enzyme present in external secretions, polymorphs and macrophages, but its physiological role is not always clear.

In a form of hereditary renal amyloidosis, the amyloid precursor protein was identified as a fibrinogen Aα chain variant (Uemichi *et al.*, 1994). This form of amyloidosis is an autosomal dominant condition characterised by proteinuria, hypertension, and subsequent azotemia in the fifth to seventh decade of life and occurred for the first time in two American kindreds of Irish descent. Later on other mutations were reported in Peruvian/Mexican and African-American kindreds, and recently another mutation was found in Europe in a family of French descent (Hamidi *et al.*, 1998).

3. Familial amyloidotic polyneuropathy (FAP)

FAP was first described by Andrade (1952), as a peculiar type of peripheral neuropathy, with an autosomal mode of inheritance that affected families living in the northern area of Portugal. Subsequently, many more patients and families were ascertained in other parts of the country (Ribeiro do Rosario *et al.*, 1961).

The disease was characterised by systemic deposition of amyloid, predominantly in the peripheral nervous system, with onset between 20 and 35 years of age and progression to death within 10 to 15 years. The major typical clinical features were: early impairment of temperature and pain sensation in the feet and autonomic dysfunction leading to varying degrees of systemic organ involvement. Extensive amyloid deposition was observed in the meninges, spinal and autonomic ganglia. Amyloid deposits were also found throughout the connective tissue and around blood vessels. No amyloid deposition was detected in the brain and very little, if any at all, in the liver (Guedes, 1976).

In 1978, Costa and colleagues reported that the amyloid fibrils in Portuguese FAP patients were constituted by a protein immunochemically related to human TTR. It was then suggested that in FAP, a genetic mutation might have occurred, leading to the production of abnormal TTR, which is abnormally degraded, bound and/or precipitated in the connective tissues as amyloid fibrils. The hypothesised genetic mutation was described some years later, in protein isolated from FAP patients, as a substitution of a methionine for valine at position 30 (Val30Met), by Saraiva *et al.* (1984). This substitution was shown to be a biochemical marker for FAP (Saraiva *et al.*, 1985) and results from a point mutation in the exon 2 of the *ttr* gene, an A for a G nucleotide change (Sasaki *et al.*, 1984).

Although several worldwide *foci* of FAP have been found, Portugal is still the largest *focus* of the disease, with more than 500 kindreds identified to date; the gene carrier frequency has been estimated as 1 in 625 (Alves *et al.*, 1997a). The second largest known Val30Met *focus* is Northern Sweden, where more than 350 families have been diagnosed (Holmgren *et al.*, 1994). Other relevant *foci* include Japan and the Island of Majorca (Ikegawa, 1991; Munar-Qués *et al.*, 1997). A few cases of homozygosity for the Met30 gene occur but do not lead to a more severe form of the disease (Holmgren *et al.*, 1988).

The reason why different TTR variants deposit in different organs is still unknown, but it is possible that different mutants interact differently with other factors involved in the pathology of this disorder. The knowledge of the molecular mechanisms

involved in this disease greatly increased in the last decade, though the role of aminoacid substitutions and other modifications on TTR amyloid formation process, as well as the phenotypic diversity associated with this disease, remain unknown.

3.1. Phenotypic diversity in FAP

FAP, Portuguese type, was thought for a long time to have a fairly uniform phenotype in Portuguese patients, with onset exclusively or predominantly in the third decade, but soon it was recognised that some heterogeneity existed, not only regarding age of onset, but also in clinical manifestations. A study performed in a considerable number of Portuguese families (Coutinho *et al.*, 1980) showed that the mode of presentation of FAP in the population was not uniform. Also, this and other studies (Sousa *et al.*, 1995), showed a later onset of the disease in women than in men; it was also reported (Sousa *et al.*, 1990) that the mean age of onset was higher for children of transmitting fathers than of transmitting mothers, resulting in earlier onset for sons of affected mothers, whereas daughters of affected fathers seemed to be the most protected.

Age of onset is perhaps the most variable factor in FAP. In Portugal, not long after the first description of FAP, some authors reported some cases with onset as late as 60 years of age (Ribeiro do Rosário *et al.*, 1961; Antunes *et al.*, 1963; Becher *et al.*, 1964). In TTR Val30Met-related FAP, mean age of onset can vary from 33.5 and 34.2 years for the Portuguese and Japanese populations, respectively (Sousa *et al.*, 1995; Ikegawa *et al.*, 1991), to 49 years in Majorca (Munar-Qués and Saraiva, 1990) and 56.7 years in the Swedish population (Sousa *et al.*, 1995). The reasons for this variability in age of onset are largely unknown and several hypotheses have been raised to explain late-onset in FAP.

One of the hypotheses, stating that late-onset patients could be carriers of another mutation was soon ruled out, since TTR Val30Met was present in late-onset kindreds, in patients showing the extremes of age of onset (Sequeiros and Saraiva, 1987) and even in some old non-manifesting carriers (Sequeiros and Saraiva, 1987; Lobato *et al.*, 1988; Sousa *et al.*, 1988; Coelho *et al.*, 1994). It was also proposed that there could be a difference in the regulatory sequences in the TTR gene. The presence of putative regulatory sequences, affecting the rate of TTR synthesis, could significantly decrease the production of the abnormal protein to low levels, while maintaining adequate synthesis of the normal TTR allele. However, studies in a family

with late-onset, showed that mutant TTR circulates at young ages in the offspring of these FAP patients (Saraiva *et al.*, 1996).

The hypothesis of gene dosage as another explanation for late-onset, was also excluded, since individuals homozygous for the TTR Val30Met allele do not differ, either clinically or in the age of onset, from the heterozygous individuals (Holmgren *et al.*, 1988). The association between a later onset, a different geographical origin of the cases and the incomplete penetrance of the gene, suggests the occurrence of an interaction between genetic and environmental factors in the expression of the FAP gene.

Differences in clinical presentation and severity of symptoms among Portuguese FAP Val30Met patients are, however, rare. Only a few cases have been reported with a more benign clinical course. DNA analysis of these individuals indicated the presence of a second mutation in the *ttr* gene that originates a substitution of a threonine for a methionine at position 119 (Coelho *et al.*, 1993).

Since the identification of the Val30Met variant, several other aminoacid substitutions were identified in the TTR molecule. Aminoacid substitutions that are not associated with amyloidosis have also been described and since some of them occur with high frequency, which might explain the existence of compound heterozygotes. One of the most interesting compound heterozygotes is TTR Val30Met/Thr119Met that has been described to have a more benign clinical course (to be discussed further in the next section).

3.1.1. Amyloidogenic variants

Amyloidogenic variants are associated with different clinical phenotypes namely neuropathy, cardiomyopathy, vitreous opacities and carpal tunnel syndrome, reflecting selective differential amyloid deposition (Table II). A few TTR mutations are related to cardiomyopathy without neurological symptoms.

Among the amyloidogenic TTR variants we can distinguish TTR Val30Met as the most frequent and widespread variant, and TTR Leu55Pro as the variant associated with the most aggressive form of TTR-related amyloidosis. TTR Leu55Pro is characterised by an early age of onset, between 15 to 20 years, and a rapid progression to death within less than 10 years (Jacobson *et al.*, 1992; Yamamoto *et*

al., 1994). In addition to neuropathy, Leu55Pro carriers have cardiomyopathy and vitreous opacities.

Table I: Amyloidogenic TTR mutations

Mutation	Clinical features	Reference
Cys10Arg	PN,AN,E	Uemichi *et al.* (1992)
Leu12Pro	LM,PN,AN	Brett *et al.* (1999)
Asp18Glu	PN,AN	Booth *et al.* (1996)
Asp18Gly	LM	Vidal *et al.* (1996)
Val20Ile	H	Jenne *et al.* (1996)
Ser23Asn	H	Connors *et al.* (1999)
Pro24Ser	H,CTS,PN	Uemichi *et al.* (1995)
Val28Met	PN,AN	Carvalho *et al.* (2000)
Val30Met	PN,AN,E	Several
Val30Ala	H,AN	Jones *et al.* (1992)
Val30Leu	PN,AN	Nakazato *et al.* (1992)
Val30Gly	LM,E	Petersen *et al.* (1997)
Phe33Ile	PN,E	Nakazato *et al.* (1984)
Phe33Leu	PN,AN	Ii *et al.* (1991)
Phe33Val	PN,AN	Booth *et al.* (1996)
Arg34Thr	PN,H	Patrosso *et al.* (1998)
Lys35Asn	PN,AN,H	Reilly *et al.* (1995)
Ala36Pro	PN,E	Jones *et al.* (1991)
Asp38Ala	PN,H	Kishikawa *et al.* (1999)
Glu42Gly	PN,AN	Ueno *et al.* (1990a)
Glu42Asp	H	Dupuy *et al.* (1998)
Phe44Ser	PN,AN,H	Klein *et al.* (1998)
Ala45Asp	H	Jacobson *et al.* (1993)
Ala45Ser	H	Janunger *et al.* (2000)
Ala45Thr	H	Saraiva *et al.* (1992)
Gly47Arg	PN,AN	Murakami *et al.* (1992)
Gly47Ala	H,PN,AN	Ferlini *et al.* (1994)
Gly47Val	PN,AN,H	Booth *et al.* (1993)
Gly47Glu	PN	Altland (1999)
Thr49Ala	H,PN	Almeida *et al.* (1992)
Thr49Ile	PN,H	Nakamura *et al.* (1999)
Ser50Arg	PN,AN	Ueno *et al.* (1990a)
Ser50Ile	H,PN,AN	Saeki *et al.* (1992)
Glu51Gly	H	Jacobson *et al.* (1999)
Ser52Pro	PN,AN.H	Booth *et al.* (1993)
Gly53Glu	LM,H	Camou *et al.* (1999)
Glu54Gly	PN,AN	Reilly *et al.* (1995)
Glu54Lys	PN,AN,H	Togashi *et al.* (1999)
Leu55Arg	LM,PN	Altland (1999)
Leu55Pro	PN,H,AN	Jacobson *et al.* (1992)
His56Arg	H	Jacobson *et al.* (1992)
Leu58His	CTS,H	Nichols *et al.* (1989)
Leu58Arg	CTS,AN,E	Saeki *et al.* (1991)
Thr59Lys	H,PN	Booth *et al.* (1995)
Thr60Ala	H,CTS	Wallace *et al.* (1986)
Glu61Lys	PN	Shiomi *et al.* (1993)
Phe64Leu	PN,CTS,H	Ii *et al.* (1991)
Phe64Ser	LM,PN,E	Uemichi *et al.* (1999)
Ile68Leu	H	Almeida *et al.* (1991a)
Tyr69His	E	Zeldenrust *et al.* (1994)

Table I: Amyloidogenic TTR mutations (continued)

Mutation	Clinical features	Reference
Lys70Asn	CTS,PN,E	Izumoto *et al.* (1992)
Val71Ala	PN,E	Almeida *et al.* (1993)
Ile73Val	PN,AN	Booth *et al.* (1998)
Ser77Phe	PN	Planté-Bordeneuve *et al.*(1998)
Ser77Tyr	PN	Wallace *et al.* (1998)
Tyr78Phe	CTS,H	Anesi *et al.* (2001)
Ile84Ser	H,CTS,E	Dwulet and Benson. (1986)
Ile84Asn	E,H	Skinner *et al.* (1992)
Ile84Thr	H,PN,AN	Stangou *et al.* (1998)
Glu89Gln	PN,H	Almeida *et al.* (1992)
Glu89Lys	PN,H	Nakamura *et al.* (2000)
Ala91Ser	PN,CTS,H	Misrahi *et al.* (1998)
Ala97Gly	H,PN	Yasuda *et al.* (1994)
Ala97Ser	PN,H	Lachmann *et al.* (2000)
Ile107Val	H,CTS,PN	Jacobson (1994)
Ile107Met	PN,H	Altland (1999)
Ala109Ser	PN	Date *et al.* (1997)
Leu111Met	H	Nordlie *et al.* (1988)
Ser112Ile	PN,H	De Lucia *et al.* (1993)
Tyr114Cys	PN,AN,E	Ueno *et al.* (1990b)
Tyr114His	CTS	Murakami *et al.* (1994)
Tyr116Ser	PN,CTS	Misrahi *et al.* (1998)
Ala120Ser	H,PN,AN	Gillmore *et al.* (1999)
Val122Ile	H	Saraiva *et al.* (1990)
Val122del	H,PN,CTS	Uemichi *et al.* (1995); Munar Qués *et al.* (2000)
Val122Ala	H,E,PN	Theberge *et al.* (1999)

AN - autonomic neuropathy; **CTS** - carpal tunnel syndrome; **E** - vitreous deposition; **H** - cardiomyopathy; **LM** - leptomeningeal amyloid; **PN** - perypheral neuropathy.

A smaller group of variants have been found associated with Familial Amyloidotic Cardiomyopathy (FAC). The disease is characterised by the deposition of amyloid fibrils predominantly in the heart, in most cases without significant neuropathic involvement, and a late age of onset. TTR Leu111Met was the first TTR variant reported associated with FAC, in which no neuropathic involvement occurred (Nordlie *et al.*, 1988). TTR Val122Ile is the most frequent of the cardiopathic TTR variants and was described in the black population with a frequency of 2% carriers, thus being considered a polymorphism in this population (Jacobson, 1992). After the sixth decade, isolated cardiac amyloidosis is four times more common among blacks than whites in the United States of America, and 3.9% of blacks are heterozygous for Val122Ile mutation. A few cases of homozygosity for this mutant have been found (Jacobson *et al*, 1997). Due to the late onset presented by TTR Ile^{122} carriers, this variant was originally thought to be related to senile cardiac amyloidosis (SSA), being found later that, in this latter condition, no TTR variant is involved and rather, it is normal TTR that deposits and forms amyloid.

Other variants, exhibit as major clinical manifestations, vitreous opacities, carpal tunnel syndrome and others. Only recently, two TTR variants (TTR Asp18Gly and TTR Val30Gly) were associated with dysfunction of the central nervous system.

Most of TTR variants have been reported in only a few families, while others (like TTR Val30Met) affect a large number of families in several countries.

3.1.2. Non-amyloidogenic variants

Several TTR mutations without pathogenic consequences have also been described (Table III). The allele frequency has been estimated in screening studies in different populations: Gly6Ser is present in about 12% of the Caucasian population and the Thr119Met mutation is found in approximately 0.8% of Portuguese and German populations investigated (Saraiva, 2001).

Table III: Non-amyloidogenic TTR mutations

Mutation	Reference
Gly6Ser	Jacobson *et al.* (1995)
Met13Ile	Altland (1999)
Asp74His	Uemichi *et al.* (1994)
His90Asn	Saraiva *et al.* (1991)
Gly101Ser	Kishikawa *et al.* (1998)
Pro102Arg	Almeida *et al.* (1991)
Arg104Cys	Saraiva *et al.* (1999)
Arg104His	Terazaki *et al.* (1999)
Ala108Ala	Palha *et al.* (1997)
Ala109Thr	Alves *et al.* (1997a)
Ala109Val	Izumoto *et al.* (1993)
Thr119Met	Alves *et al.* (1997a)
Pro125Ser	Ferlini *et al.* (1996)

Adapted from Saraiva (2001)

Also, several compound heterozygous individuals are found in the Portuguese population, carriers of two TTR variants (Alves *et al.*, 1996). Compound heterozigosity of non-amyloid and amyloid mutations usually occurs in different alleles. In some cases the presence of an "extra" non-amyloidogenic mutation affects the phenotype usually exhibited in patients carrying an amyloidogenic mutation and a normal copy of the TTR gene.

The presence of TTR Thr119Met seems to exert a "protective effect" on individuals also carriers of TTR Val30Met. To elucidate the mechanism by which, in

TTR Val30Met carriers, the presence of TTR Thr119Met might protect against the development of FAP, both biochemical and metabolic approaches were followed. Thyroxine binding studies showed a higher binding capacity in the TTR Thr119Met carriers (Curtis *et al.*, 1994), that could be attributed to the higher plasma TTR levels presented by these individuals. X-ray crystallographic studies of recombinant TTR Thr119Met variant, revealed structural alterations, mainly at the level of residue Leu^{110}, allowing a closer contact between the hormone and the protein, thus explaining the increase in T_4 binding (Almeida *et al.*, 1997). Also, *in vivo* comparative catabolism studies revealed a slower plasma clearance of TTR Thr119Met, in contrast to a faster clearance of homotetrameric TTR Val30Met, and a normal behaviour of the heterotetrameric TTR Val30Met/Thr119Met (Alves *et al.*, 1997b). The reasons for the decreased catabolism of TTR Thr119Met are not known; it seems likely that the Met^{119} residue affects TTR stability or its interaction with cellular sites, possibly receptors that might be involved in its disposal. Also, higher TTR levels were found in carriers of TTR Thr119Met mutation, which taken together with another observation concerning decreased TTR plasma levels in FAP Val30Met patients (Saraiva *et al.*, 1983), led to the hypothesis that at least in part, a different clearance could account for the differences in circulating plasma levels observed for each of the mutations. Furthermore, comparative studies of TTR stability of Met^{119} containing species towards dissociation by urea, suggested a higher resistance of the TTR tetrameric structure by the presence of the Met^{119} monomers. This is obvious in heterozygous individuals and on compound heterozygous also carriers of TTR Val30Met, indicating that the apparent protective effect of TTR Thr119Met on the TTR Val30Met carriers is associated with a stabilisation of the TTR tetrameric structure.

A more recent non-pathogenic mutation, TTR Arg104His, has been described in heterozygous and compound heterozygous individuals from a Japanese family with FAP (Terazaki *et al.*, 1999). Carriers of Val30Met and Arg104His mutations present a very mild form of FAP with slow progression of the disease. The His^{104} mutation has the same stabilising effect on tetrameric TTR as the previously described Met^{119} substitution, showing an increased resistance to dissociation into monomers. Concerning T_4 binding, TTR Arg104His presents a binding affinity lower than that of Thr119Met, but still higher than normal TTR. However, TTR from the compound heterozygotic carrier of TTR Val30Met/Arg104His presented a T_4 binding affinity lower than normal (Almeida *et al.*, 2000). These results indicated that the His^{104} substitution induces structural alterations that increase the stability of the tetramer in compound heterozygotes for TTR Val30Met despite a lower affinity for T_4 binding. This indicates

that the structural alterations induced by both mutations are different, as would be expected by their different localisation in the molecule. Contrary to residue 119, aminoacid 104 is not located in the T_4 binding channel and may not interact directly with T_4. TTR and RBP levels were also measured and found to be increased in these patients serum.

The polymorphic Gly6Ser mutation has been described in compound heterozygotes in association with different amyloid mutants (Val30Met; Phe33Ile; Ala45Asp; Ser77Tyr; Tyr114Cys; Thr119Met and Val122Ala). The presence of TTR Ser6 does not seem to influence the onset and development of FAP, neither in the Portuguese nor in the Swedish populations, in particular for Met^{30} carriers (Alves *et al.*, 1996). The neutral behaviour of this mutation contrasts with the protective effect of Thr119Met and Arg104His described above.

3.2. Models of amyloidogenesis in FAP

The mechanisms of amyloid fibril formation are still largely unknown, but it is clear that several factors interact in this process. The morphological similarity presented by amyloid fibrils is in contrast with the structural diversity of their protein precursors, presenting little or none primary sequence homology; it has, thus, been suggested that some common molecular intermediate amyloidogenic species is formed. Two types of factors can be considered to take place in the amyloid formation process: the amyloidogenic potential of the protein and the presence of tissue/circulating factors that can also lead to fibril formation and/or deposition.

Most types of fibrils have been produced *in vitro* from their normal precursors, suggesting that the amyloidogenic potential of the proteins is the most important amyloidogenic determinant. However, the presence of external factors is necessary to explain aspects as the selective deposition of amyloid in several organs and differences in age of onset, among others. Tough wild type proteins are able to form fibrils *in vivo*, this amyloid formation process seems to be accelerated by the presence of mutations in the precursor molecules and/or by other external factors.

Two main hypotheses have been raised to explain amyloid formation in TTR related amyloidosis: the conformational hypothesis and the proteolytic hypothesis.

3.2.1. Conformational hypothesis

This hypothesis is based on the formation/exposure of new structural domains in the TTR molecule, due to aminoacid substitutions and other factors, originating a molecule with an increased amyloidogenic potential and therefore with an increased tendency to self-assembly into fibrils (Saraiva and Costa, 1991; McCutchen *et al.*, 1993).

The structure of the protein is an important determinant in the amyloid formation process. Most proteins, which are precursors of amyloid fibrils, are rich in β-sheet secondary structure, as is the case of TTR (Blake *et al.*, 1978b). This is not, however, true for all amyloid precursors: the native form of lysozyme is predominantly α-helix, with a small amount of β-structure (Artymiuk and Blake, 1981) and the predicted structure for apoAI is also mainly α-helix (Segrest *et al.*, 1994). The occurrence of a conformational modification is thus thought to be necessary for amyloid formation.

Although they do not represent a prerequisite for fibril formation, several point mutations in genes encoding amyloid precursor proteins have been detected in different types of familial amyloidoses. These changes in the primary structure of the protein may increase its inherent fibrillogenicity accelerating the process of fibril formation, which in the absence of these substitutions, occurs at much slower rates. Supporting this hypothesis is the fact that SSA, a condition in which amyloid is formed by non-mutated TTR, appears only in elderly people, whereas patients with FAP or FAC present an earlier, although very variable, age of onset.

The mechanisms by which aminoacid substitutions alter the intrinsic amyloidogenic potential of TTR are not known and the comparative study of amyloidogenic and non-amyloidogenic TTR variants, both at structural and functional levels, is an attempt to identify conformational changes rendering the molecule vulnerable to aggregation.

3.2.1.1. Mutations and Conformation

The effects introduced by amyloidogenic and non-amyloidogenic mutations have been the subject of intensive analyses by X-ray crystallography, but with the exception of Leu55Pro mutation their structures did not reveal drastic changes, as summarised next.

X-ray crystallographic analyses of serum and recombinant homotetrameric TTR Val30Met (Terry *et al.*, 1993), showed that the effect of the substitution at position 30 was transmitted through the protein core to Cys^{10} (the only cysteine in the molecule), that becomes slightly more exposed than in the native molecule.

Regarding TTR Ile122Val, the largest deviations from the normal structure occur in surface loops in the region of the substitution (Damas *et al.*, 1996). Small changes were observed in the region associated with the intra and inter-dimer interactions, corresponding to a weaker bonding.

Hamilton and co-workers (1996) determined the X-ray crystal structure of another TTR variant: Ile84Ser. The substitution at position 84 resulted in changes in shape of the putative site for complex formation with RBP, thus explaining the low RBP levels shown in individuals carriers of this mutation (Benson *et al.*, 1993); however, once more, compared to the WT structure, the crystal packing exhibited very subtle modifications.

So far, the solved structures did not show dramatic changes in the tetrameric structure of the protein. The only abnormal crystal packing was observed for the TTR Leu55Pro mutant. Studies by Sebastião *et al.* (1998) showed significant changes, resulting in the presence of eight TTR monomers forming the building blocks of an asymmetric repeating unit, consistent with data from synchrotron analyses of *ex vivo* fibrils (Inoue *et al.*, 1998) where the dimensions of the TTR filament suggest that its basic unit is a modified monomer. A displacement of the D strand was observed and as a result, the interactions between strands D and A were weakened. Due to an elongation of the hydrogen bonds between the two dimers, a less stable tetrameric structure arises, showing a disrupted D strand which becomes part of a long loop connecting strands C and E (Figure 6).

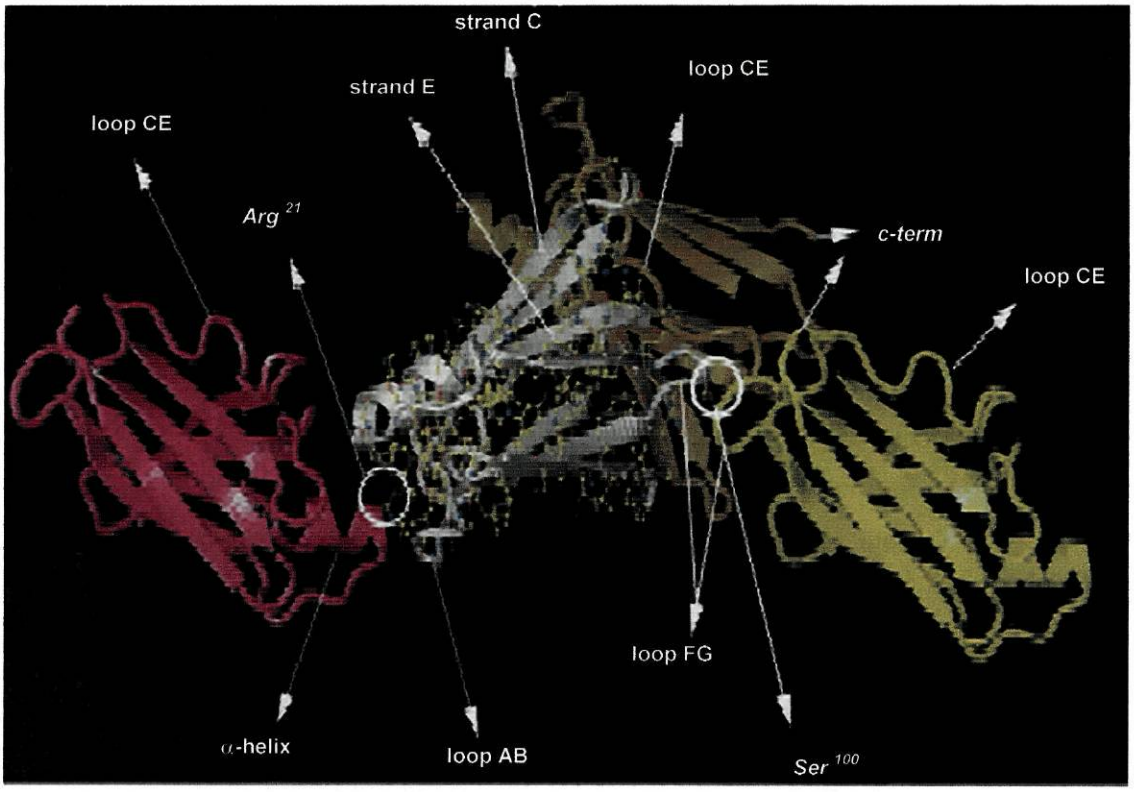


Figure 6. Leu55Pro TTR monomer-monomer interactions: loop FG ----- loop FG, and loop AB ---- α-helix. (Sebastião *et al.*, 1998).

These results helped explaining the higher degree of amyloidogenicity displayed by this variant and this asymmetric unit observed in the crystal, showing several channels running parallel to each other, suggest a tubular structure that might be similar to the TTR amyloid fibril structure. Therefore, this crystal structure may resemble the amyloidogenic intermediate in the biochemical pathway that leads to amyloid fibril formation. The disruption of D strand affects the hydrogen bonding with the A strand, exposing new surfaces involved in aggregation. In particular, the contacts between the α-helix and the AB loop are different, suggesting that these regions are important in amyloidogenesis. It seems likely that soluble TTR Leu55Pro exists in an amyloidogenic conformation, with an increased tendency for self-association into amyloid fibrils, even at conditions where the wild-type protein remains stable.

Serpell *et al.* (1996) had already reported that the amyloidogenic mutations had an elevated occurrence in the D strand and this domain was therefore predicted to be "a hot-spot" area for amyloidosis. In fact, engineered deletions or multiple substitutions in the D strand led to highly amyloidogenic mutants; furthermore, when monoclonal antibodies (Mabs) were raised against these highly aggressive TTR forms, two Mabs

did not recognise native TTR but only TTR fibrils (Goldsteins *et al.*, 1999). Two cryptic epitopes were thus mapped to a domain of TTR, where most mutations associated with amyloidosis occur and which the authors proposed to be displaced at the initial phase of amyloid formation, opening up new surfaces necessary for auto-aggregation of TTR monomers. These results provided direct biochemical evidence for structural changes in a putative amyloidogenic intermediate of TTR.

3.2.1.2. Monomeric Intermediates

Colon and Kelly (1992) were the first to propose the existence of an intermediate monomeric structure in the amyloid formation pathway. Based on the *in vitro* production of amyloid fibrils from native TTR by acid mediated denaturation, they suggested that amyloid formation would occur during the normal denaturation or folding pathways of the TTR molecule, by self-assembly of a denaturing/refolding intermediate. They proposed a model where partial acid denaturation of native TTR tetramer was achieved in the lysosomal environment, producing a structurally rearranged amyloidogenic monomer. Subsequent studies (Lai *et al.*, 1996) suggested that at physiological concentrations, WT tetrameric TTR remained associated from pH 7 to pH 5 and was unable of amyloid fibril formation. Tetrameric TTR would dissociate to a monomer, in a process dependent on both pH and protein concentration, below pH 5. The extent of amyloid formation would thus correlate with the concentration of the TTR monomer, having an altered, but defined, tertiary structure over the pH range of 5.0 to 3.9. This structurally defined monomeric intermediate begins to adopt alternative non-amyloidogenic conformations around pH 4.0, ultimately forming an A-state conformation below pH 3.0 (Figure 7).

Biophysical studies, as well as pH dependent proteolysis sensitivity data, suggested that the region comprised by [C strand-CD loop-D strand], undergoes a rearrangement between pH 5.1 and 4.0, leading to a monomeric intermediate structure having strands A, B, F and H exposed. Since the C and D strands interact weakly with the β-sheet core of TTR, mild denaturing conditions could convert the [C strand-CD loop-D strand] region into a large unstructured domain, therefore exposing the A and B strands.

This amyloidogenic intermediate was thus postulated to contain most of the native structure except for the rearrangement involving strands C and D. In this model, FAP amyloidogenic mutations would not affect the structure of the folded state

(tetramer) but would favour the denaturation pathway and/or degradation pathways for TTR turnover, by either destabilising the tetramer and/or making the intermediate form more accessible under milder acidic conditions.

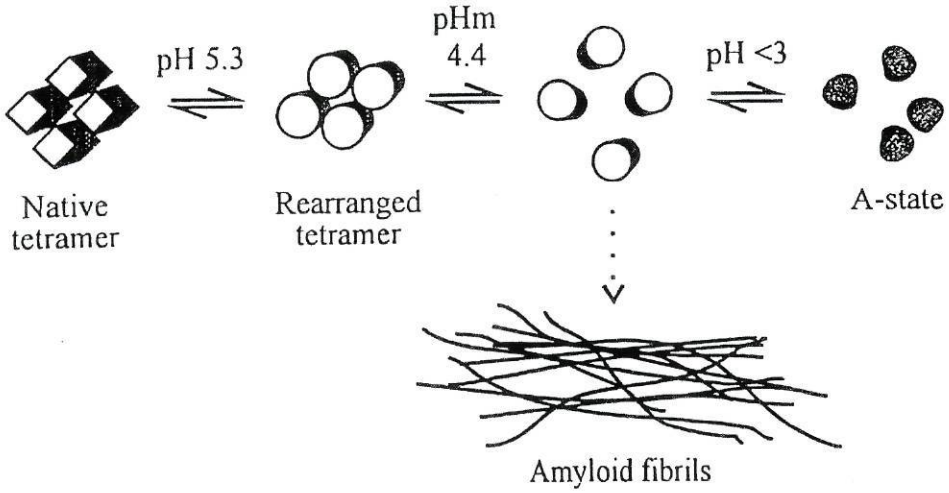


Figure 7. Acid mediated denaturation pathway of native TTR with possible fibril formation.
The partial acid denaturation of the native TTR tetramer, achieved in the lysosomal environment, produces a structurally rearranged amyloidogenic monomer, retaining most of its native secondary and some native tertiary structures, that polymerises into amyloid fibrils. Further denaturation (by lowering pH) yields a monomer in the so called "A-state", with no native tertiary structure and incapable of self-associating into fibrils. PHm – midpoint of tetramer to monomer equilibrium.
(Adapted from Lai *et al.*, 1996)

It has been shown that the amyloidogenic potential of the TTR variants is related with decreased stability of the TTR tetramer, culminating in dissociation to monomeric species as demonstrated for the Val30Met and Thr119Met TTR variants (Alves *et al.*, 1997; Quintas *et al.*, 1997). The latter study shows that all WT, Val30Met, Leu55Pro and Thr119Met tetramers dissociate to monomers upon dilution at pH 7.0 and nearly physiological ionic strengths. The amyloidogenic proteins show a complex equilibrium between monomers, tetramers and high molecular weight soluble aggregated species and it was suggested that these soluble aggregates, at a later stage, would give rise to insoluble fibrils. Substitutions like Arg104His and Thr119Met, may contribute to the increase of stability of the TTR tetramer or monomer inhibiting the formation of a non-native monomeric amyloidogenic intermediate.

More recently, Quintas *et al.* (1999) proposed that the monomers resulting from the dissociation are non-native monomers that begin the process of amyloid formation and cannot re-associate into tetramers (Figure 8). Their model for TTR fibrillogenesis is based on tetramer dissociation naturally occurring under commonly observed physiological solutions conditions, not involving the lysosome and compatible with the environment of the interstitial milieu.

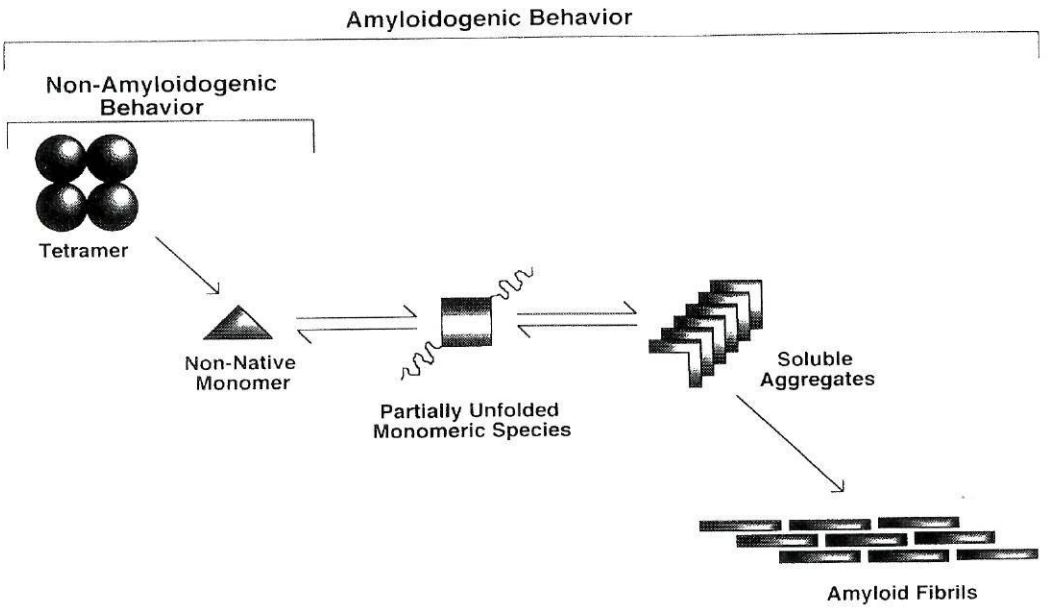


Figure 8. Model for amyloid fibril formation by TTR at commonly encountered physiological conditions.

At 37^{0}C and pH 7, tetrameric TTR may dissociate to a non-native monomer, which in turn depending on its conformational stability, may undergo partial unfolding that leads to aggregate formation and eventually amyloid fibril assembly. Both amyloidogenic and non-amyloidogenic TTR variants dissociate to a non-native monomer. However, only the amyloidogenic variants produce large amounts of partially unfolded monomeric species as a consequence of the marginal conformational stability of the non-native monomer.

(Quintas *et al.*, 2001)

3.2.1.3. Oligomeric intermediates

Several authors have questioned the monomer as the building block of amyloid fibrils and oligomeric intermediates such as the tetramer or the dimer have been suggested.

TTR tetramers were proposed to assemble into amyloid fibrils by inter-molecular disulphide bridges, based on two observations: that in the crystal structure of TTR Val30Met, Cys^{10} was slightly more exposed than in native TTR, and on the existence of disulfide bridges in some amyloid fibrils (Felding *et al.*, 1985; Thylen *et al.*, 1993; Terry *et al.*, 1993). However, the existence of an amyloidogenic variant lacking this Cys residue, TTR Cys10Arg (Uemichi *et al.*, 1992) and the *in vitro* production of amyloid-like fibrils from a recombinant TTR where a Cys^{10} was replaced by an Ala (McCutchen and Kelly, 1993), could not be explained by this model.

The possibility that TTR amyloid fibrils could be built from dimeric/tetrameric building blocks was recognised by Blake and Serpell (1996) based on the interpretation of the high resolution X-ray diffraction analysis of fibrils. The semblance of continuous intermolecular β-sheets, in a helical form, suggested that some aspects of the natural dimeric association could be retained in the fibrillar form. In this model, the core of the fibrils has a helical arrangement and is composed of units with 24 β-strands and a path of 115.5 Å along the direction of the fibril axis; the strands in each β-sheet interact through hydrogen bonding and these sheets are stacked in a parallel fashion. This structure was considered to be independent of the precursor protein and therefore is shared by all amyloid fibrils (Sunde *et al.*, 1997). Inouye *et al.* (1998), based on the same X-ray diffraction analysis data, envisaged a different interpretation, stating that it was very unlikely that TTR amyloid fibrils derived from tetrameric protein subunits, since it was impossible to align native tetramers into the established sized anti-parallel β-pleated sheet model. They proposed a different model, based on building subunits with a tertiary structure close to the conformation of the TTR monomer, with reorganised or truncated β-sheets. These two different interpretations indicated that more accurate data are necessary for a complete description of the arrangement of the amyloid fibrils.

Recently, Eneqvist and Sauer-Eriksson (2001) have also suggested that a tetramer might be the building block of TTR amyloid fibrils, based on molecular packings observed in the crystal structure of a highly amyloidogenic variant of TTR, previously produced and characterised by Goldsteins *et al.* (1997). The structure of this designed, highly amyloidogenic triple D-strand mutant

(TTRGly53Ser/Glu54Asp/Leu55Ser) revealed a conformational change of the CD-loop, D-strand and DE-loop, denoted as the β-slip (Eneqvist *et al.*, 2000). This creates new protein-protein interactions at a potential amyloid packing site, which led these authors to propose a new model for amyloid formation based on distorted but intact tetramers comprising the basic building blocks for TTR amyloid fibrils. New packing interactions include residues from the BC-loop, DE-loop, FG-loop and part of the RBP-binding site and therefore weaker RBP-binding would increase the amount of TTR tetramers that are free to bind to β-slips. Presuming that the β-slip can only occur in an empty hormone-binding channel, mutations influencing hormone binding would also affect the frequency of β-slips. Eneqvist and Sauer-Eriksson (2001) suggest that most FAP mutants should have only a minor effect on stability, supporting models that propose that amyloid is formed from distorted but intact tetramers. However, this model is based on the structure of a highly artificial TTR mutant and looking at naturally occurring mutations it is known that at least three of the FAP mutants, TTR Val20Ile, TTR Val30Met and TTR Leu55Pro were reported to have decreased tetramer stability (McCutchen *et al.*, 1995; Jenne *et al.*, 1996; Quintas *et al.*, 1997) and therefore, under several conditions tested, the tetramer easily dissociates into monomers, compared to other mutants or the WT protein.

Using hydrostatic pressure and spectroscopic techniques, Ferrão-Gonçalves *et al.* (2000) showed that pressure could convert native TTR tetramers into an altered state sharing properties with an amyloidogenic intermediate, probably involved in the aggregation pathway. They proposed that under physiological conditions during TTR's lifetime, any alteration in tetrameric structure exposes hydrophobic patches (such as aging) and loosening of the subunits interactions could be a starting point for the aggregation process. This speculation however does not include the effects of the same pressure effects on single-aminoacid mutant TTR forms associated with FAP.

Recently, the substitution of two aminoacids in the hydrophobic core of the A-strand of TTR, as an approach to significantly destabilise the native fold of the tetramer, led to a mutant that is very prone to form amyloid (Olofsson *et al.*, 2001). Renaturation of this mutant at low temperature facilitated the isolation of an amyloid-forming intermediate state having the apparent size of a dimer, with a high content of random coil. The authors considered that this discovery was in agreement with the findings of Serag *et al.* (2001) that were able to form amyloid fibrils out of cross-linked engineered TTR mutants with extra cysteines. They suggest that the native dimeric interactions are preserved within the amyloid fibril and propose a mechanism for

fibrillogenesis involving an oligomeric intermediate that is either a dimeric building block or a multiple of such.

3.2.2. Proteolytic hypothesis

Proteolysis has always been thought to play an important role in most types of amyloidoses, due to the generation of amyloid peptides by proteolytic cleavage of the corresponding protein precursor. There is no specific pattern for cleavage: some amyloid subunits are N-terminus degradation fragments (immunoglobulin light chains in AL, AA in secondary amyloidosis), some are C-terminus segments (cystatin-C variant in HCHWA-D) and others are internal proteolytic fragments (Aβ in Alzheimer's disease and Down's syndrome, Aβ-variant in HCHWA-I, gelsolin in FAP).

Proteolysis does not seem to be necessary to amyloid formation in TTR-related amyloidosis. However, TTR peptides, in addition to intact protein, have been found in amyloid fibrils from SSA and some FAP patients, fact that raised the hypothesis of proteolysis as a process resulting of physiological mechanisms, like aging, capable of triggering fibril formation by releasing amyloidogenic fragments (Saraiva and Costa, 1991).

The observed positions of cleavage originating these fragments vary slightly, according to the TTR variant involved but concentrate in four aminoacids, mainly: positions 46, 49, 52 and 59. The importance of this phenomenon in TTR amyloidosis is still not established, nor the timing of its occurrence. According to the model proposed by Colon and Kelly (1992), proteolysis at the referred positions would support the hypothesis that there was a higher exposure of this region (strand C-CD loop-strand D), due to conformational modifications leading to the formation of an amyloidogenic intermediate.

The fibril forming capacity of synthetic peptides representing parts of human TTR sequence was investigated by Gustavsson *et al.* (1991). Most of the synthetic peptides that were fibrillogenic in this study, represent parts of the TTR sequence that reside mostly in β-strands in the tertiary structure of TTR. A peptide corresponding to the α-helix was also strongly fibrillogenic *in vitro* as was a peptide representing the DE-loop. In SSA and in FAP, the fibrils contain C-terminal fragments of TTR starting just before the D-strand. It was then suggested that the cleavage of the TTR molecule by exposing a highly fibrillogenic sequence would possibly be important for *in vivo* fibrillogenesis.

3.2.2.1. Truncated dimers as building blocks

A new model for amyloid fibril formation based on the formation of fibrils form N-terminal truncated dimers as building blocks has been proposed by Schormann *et al.* (1998). Proteolytic cleavage was postulated to be the initiation step in amyloid fibril formation. All amyloidogenic variants showed an increased main chain solvent exposure when compared to normal and non-amyloidogenic TTR variants, which was postulated to result in increased susceptibility to proteolysis: after limited proteolysis, dimers are incapable of re-association into native tetramers. This assumption is in agreement with the fact that peptide fragments as well as full-length TTR were found in Val30Met TTR amyloid fibrils (Thylen *et al.*, 1993); Dwulet and Benson, 1986). However, this is not true for all samples of Val30Met fibrils, as described in other studies, where no fragments were found (Saraiva *et al.*, 1984; Tawara *et al.*, 1983).

The involvement of proteolysis in TTR amyloidosis is, however, still not established and it is unclear whether is occurs before, during or after amyloid formation.

3.2.3. *In vivo* models of FAP

The pathological process leading to the aggregation of soluble TTR into insoluble amyloid fibrils is not fully understood and no effective therapy has been devised. If however an efficient animal model can be established for FAP, it would help elucidate the disease process. Several groups tried to generate transgenic mice carrying the human *ttr* Val30Met gene. A considerable degree of homology was found between the aminoacid sequences of mouse and human TTRs (Wakasugi *et al.*, 1985). Among the 127 aminoacid residues of the mature protein, 25 are replaced by different aminoacids in the mouse TTR. Interestingly, 24 out of the 25 substitutions are located in the outer surface of the protein. Thus, the regions corresponding to functional domains of the proteins are highly conserved between mouse and human. In addition to this, the sequences of the 5'-flanking region are highly conserved and the homology from the putative TATA box to ~189 region was 85% (Wakasugi *et al.*, 1986). These observations first suggested that the human *ttr* gene could be transcribed to produce a functional human TTR protein in the mouse.

To study the control mechanism of TTR gene expression and to construct a mouse model for FAP, Yamamura *et al.* (1987) produced transgenic mice by

microinjecting cloned human mutant TTR genes into fertilised eggs of the C57BL/6 mouse, testing whether the human TTR gene could be expressed in mouse tissues. In this system the human TTR gene was expressed in liver and yolk sac but not in brain, suggesting that the control region of TTR gene expression in liver and yolk sac is retained within the 500 bp upstream region of this gene and that a regulatory element directing the TTR gene expression in brain might exist in the more upstream region. They mentioned that the level of expression in this transgenic mouse model was only one-tenth of mouse TTR and might be too low to cause the disease in this animal.

Mice models for FAP, using inducible heterologous promoters were the first to be developed by two groups of researchers. Sasaki and co-workers (1986) reported the breeding of transgenics for TTR Val30Met in the hybrid background (C57B1/6XC3H) fertilised eggs with BALB/c sperm. The mouse metallothionein promoter (MT) was fused to a human *ttr* gene containing the Val30Met mutation (MT1-TTRMet30). The MT gene was expressed in many tissues, particularly in the liver, and this expression was zinc-inducible, except for the testis. Amyloid deposition however, was not observed (Sasaki *et al.*, 1989) and TTR expression was relatively low (5-10 μg/ml).

Shimada *et al.* (1989) also reported the production of TTR Val30Met transgenic mice using the same promoter. These transgenics were made in the C57B1/6J background and the structural portion of the human *ttr* Met30 gene was also ligated to the mouse MT promoter. The MT-TTRMet30 gene was expressed in various tissues including liver, brain, heart, skeletal muscle, kidney and lung and TTR levels in the serum were 10-60 μg/ml. Though by the age of 6 months amyloid deposition could be seen in the *mucosa* and small intestine, and at 12 months, deposits were also evident in 10% of renal *glomeruli*, heart and thyroid, amyloid deposition was never observed in the brain, bone marrow, spleen, liver, limph nodes, choroid plexus or peripheral nerve (Yi *et al.*, 1991; Araki *et al.*, 1994).

Other researchers turned their efforts to the creation of models using homologous promoter regions, replacing the MT promoters for transgenic generation. Using the entire human *ttr* Met30 gene with 600 bp of upstream sequence including the native promoter (0.6-TTRMet30), Yamamura *et al.* (1987) verified that gene expression was confined to the liver and that these mice showed a low level of TTR mRNA expression (1/10 of the endogenous murine TTR level) in the liver and a low serum concentration (2-30 μg/ml). Furthermore, Congo red staining for amyloid in various tissues was negative until 12-15 months of age (Shimada *et al.*, 1989).

In 1996, Yokoi and co-workers used a more complete DNA fragment to produce their transgenic mice, containing about 6 kb of the upstream region and the entire human *ttr* Met30 gene (6-TTRMet30) instead of the 0.6 kb sequence used before. This was based on the identification of a distant enhancer element in the mouse *ttr* gene, by Costa *et al.* (1990) upon the observation that mouse *ttr* gene expression in cultured human hepatocytes was increased about 10-fold by the presence of that element. The animals had variable serum levels of TTR ranging from 0 to 170 μg/ml. Amyloid deposition was observed, starting in the gastrointestinal tract, cardiovascular system and kidneys, 6 months after birth and extended to various other organs and tissues with advancing age (Nagata *et al.*, 1995). At 24 months of age, the pattern of amyloid deposition was similar to that observed in human autopsy cases of FAP, except for its absence in the choroid plexus and in the peripheral nervous system (Takaoka *et al.*, 1997). These results suggested that intrinsic environmental factors other than the mutant gene were involved in the late-onset deposition of amyloid fibrils.

In these TTR transgenics, TTR expression occurs both in the liver and in the choroid plexus, as in humans. The fact that human TTR molecules produced in the mouse liver are expected to form hybrid tetramers with mouse TTR molecules, was first thought as responsible for the slower and lesser degree of deposition in the transgenics expressed under the control of their own regulatory elements. When mice lacking the endogenous *ttr* gene and carrying human mutant TTR Val30Met were generated (Maeda *et al.*, 1996; Yokoi *et al.*, 1996), it was observed that these animals had a higher average level of circulating human TTR Val30Met than FAP patients (200-600 μg/ml). However, the pattern of amyloid deposition was identical to the one described for the 6-TTRMet30 transgenics.

To study the process of age-related fibrillogenesis, a feature of senile systemic amyloidosis (SSA), where normal, non-mutated TTR deposits in the heart of old people, transgenic mice over-expressing human WT TTR have been produced (Teng *et al.*, 2001). These authors observed that 84% of the mice (C57Bl/6xDBA/2) older than 18 months, developed TTR deposits occurring primarily in the heart and kidney. In most of the animals, the deposits were non-fibrillar and non-Congophilic, but 20% of the animals had cardiac amyloid deposits identical to the lesions seen in SSA. It was the first time in any form of amyloidosis that non-fibrillar deposits have been shown to systematically occur temporarily before the appearance of fibrils derived from the same precursor in the same tissues. The hypothesis that the non-Congophilic material is either a pre-amyloid state or an alternate form of tissue deposition was raised and that

in vivo factors, perhaps associated with aging, impact on both precursor deposition and amyloid fibril formation.

An efficient animal model for FAP with generalised amyloid deposition, including deposition in the peripheral nerve within a reasonable time frame remains, to be produced.

4. Therapeutic approaches to FAP

Knowledge of the intermediate structure of the aggregates forming the amyloid fibrils would allow the design of drugs for preventing protein association or the occurrence of an amyloidogenic intermediate. Assuming that the intermediate structure is a monomer with an altered conformation, a drug capable of binding to the TTR tetramer and inhibiting its dissociation should be effective in preventing the formation of amyloidogenic intermediate species. Alternatively, if the interfaces of protein assembly in the amyloid fibrils are known, the design of molecules capable of disrupting the fibril structure, by interfering with those interfaces, would be facilitated (Damas and Saraiva, 2000). Trials on prevention or reversal of amyloid fibril formation must also be based upon a clear understanding of how the biophysical properties of proteins isolated from organs of patients afflicted with amyloidosis relate to the actual secondary structure of these fibrils in tissues.

Removal or reduction of the amyloidogenic precursor pool has been the subject of several therapeutic trials. In FAP patients, liver transplantation has been shown to replace the circulating variant TTR with normal protein and the gradual reduction of amyloid load has been reported (Holmgren *et al.*, 1993), with regression of the amyloid deposits after 1-2 years of transplantation. However, local fibril formation still occurs in other organs like choroid plexus and the eye, which keep on synthesising the mutant protein. The autonomic nerve function can improve but the peripheral neuropathy seems to recover very slowly.

To prevent the formation of an amyloidogenic intermediate structure by stabilising the tetrameric TTR is a possible intervening method. The potential use of small molecules that bind in the TTR channel might stabilise the native fold of the protein, preventing its dissociation. Some drugs seem capable of stabilising the TTR tetrameric structure, as seen with sulphite by Altland and Winter (1999). These authors, in previous studies observed that oxidation of Cys^{10} by sulphite leads to more stable monomers. This effect was tested *in vitro* and *in vivo* and in addition to the stabilising effect, increase in the tetramer/monomer ratio was also detected. Based on these observations, sulphite was presented as being a potential candidate for delaying the onset and progress of TTR amyloidosis.

Disruption of fibrils is another alternative currently being studied *in vitro*. Recently, Palha *et al.* (2000b), showed that 4'-deoxy-4'-iododoxorubicin (IDOX)

strongly interacts with TTR amyloid fibrils and is capable of disrupting the fibrillar structure of amyloid into amorphous material. It had previously been reported that the administration of this cytotoxic drug (that binds specifically to several types of amyloid), reduced splenic amyloid deposits in the murine AA model (Merlini *et al.*, 1995). This same drug was also used in AL patients, and reabsorption of amyloid deposits was observed in some cases. The mechanism by which the drug acts was not known, but it appeared that IDOX prevented subsequent deposition of precursor after its binding to amyloid fibrils, facilitating or inducing proteolytic reabsorption of deposits (Gianni *et al.*, 1995). Recently, Sebastião *et al.* (2000), proposed a theoretical model concerning the interaction of Leu55Pro TTR with IDOX, starting with the observation that crystals of that highly amyloidogenic variant, soaked with IDOX, underwent rapid dissociation, whilst WT TTR crystals under the same conditions, were quite stable. The 3D model of the Leu55Pro TTR-IDOX describes the binding of the iodinated sugar ring of the drug into a region between the two β-sheets of the amyloidogenic monomer, causing disturbances in the amyloid-like structure of Leu55Pro TTR, leading to its dissociation.

Other type of anti-amyloid drugs is directed towards the interaction of the amyloid precursor with other components of amyloid deposits. Small molecules which mimic elements of the structure of heparan sulphates can interfere with both the *in vivo* induction and persistence of AA amyloid deposits and, furthermore, with the heparan sulphate induction of Aβ β-sheet formation and Aβ fibrillogenesis *in vitro* (Kisilevsky *et al.*, 1995). Several attempts have been performed to identify pharmaceutically acceptable molecules that inhibit and reverse the binding of SAP to amyloid fibrils *in vivo* (Pepys *et al.*, 1996), since a prophylactic effect of sulphated glycans has been shown in animal models of scrapie (Priola and Caughey, 1994). This approach has potential also in FAP.

Several tools are available to study the mechanisms of amyloidogenesis and for the development of therapeutic strategies and/or drugs, capable of interfering with this process. *In vitro* studies represent the first approach to elucidate structure and give indications of factors affecting fibril formation.

5. Objectives

The general aims of the present study were to characterise amyloidogenic determinants in FAP, envisioning a contribution to the elucidation of the mechanism of fibrillogenesis. The work focused on the following questions:

A. Examination of protein stability and propensity to *in vitro* amyloid formation of recombinant designed TTR mutants, with substituted residues in areas of the molecule that might represent key surfaces:

- Is a particular part of TTR more important for tetramer de-stabilisation?
- Is a particular part of TTR more important for fibril formation?
- Can stable TTR dimers form fibrils?
- Can stable TTR monomers form fibrils?

B. Investigation of altered conformations that might represent intermediate structures in transthyretin fibrillogenesis:

- Do soluble tetrameric mutant TTR forms, express cryptic epitopes common to TTR amyloid fibrils?
- Do different forms of TTR amyloidogenic intermediates express cryptic epitopes common to TTR fibrils?

6. Conclusions

A. Examination of protein stability and propensity to in vitro amyloid formation of recombinant designed TTR mutants, with substituted residues in areas of the molecule that might represent key surfaces

Dissociation of the TTR tetramer is a pre-requisite for amyloid formation *in vitro* and involvement of monomers and/or dimers in fibril formation has been suggested by structural studies. We have designed five mutations with the purpose of stabilising (Val30Cys/Leu55Cys, Ser117Cys and Glu92Cys) or destabilising (Asp18Asn and Leu110Ala) the monomer/dimer/tetramer interactions in TTR, aiming at elucidating structural determinants in amyloidogenesis.

Mutants' resistance to dissociation was analysed through HPLC studies of diluted TTR preparations. All "stabilised mutants" migrated as tetramers and upon dilution no other TTR species was observed, confirming their increased resistance to dissociation. For the "destabilised mutants", a mixture of both tetrameric and monomeric forms coexisted at low dilution and the latter increased upon 10-fold dilution. Both "destabilised mutants" formed amyloid *in vitro* by acidification. This result indicated that both the AB loop of TTR, destabilised in Asp18Asn and the hydrophobic interactions affecting the dimer-dimer interfaces in Leu110Ala are implicated in the stability of the tetrameric structure.

The "stabilised mutants" Ser117Cys and Glu92Cys, which were dimeric in nature through disulphide bonding, were unable to polymerise into amyloid even at pH 3.2. The same was observed for the double mutant Val30Cys/Leu55Cys, monomeric at pH 3.2 and still unable to form fibrils due to the disulphide bonding. When the amyloid formation assay was repeated in the presence of β-mercaptoethanol we observed that upon disruption of the S-S bridges on these stable dimers and monomers amyloid fibrils could be formed. These experimental evidences suggest that monomers rather than dimers are the repeating structural subunit composing the amyloid fibrils, and that partially unfolding or destabilisation of monomers is requested for fibrillogenesis.

B. Investigation of altered conformations that might represent intermediate structures in transthyretin fibrillogenesis:

The TTR tetramer is thought to dissociate into monomeric intermediates and subsequently polymerise into the pathogenic amyloid form. We characterised

intermediate TTR structures in the *in vitro* amyloidogenesis pathway, by trying to destabilise the TTR tetrameric fold, based on the known crystallographic structure of a Leu55Pro transthyretin variant. That study revealed conformational changes in the tetrameric structure of the protein, possibly responsible for its instability and for the exposure of new molecular surfaces involved in TTR polymerisation. The refinement of this crystal structure showed that the pro → leu substitution disrupted the hydrogen bonds between strands D and A, resulting in significantly different interface contacts from those of the WT protein. The packing interactions present in this crystal structure are significant and it is interesting to observe that one of them corresponds to the interaction of the side chain of Arg^{21} with the main chain of Gly^{83}, from the α-helix and belonging to a different monomer. Based on these novel findings we designed a mutant with a tyr → phe substitution at position 78. Tyr^{78} belongs to the α-helix region and is hydrogen bonded to Asp^{18} from the AB loop. When this interaction is disrupted it is possible that a subtle change occurs in the helix and a more drastic alteration happens on the AB loop.

Surprisingly, we generated a soluble tetrameric form of TTR that is recognised by a monoclonal antibody, previously reported to react only with highly amyloidogenic mutant proteins lacking the tetrameric native fold and with amyloid fibrils. BIAcore system analysis showed that Tyr78Phe had similar binding properties as synthetic fibrils. The affinity of this interaction was 10^7 M^{-1}. We suggest that the tetrameric structure of Tyr78Phe is altered due to the loosening of the AB loops of the tetramer, leading to a structure that might represent an early intermediate in the fibrillogenesis pathway, exposing a common cryptic epitope to amyloid fibrils.

To further investigate conformational intermediates with exposed cryptic epitopes that might reveal amyloidogenic surfaces, we performed a genetic screening using peptide aptamers, from a 20-mer-aptamer library, in an adapted two-hybrid system. Two TTR target molecules were employed as *baits*, in order to identify *in vivo* interactors out of the library, specific for soluble WT and aggregated TTR Leu55Pro. Y190 yeast cells expressing WT TTR or Leu55Pro TTR fused to the GAL4 DNA-binding domain were transformed with the activation-domain-tagged peptide aptamer library. Using a polyclonal antibody and an amyloid specific monoclonal antibody (Mab 15) we succeeded in differentiating soluble from aggregated TTR forms through the comparison of their recognition patterns when analysing yeast colonies. Thirty positive interacting clones were isolated in this screen and aptamers were organised into seven groups according to the different recognition patterns obtained. We selected 5

aptamers corresponding to the most representative set of different situations: i) 29B - interactor with soluble TTR *bait* (positive for Pab interaction and negative for Mab 15), causing no change in antibody recognition pattern; ii) three interactors with "type I aggregates" *bait* (positive for Mab 15 interaction and negative for Pab): C45 - producing no effect in the recognition patterns; C26 - reverting both recognition patterns; 67 - reverting the recognition pattern of the Pab only, and iii) one interactor with "type II aggregates" *bait* (positive for the interaction of both Abs): 40W - reverting the recognition pattern of Mab 15 and not affecting the Pab's reactivity.

In vitro studies were performed in order to validate the interactions between the selected aptamers and TTR forms (WT and mutant) as well as evaluating their specificity and inhibition activities towards fibrillogenesis. BIAcore competition studies and kinetic determinations for the affinity of interactions between antibodies/aptamers and several TTR forms (WT, Val30Met, Tyr78Phe and Leu55Pro soluble and Leu55Pro amyloid fibrils) were performed. BIAcore detection revealed new interactions for some of the peptides, namely towards Tyr78Phe soluble TTR and the soluble form of Leu55Pro, leading to the suggestion of the following epitopes:

1. Expressed on WT TTR and abolished in the conversion of native into fibrillar structures.
2. Cryptic epitopes:
    2a. Expressed only on TTR amyloid fibrils.
    2b. Expressed in TTR amyloidogenic intermediates and on TTR amyloid fibrils, involving three different regions of the molecule.

Additional studies must be undertaken in order to identify these epitopes and test their action in preventing fibrillogenesis.

PART II

EXPERIMENTAL WORK

EXPERIMENTAL WORK

Chapter I

Designing transthyretin mutants affecting tetrameric structure: Implications in amyloidogenicity

1. INTRODUCTION

Abnormal self-assembly of transthyretin into amyloid fibrils has been implicated as the causative agent in Familial Amyloidotic Polyneuropathy. The majority of the transthyretin-associated amyloidoses is due to single aminoacid substitutions (Saraiva *et al.*, 1996).

Plasma TTR is a homotetrameric protein, with a physiological role in the transport of the hormone thyroxine and retinol. It was established by X-ray crystallography that two binding sites exist for thyroxine; they are located in a central channel formed by the interaction of the four monomers (Wojtczak *et al.*, 1996). Although both binding sites are similar, they present different binding affinities for T_4 due to negative cooperativity of binding. Retinol binding protein, which binds TTR for the transport of vitamin A, attaches to the exterior of the tetramer as revealed by the structure of the complex (Monaco *et al.*, 1995). TTR has an extensive β-sheet structure making the molecule potentially amyloidogenic and mutations in its sequence increase the susceptibility to amyloid formation (Bonifácio *et al.*, 1996).

The molecular mechanism leading to the polymerisation of TTR and its deposition as amyloid fibrils is unknown, although evidences support the notion that fibril formation may develop from destabilisation of the tetrameric form of the protein. This was revealed by crystallographic studies of amyloidogenic variants, namely Val122Ile and Leu55Pro (Damas *et al.*, 1996; Sebastião *et al.*, 1998) and in biophysical *in vitro* amyloid formation studies (Lai *et al.*, 1996). In order to elucidate more directly the fibril structure in FAP, X-ray diffraction patterns from native vitreous TTR amyloid fibrils have been analysed. While some authors proposed a model based on a helical protofilament with dimensions suggesting a dimer/monomer size-unit for the fibril (Blake *et al.*, 1996), others advanced that the TTR monomer would constitute the unit-structure of the protofilament (Inouye *et al.*, 1998). Therefore, information is needed on the importance of specific residues and/or domains that stabilise the tetrameric structure as well as on the unit structure of fibrils. Observations resulting from several studies lead us to design TTR mutants with weakened or strengthened dimer/tetramer interactions in order to address these issues.

It has been suggested that the substitution Thr119Met might have a protective role concerning amyloidogenesis, indicated by the slower progress of the disease in heterozygote patients of TTR Val30Met-Thr119Met (Coelho *et al.*, 1996). Studies on the Thr119Met variant (Alves *et al.*, 1997b) indicated that, in opposition to most TTR variants, the substitution might induce the stabilisation of the tetrameric structure

resulting on lower amyloidogenic potential. In fact, comparative investigations by isoelectric focusing (IEF), on the resistance of TTR to dissociation into monomers, on serum samples from carriers of mutations TTR Val30Met and TTR Thr119Met and compound heterozygotes for the two mutations, documented a striking difference of resistance to dissociation.

In the present study we have designed five TTR mutants with the purpose of destabilising (Asp18Asn and Leu110Ala) or stabilising (Val30Cys/Leu55Cys, Ser117Cys and Glu92Cys) the monomer and dimer interactions in TTR, aiming at elucidating the major determinants in the fibrillogenesis cascade.

2. MATERIALS AND METHODS

2.1. Design of domain interface variants

The computer graphics software Turbo-Frodo (Roussel and Cambillau, 1989) was used to predict the possible structural changes associated with specific mutations: Asp18Asn, Val30Cys, Leu55Cys, Glu92Cys, Leu110Ala and Ser117Cys. By inserting the variant residues into the known molecular structures of the WT, Leu55Pro (Sebastião *et al.*, 1998) and Thr119Met (Almeida *et al.*, 1997) TTR molecules, we assessed the changes in hydrogen bonding and/or close contacts with other main chain or side-chain atoms.

2.2. TTR *in vitro* mutagenesis and expression

The WT transthyretin cDNA cloned into the pINTR vector, preceded by a sequence encoding the OmpA leader signal and flanked by *BamHI* and *PstI* sites (Furuya *et al.*, 1991), was used to produce mutant TTR molecules by site-directed mutagenesis (Quick change site directed mutagenesis kit, Stratagene). These mutants were constructed using two mismatched primers, introducing a 1-3 base substitution in the original sequence:

Asp18Asn: 5' C-A-A-A-G-T-T-C-T-A-A-A-T-G-C-T-G-T-C-C-G 3'
5' C-G-G-A-C-A-G-C-A-T-T-T-A-G-A-A-C-T-T-T-G 3'
Val30Cys: 5' G-C-C-A-T-C-A-A-T-G-T-G-G-C-C-T-G-T-C-A-T-C-T-G-T-T-C 3'
5' G-A-A-C-A-C-A-T-G-A-C-A-G-G-C-C-A-C-A-T-T-G-A-T-G-G-C 3'
Leu55Cys : 5' G-A-G-T-C-T-G-G-A-G-A-G-T-G-T-C-A-T-G-G-G-C-T-C-A-C-A 3'
5' T-G-T-G-A-G-C-C-C-A-T-G-A-C-A-C-T-C-T-C-C-A-G-A-C-T-C 3'
Glu92Cys: 5' G-A-G-C-A-T-G-C-A-T-G-C-G-T-G-G-T-A-T-T-C 3'
5' G-A-A-T-A-C-C-A-C-G-C-A-T-G-C-A-T-G-C-T-C 3'
Leu110Ala: 5' C-A-T-T-G-C-C-G-C-C-G-C-G-C-T-G-A-G-C-G-C-C 3'
5' G-G-G-G-C-T-C-A-G-C-G-C-G-G-C-G-G-C-A-A-T-G 3'
Ser117Cys: 5' C-T-A-C-T-C-C-T-A-T-T-G-T-A-C-C-A-C-G-G-C 3'
5' G-C-C-G-T-G-G-T-A-C-A-A-T-A-G-G-A-G-T-A-G 3'

All PCR amplified products were treated with *DpnI* to digest non-mutated parental DNA template, and 1/10 of the reaction volume containing the circular,

mutated dsDNA, was used to transform Epicurian coli XL1-Blue supercompetent cells. Small DNA plasmid preparations were produced and tested by sequencing. Mutagenised TTR was expressed in an *Escherichia coli* expression system as described in Furuya *et al.* (1991).

2.3. Isolation and Purification of TTR variants

E. coli strain BL-21 was transformed with individual expression plasmids and the periplasmic space contents were obtained by osmotic shock. The supernatant was fractionated on DEAE-Sephadex and TTR containing peaks were dialysed overnight (O/N) against water and lyophilised. Further purification was achieved by preparative gel electrophoresis, followed by a gel filtration using a 1.5x50 cm BioRad column packed with Biogel P-100 (BioRad) equilibrated with appropriate buffer (0.1 M KH_2PO_4, 0.1 M Na_2SO_4).

2.4. Gel electrophoresis

Proteins were analysed either in native or denaturing conditions. Native electrophoresis was carried out on an 8% acrylamide gels system and on a gradient system (4 - 16%). Electrophoresis under denaturing conditions was performed in SDS-PAGE gels (15% acrylamide, 0.1% SDS) with or without heat treatment of samples and with or without addition of β-mercaptoethanol (0.1 M). Isolelectric focusing gels (7% acrylamide) were also performed, under semi-denaturing conditions (4M urea) (Alves *et al.*, 1997b).

2.5. Western blots

Proteins were transferred from gels into nitrocellulose membranes (Hybond ™ - C pure, Amersham), using a Tris-Glycine system, for 1 hour, at 1 mA/cm^2 of membrane. Native gels were transferred to nitrocellulose membranes using a Tris-Boric Acid system, for 90 minutes, at 100 volts. After blocking and washing with PBST, the membrane was incubated with rabbit anti-TTR polyclonal antibody (DAKO), 1:1000 dilution in PBS, for 1 hour at room temperature (RT). After several washes and a last incubation with anti-rabbit Immunoglobulins - horseradish peroxidase conjugated

(Binding Site), 1:1000 dilution, for 1 hour at RT, TTR was visualised using the ECL method (Pierce).

2.6. T_4 Binding assay

0.5 - 2 μg of soluble TTR samples were incubated with radiolabelled T_4, for 1/2 hour, before loading on 8% acrylamide gels, as described in Saraiva *et al.* (1988). The gels were dried and autoradiography performed.

2.7. Chromatography

High performance liquid chromatography (HPLC)

All TTR preparations destined for gel filtration chromatography were diluted more than 10 times in gel filtration chromatography buffer (20 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0) according to Quintas *et al.* (1997). Protein concentration of TTR preparations destined for gel filtration chromatography was determined spectrophotometrically, at 280 nm, using an extinction coefficient of 7.76 x 10^4 M^{-1} cm^{-1} based on a 55kDa molecular weight for TTR [A_{280} (1%) = 14.1 mg^{-1} ml cm^{-1}]. HPLC was performed on a Beckman Ultraspherogel SEC2000 column, coupled to a Gilson high precision pump P-302 and a Gilson holochrome-280 UV detector. Gel filtration chromatography buffer was used as eluent at a flow rate of 0.5 ml/min and each TTR preparation was injected in a volume of 200 μl. Before injection, the column was allowed to equilibrate with 2-5 column volumes of buffer and was cleaned with 0.5 M NaOH between injections. The protein standards used in calibration were: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Size-exclusion chromatography

Gel filtration chromatography was performed on a Sephacryl S-200 high resolution column (Bio-Rad), 1.0 x 100 cm, in 0.05M sodium acetate buffer, pH 6.3 or pH 3.2. This system was previously calibrated using four protein standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

2.8. Preparation of amyloid fibrils

100 μg of each mutant in a 0.2 mg/ml concentration, were incubated in 0.05 M sodium acetate/0.1 M KCl buffer on a pH scale from 2.6 to 6.8, for 48 hours, at RT, to form amyloid fibrils (Bonifácio *et al.*, 1996; Lai *et al.*, 1996). Glycine-NaOH 50 mM, pH 9.0 buffer was then added to each solution and the amyloid fibrils were tested for ThT fluorescence. Excitation spectra were recorded on a Jasco FP-770 spectrofluorometer at 25^{0}C with 30 μM ThT (Fluka) in 50 mM Glycine-NaOH buffer, pH 9.0 in 1 ml assay volume. Excitation and emission slits were set to 5 and 10 nm, respectively. Electron microscopy was performed in samples of amyloid fibrils prepared from the WT TTR, as described in Bonifácio *et al.* (1996).

2.9. Alkylation of TTR

1 mg of soluble TTR samples was reduced and alkylated as described in Saraiva *et al.* (1984).

3. RESULTS

3.1. Modelling

Five TTR mutants were designed to weaken or strengthen dimer/monomer interactions and their structural-functional properties were analysed. Two of these mutants, here named as "destabilised mutants" were constructed to be unstable, due to a fast dissociation into monomeric units (Figure 9, A). In the WT protein, the side chain of Asp^{18} is H-bonded to the NH group of Asp^{21} of the other dimer in the tetramer. In view of this, we reasoned that by replacing an asparagine in position 18 by a similar but not charged aminoacid, this link should disappear loosening the AB loop area. In variant TTR Thr119Met, there is a van der Waal's interaction between aminoacids Met^{119} and Leu^{110} belonging to different dimers, which strengthens the interaction between dimers in the thyroxine-binding pocket (Almeida *et al.*, 1997). In the WT protein, Leu^{110} projects into the channel forming hydrophobic interactions with aminoacids from another dimer. Introducing an alanine in position 110 should weaken those interactions, promoting dissociation of the molecule.

The other three mutants, referred to here as "stabilised" were designed with the purpose of creating stronger tetramers through very stable dimers (Figure 9, B) or stable monomers (Figure 9, C). In tetrameric TTR each monomer is composed of two four-stranded β-sheets (DAGH and CBEF) and the association of two monomers in a dimer results in two eight-stranded β-sheets (DAGHH'G'A'D') and (CBEFF'E'B'C'). The introduction of cysteines in positions 92 and 117, of strands F and H respectively, should create disulphide bridges between the two monomers from each dimer, stabilising the tetramer through stronger dimers. Furthermore, by replacing residues Val^{30} and Leu^{55} with two cysteines, disulphide bridges should be formed between these nearby residues in the same monomer, creating an unusually stable subunit.

We report here different physical-chemical characteristics of "stabilised/destabilised" mutants.

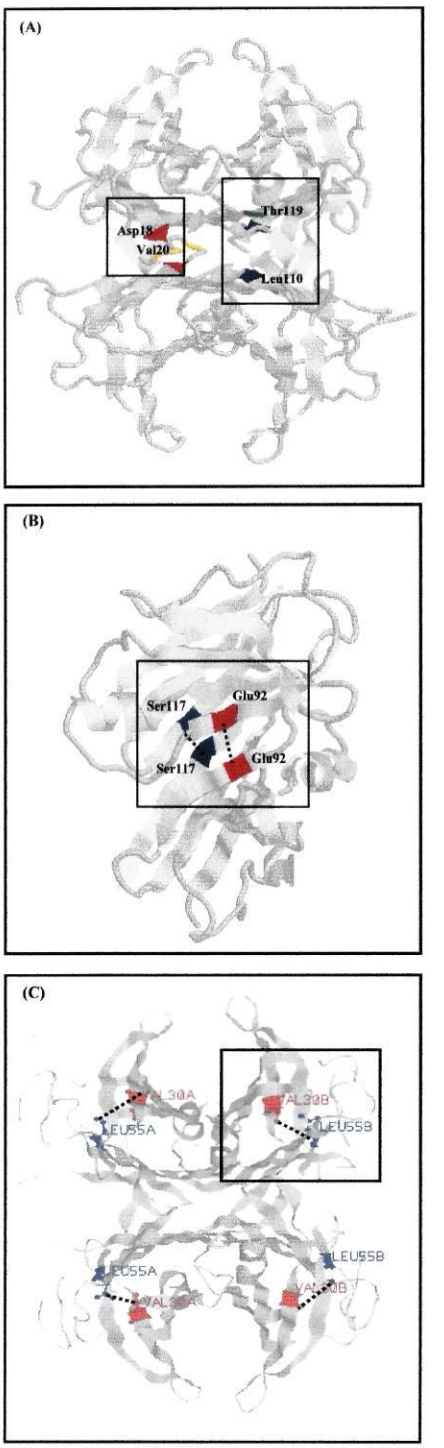


Figure 9. Dimer-dimer and intra-monomeric interfaces in transthyretin.

(A) Shows a close proximity of residues 18 and 20 (part of the AB loop), as well as 119 and 110 (facing the T_4 binding pocket). **(B)** Shows that Glu^{92} and Ser^{117} are close together in the dimer, which enables the formation of disulphide bridges when these residues are substituted by cysteines. **(C)** Shows the proximity of residues Val^{30} and Leu^{55} in the monomer, enabling the formation of disulphide bridges, upon the introduction of cysteines in these positions. The ribbon representation of the WT human TTR was retrieved from the Swiss Protein Data Bank (1TTA pdb).

3.2. Physical-chemical characterisation of TTR mutants: tetramers, dimers and monomers

3.2.1. Functional tetramers

To assess whether the mutants were still functional in what concerns T_4 binding, preparations of recombinant protein were incubated with radiolabelled thyroxine (section 2.6. Materials and Methods) and its behaviour compared with the WT protein in a human serum sample (Figure 10). We observed that two of the mutants containing extra cysteines (Ser117Cys and Glu92Cys) had a tetrameric structure and kept their ability to bind T_4. This experiment also shows that Cys^{10} did not form disulphide bonds with either Cys^{92} or Cys^{117} since both tetramers were functional (this experiment was not performed for the double mutant Val30Cys/Leu55Cys). As for Asp18Asn and Leu110Ala mutants, both designed to create instability in the TTR tetramer, exhibited contrasting behaviours. The first one was not affected in its ability to bind T_4 whilst the second did not bind the ligand. This result was not surprising, since in the WT protein Leu^{110} is highly involved in T_4 binding.

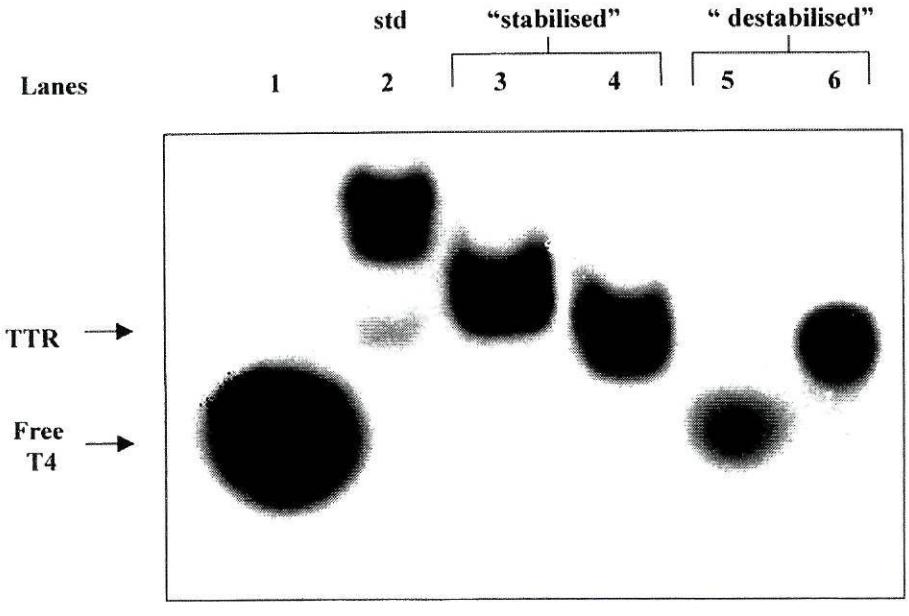


Figure 10. Functionality of mutant tetramers, concerning T_4 binding.
Native-PAGE of four recombinant TTR mutants previously incubated with radiolabelled T_4. Lanes 1-6: free T_4; human serum; Glu92Cys; Ser117Cys; Leu110Ala; Asp18Asn.
T_4 - Thyroxine; TTR – transthyretin; std – standard human serum.

3.2.2. Resistance to dissociation into monomers

We have investigated the resistance to dissociation of both "stabilised" and "destabilised" mutants. Comparing the elution pattern of 3 μM and 0.3 μM solutions of the mutants with the WT TTR, a marked difference was observed. For the WT protein, considering the highest concentrated solution (3 μM), the only isolated form obtained by chromatography corresponds to a tetrameric form of the protein, with a total absence of aggregates and/or monomers. When the preparation is diluted 10-fold, a second form is eluted, with a size of 14 kDa, corresponding to the monomeric form of the protein, as reported by Quintas *et al.* (1997).

The behaviour of the "destabilised mutants" was quite different, in particular Leu110Ala. When a 3 μM solution was tested by gel filtration and the eluted peaks analysed on a native-PAGE, it could be seen that a mix of the two forms already coexisted at low dilution (Figure 11, middle and lower panels). This observation contrasted with the elution pattern obtained for the WT TTR (Figure 11, upper panel). Also, after a 10-fold dilution, the proportion of monomeric form relative to the tetrameric one, rises from 0.25 to 0.4 (data not shown), increasing the equilibrium flow in the direction of the monomeric form in solution. This suggests that in this mutant the hydrophobic interactions are reduced affecting the dimer-dimer interfaces, which are unstable and dissociate into monomers much more easily than in the WT TTR.

A similar elution pattern was obtained for Asp18Asn, although the increase of monomeric form relative to the tetrameric one, upon 10-fold dilution, was not as marked as for Leu110Ala (data not shown), implicating the AB loop in the stabilisation of the tetramer.

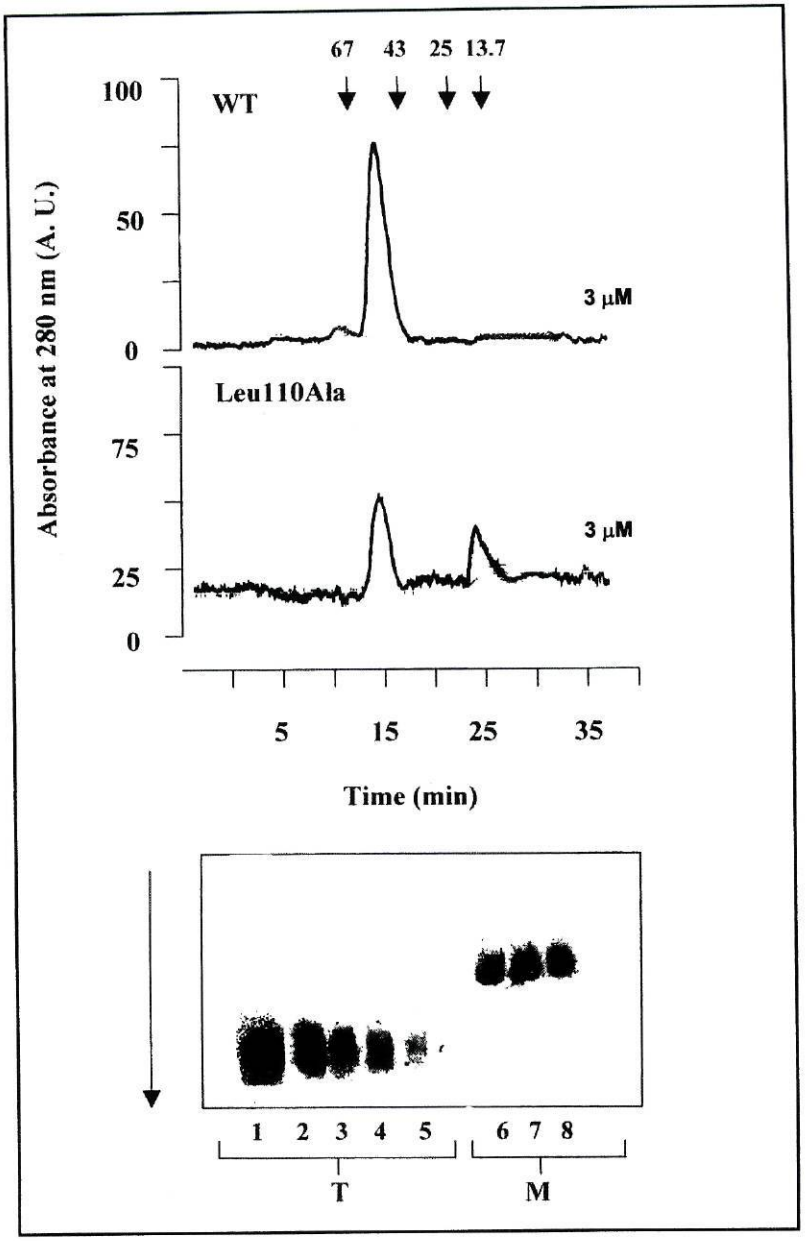


Figure 11. Dissociation into monomers is favoured in the "destabilised mutants".
HPLC elution patterns of WT TTR and Leu110Ala, at 3 μM concentration. The protein standards used to calibrate the column are indicated: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The lower panel shows the eluted fractions of recombinant Leu110Ala run on a native-PAGE. Lane 1, WT TTR; lane 2-5, Leu110Ala tetramer elution (13-16 minutes); lane 6-8, Leu110Ala monomer elution (22-28 minutes). The arrow indicates the direction of electrophoresis. T - tetramer; M - monomer.

The resistance of the "stabilised mutants" Ser117Cys and Glu92Cys, to dissociate into monomers is evident in Figure 12 where it is observed that not even a 10-fold dilution is able to produce monomeric forms; this behaviour was already expected, since under semi-denaturing IEF (4 M urea), the two cysteine-linked mutants

migrated as tetramers, whereas WT TTR presented both tetramers and monomers (data not shown).

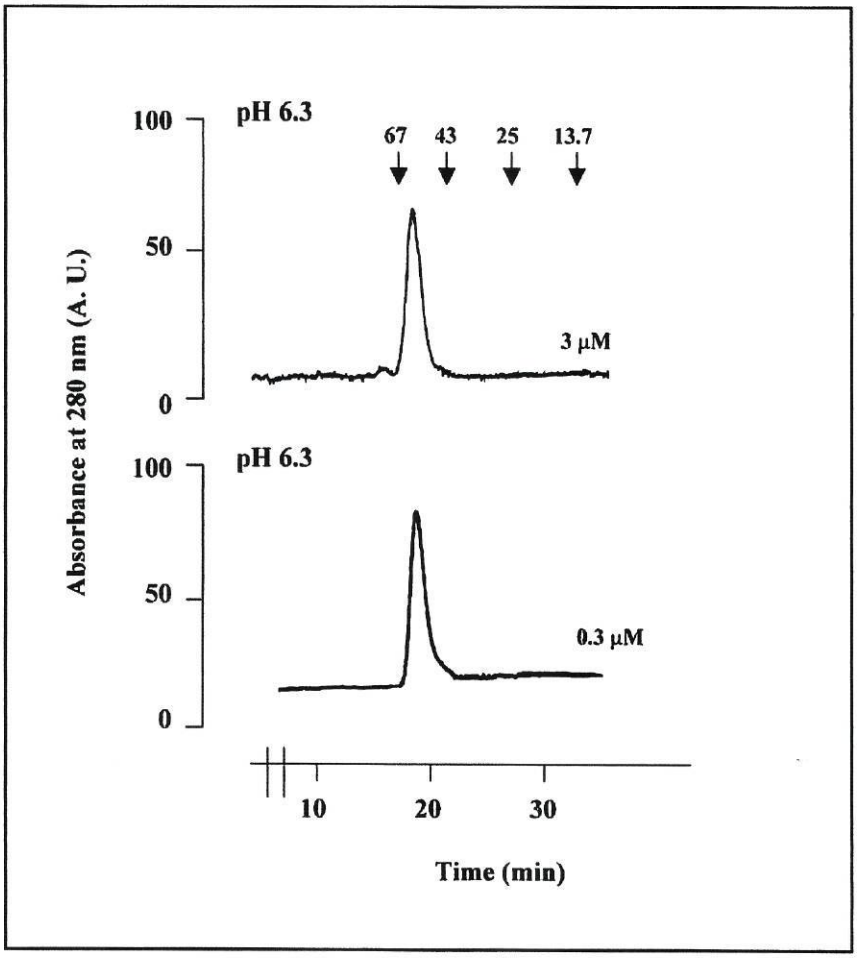


Figure 12. "Stabilised mutants" are resistant to dissociation into monomers.
Gel filtration chromatograms of Ser117Cys TTR run at pH 6.3, 3 μM and 0.3 μM concentrations, respectively. The tetrameric form of the mutant is eluted at 20 minutes for both concentrations tested. Similar data were obtained for Glu92Cys (not shown). The protein standards used to calibrate the column are indicated: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

3.2.3. Dimeric TTR species

The dimeric nature of Ser117Cys and Glu92Cys TTR variants was determined by gel filtration chromatography, comparing the elution pattern of protein solutions incubated at pH 3.2, with others incubated at pH 6.3. At pH 6.3 all the proteins elute in the form of tetramers. Figure 13 (upper panel), shows that at pH 3.2 the only isolated

species of the "stabilised mutants", eluted at 28 minutes corresponds to the dimer, with a size of 28 kDa, with a total absence of aggregates and/or monomers. Under acidic pH, both "stabilised mutants" are dimers, whilst WT TTR dissociates into monomers and precipitates as amyloid, as published by Bonifácio *et al.* (1996) and Lai *et al.* (1996). As a control, to assess whether the behaviour of these two mutants was being affected in any other way apart from the expected due to the extra disulphide bridges, the same 10-fold dilution was performed after reducing and alkylating the protein preparations. When formation of disulphide bridges was prevented, both "stabilised" mutants behaved as the WT TTR, exhibiting the tetrameric and monomeric forms (Figure 13, lower panel).

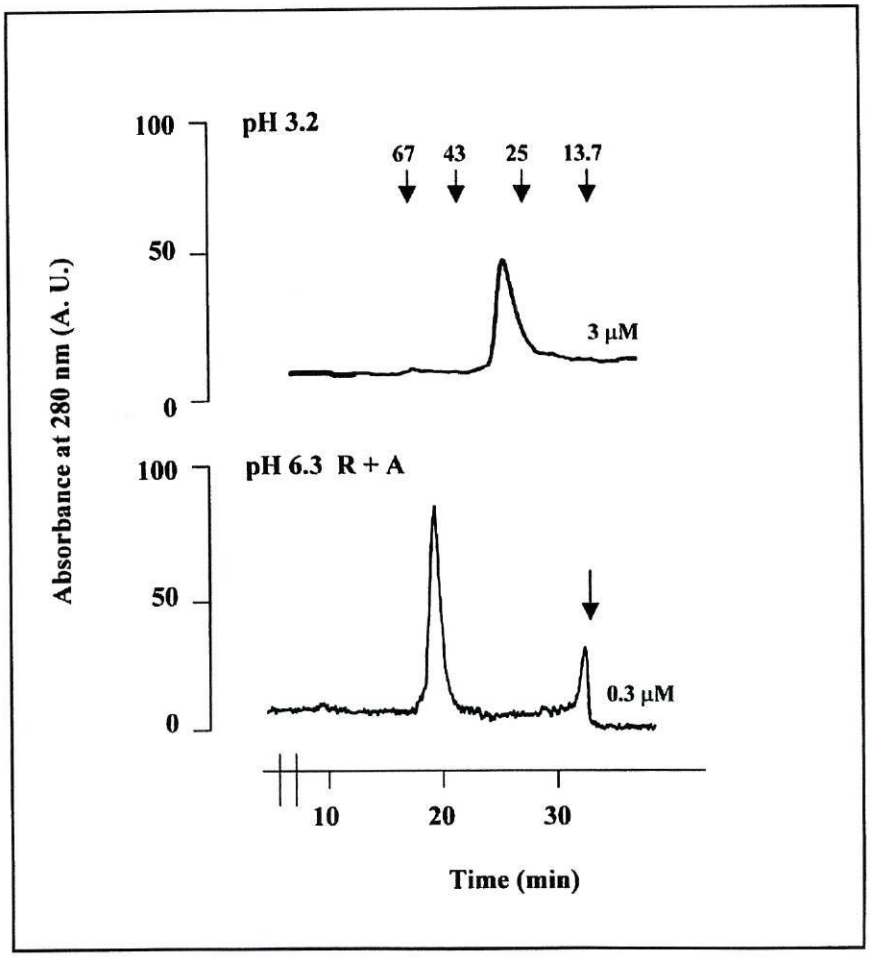


Figure 13. Dimeric nature of "stabilised mutants" at pH 3.2.

Gel filtration chromatograms of Ser117Cys TTR run at pH 3.2, 3 μM concentration and at pH 6.3, 0.3 μM concentration. The dimeric form of the mutant is eluted at 28 minutes (upper panel). A reduced an alkylated preparation of the mutant protein (lower panel) presented two TTR forms: a tetrameric form eluted at 20 minutes and a monomeric form at 31 minutes, indicated by an arrow. Similar data were obtained for Glu92Cys (not shown). R + A - Reduced and alkylated protein preparation.

3.2.4. Stabilised monomeric species

The monomeric stabilisation of the double mutant Val30Cys/Leu55Cys was assessed by gel filtration chromatography, under acidic conditions, as shown in Figure 14. Val30Cys/Leu55Cys eluted as a tetramer at nearly physiological pH (upper panel), behaving similarly to the WT protein that under the same conditions exhibits only a tetrameric form. On the other hand, at pH 3.2 the only isolated form obtained by chromatography corresponds to a monomeric form of the protein (lower panel), showing that there are no disulphide bridges formed between the two introduced cysteines and Cys^{10} in the A-strand.

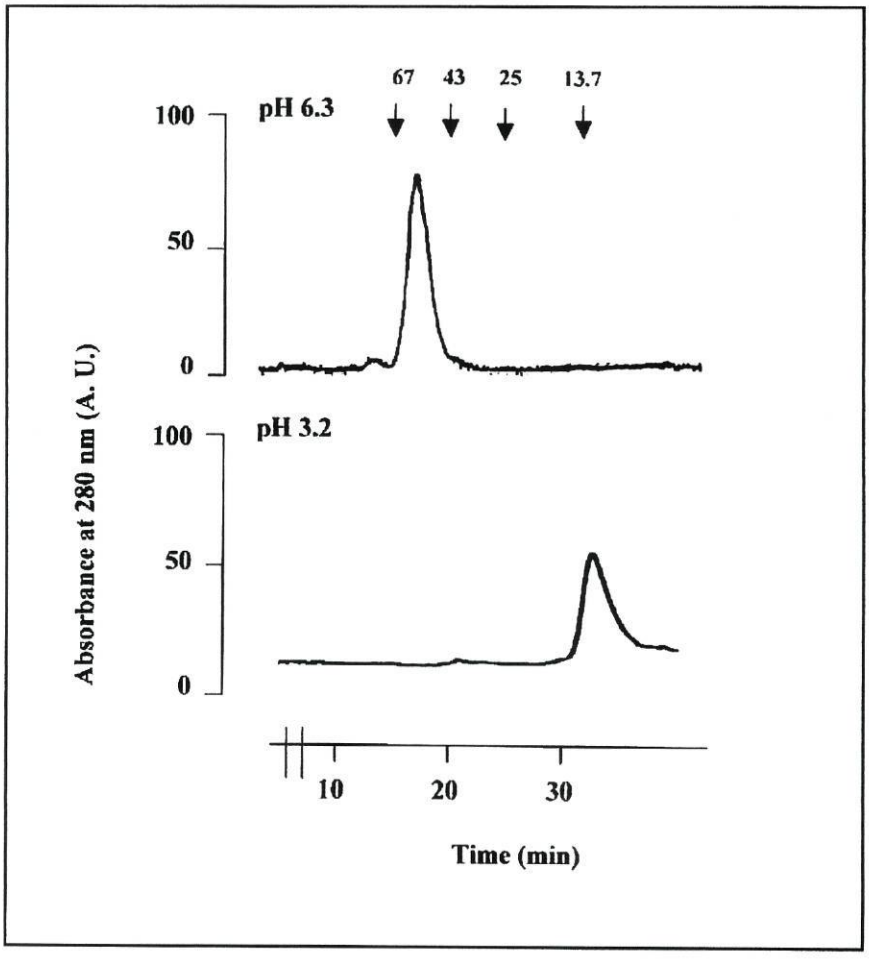


Figure 14. Monomeric nature of Val30Cys/Leu55Cys double mutant at pH 3.2.
HPLC elution patterns of the double mutant run at pH 6.3 (upper panel) and pH 3.2 (lower panel). The tetrameric form of the mutant is eluted at 16 minutes and the monomeric form at 35 minutes. All elution patterns were obtained at 3 μM concentration. The protein standards used to calibrate the column are indicated: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

3.3. Susceptibility to amyloid formation

All five mutants were tested for their susceptibility to amyloid formation upon acidification (leading to dissociation and aggregation into fibrils), measuring the fluorescence of protein solutions previously incubated at different pHs (2.3 - 6.3) after addition of ThT.

It was observed that both "destabilised mutants" TTR Asp18Asn and TTR Leu110Ala, had a similar susceptibility to amyloid formation as the WT protein, requiring an acidic pH below 4.5 to form fibrils (Figure 15).

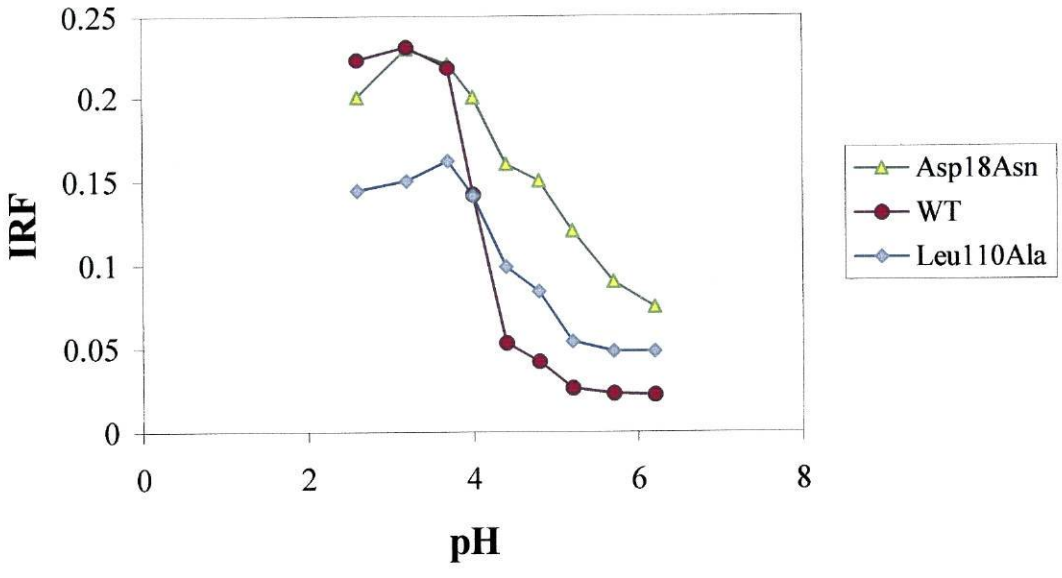


Figure 15. Highly unstable amyloidogenic tetrameric structures.
Graphic representation of the data obtained in amyloid formation for protein preparations of WT TTR, TTR Asp18Asn and TTR Leu110Ala, upon incubation at pH range 2.3-6.2. IRF - intensity of relative fluorescence.

When TTR Glu92Cys and TTR Ser117Cys were tested for their susceptibility to amyloid formation, on the contrary, no fibrils were observed even at pH 3.2. However, when we repeated the amyloid formation assay, in the presence of β-mercaptoethanol to disrupt the dimers, amyloid fibril formation occurred (Figure 16), indicating that monomers rather than dimers are necessary for fibrillogenesis.

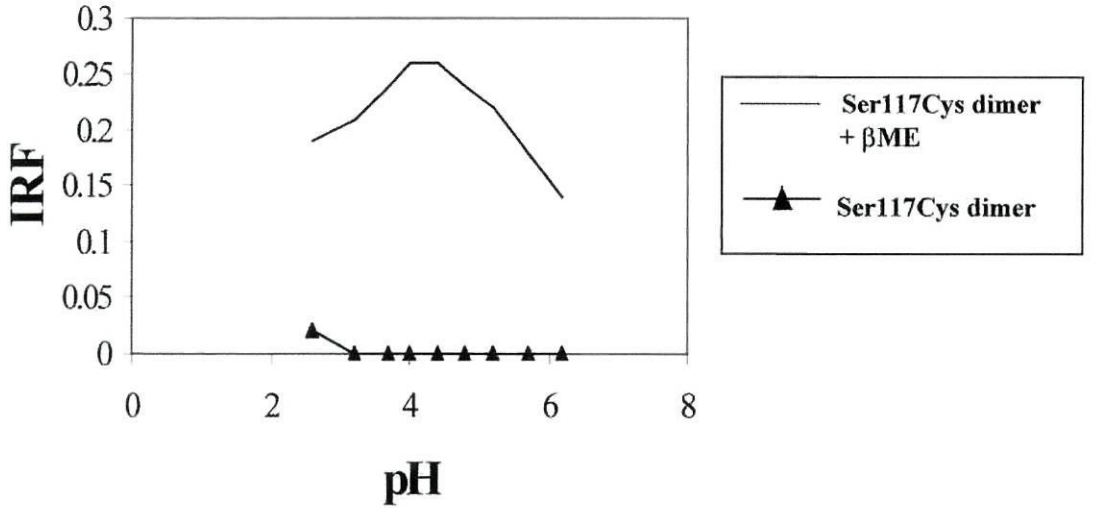


Figure 16. Monomers are required for amyloid formation.
Graphic representation of the data obtained in amyloid formation in protein preparations of Ser117Cys, upon incubation at pH range 2.3-6.2, with or without β-mercaptoethanol and tested for ThT fluorescence. Each point corresponds to the maximum peak obtained at 440 nm upon ThT binding. Similar data were obtained for Glu92Cys (not shown). IRF - intensity of relative fluorescence.

The same experiment was performed with the double mutant Val30Cys/Leu55Cys and it was seen that though this mutant eluted as a monomer at pH 3.2, no fibril formation occurred at any of the pHs assayed. Apparently, the existence of a disulphide bond, making tight monomers, prevented them from associating into fibrils, and these TTR subunits remained in solution. When we repeated the amyloid formation assay, in the presence of β-mercaptoethanol to disrupt the intramonomeric disulphide bonds, amyloid fibril formation did occur (Figure 17), indicating that unstable/disorganised monomers are necessary for fibrillogenesis.

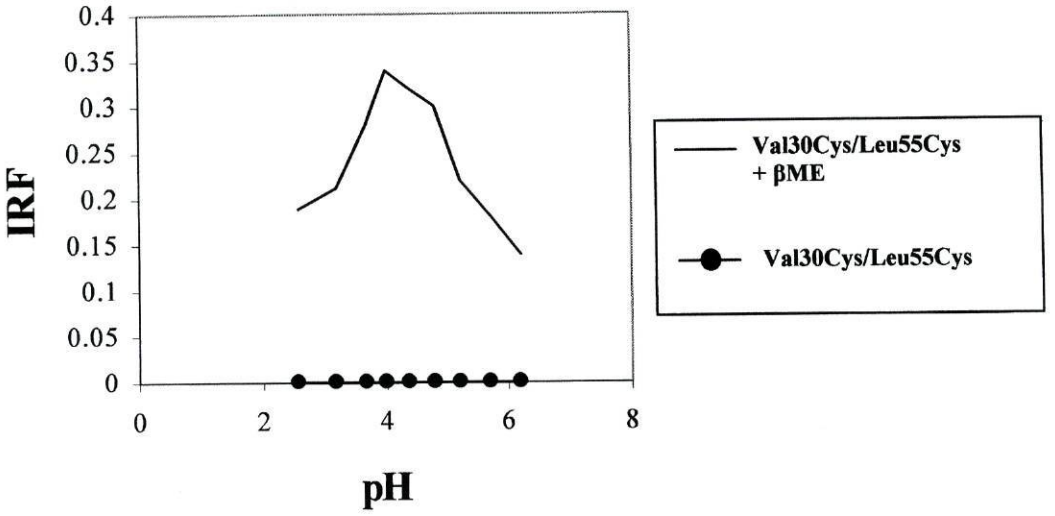


Figure 17. Unstable/Disorganised monomers are required for amyloid formation.
Graphic representation of the data obtained in amyloid formation in a protein preparation of Val30Cys/Leu55Cys, upon incubation at pH range 2.3-6.2, with or without β-mercaptoethanol and tested for ThT fluorescence. IRF - intensity of relative fluorescence.

4. DISCUSSION

To date, no unifying model for TTR amyloid formation at physiological conditions was developed, but all current hypotheses assume that the mutations in TTR affect the structural equilibrium of the molecule, destabilising the tetramer in favour of intermediates, which exhibit amyloidogenic potential. Colon and Kelly (1992) first presented biochemical evidences of the existence of amyloidogenic intermediates after partial acid denaturation, creating a model for fibril formation. They proposed that TTR could self-assemble into amyloid like fibrils in an acidic environment (simulating the lysosome), with the more amyloidogenic variants requiring less extreme conditions. X-ray diffraction studies on amyloid fibrils extracted from humor vitreous led to different models. In one case the authors explicitly say that monomers are the composing units of the fibrils (Inouye *et al.*, 1998) while in a previous work a continuous β-sheet helix composed of monomers or dimers was proposed (Blake *et al.*, 1996).

Our work aimed at clarifying the structure of the amyloid forming-units. Based on X-ray data on several TTR structures, we built highly stable tetramers (two of the "stabilised mutants") with covalent disulphide bridges between monomers from the same dimer.

The association of the two transthyretin dimers in the tetrameric structure, forms a 16-stranded β-barrel 55 Å long (Blake *et al.*, 1978b). TTR has only one cysteine (Cys^{10}) per identical subunit and its location is near the entrance of the central channel of the molecule. Both isolated recombinant TTR "stabilised mutants" bound T_4 in qualitative assays (Figure 10), which clearly shows that there are no extra disulphide bridges between the cysteines introduced in these mutants and the native Cys^{10} at the entrance of the T_4 binding pocket. The dimeric structure was conserved even at very low pH and no amyloid fibrils were formed *in vitro* from these dimeric species. Thus, our studies demonstrated that monomers, rather than dimers are the repeating structural subunit composing the amyloid fibrils, corroborating the model described by Inouye *et al.* (1998) based on X-ray analyses of vitreous fibrils from FAP patients.

Quintas *et al.* (2001) conducted aging experiments of tetrameric TTR and chemically induced protein unfolding, showing that tetramer dissociation and partial unfolding of the monomer precedes amyloid fibril formation. They concluded that amyloid fibril formation by some TTR variants might be triggered by tetramer dissociation to a compact non-native monomer with low conformational stability, which in turn would originate partially unfolded monomeric species with a higher tendency for ordered aggregation into fibrils. By tightening monomeric interactions with the

introduction of two cysteines replacing Val^{30} and Leu^{55}, we created a mutant, which under acidic conditions was stable as a monomer. The disulphide bonds in this TTR subunit might have prevented its unfolding, making impossible the formation of fibrils. Therefore, our results support the hypothesis that a partially unfolded or unstable monomer is necessary for the formation of amyloid fibrils.

Exploring the new surfaces exposed in the packing contacts revealed by the crystal structure of TTR Leu55Pro (Sebastião *et al.*, 1998), we designed a mutant with an asparagine for an aspartate substitution at position 18. Asp^{18} has close contacts with aminoacids in positions 20 and 21, holding the tetrameric structure closely packed (Figure 9, A). When an uncharged aminoacid is introduced, the AB loop must become looser, untied to the body of the tetramer, which might explain the occurrence upon dilution, in the sub-micromolar range (3 μM), of a mix of tetrameric and monomeric forms. This tetramer is unstable, although it still binds T_4 and therefore no large alterations occurred in the binding channel. The AB loop seems to represent one of the key domains in the stabilisation of the TTR tetramer; the slightest alteration that might displace this area of the molecule affecting the native fold of the structure, promotes dissociation into monomers. Two amyloidogenic mutants affecting Asp^{18}, associated with clinical phenotype (Asp18Glu and Asp18Gly) have been described. Mutations in other parts of the molecule might cause a modification in this area as Leu55Pro does.

So far, there are more than 80 mutations described for TTR. The variant TTR Thr119Met is of particular interest with regard to T_4 binding. In this variant, a methionine appears in place of a threonine at aminoacid 119 in the TTR monomer. The aminoacid at position 119 is located deep inside the T_4 binding channel and is postulated to interact with both the 4'-hydroxyl group and the 5'-iodine of T_4 (Blake *et al.*, 1978a). Substitution of a polar aminoacid for a non-polar one may allow the T_4 molecule to interact more closely with the other aminoacids involved in the binding cleft, and thus, lead to the observed stronger binding. In addition, the three-dimensional structure of variant TTR Thr119Met shows that the substitution of a methionine for a threonine at position 119 induces a movement of residue Leu^{110}, pushing it into a position closer to the ligand. This structural alteration may strengthen the binding interactions of TTR with the T_4 phenolic ring, leading to the observed increase in the binding affinity of the protein (Almeida *et al.*, 1997).

Our results concerning the susceptibility to dissociation into monomers of Leu110Ala, showed that at 3 μM dilution, and by comparison with the WT protein, two clear forms coexisted in solution in relative proportions of 0.75 of tetrameric form to 0.25 of monomeric form (Figure 11). When the preparations were diluted 10-fold, these

relative values were altered to 0.6 and 0.4, corresponding to the tetrameric and monomeric forms in solution, respectively. These results reflect the importance of Leu^{110}, not only in maintaining the T_4 binding properties of native TTR, but also in strengthening the contacts between dimers. The location of this residue, deep in the hydrophobic core of the tetramer, in the dimer-dimer interface, favoured a fast dissociation into monomers upon the substitution of a leucine for an alanine, suggesting that a high number of bonds were broken or severely affected.

The experimental evidences of this work show that monomers, rather than dimers, are the repeating structural sub-units composing the amyloid fibrils and that a rearrangement in the monomeric tertiary structure must precede fibrillogenesis. The importance of specific areas and residues for stabilising the tetrameric structure of TTR is highlighted. In particular, contacts between dimeric surfaces and destabilisation of the AB loop seem pivotal. Monomeric instability might also involve rearrangement of the C and D strands, as suggested by the occurrence of stable monomers, which did not form amyloid (carrying cysteines at positions 30 and 55). This information will help designing strategies for therapeutic intervention in FAP.

Chapter II

Search for intermediate structures in transthyretin fibrillogenesis:

I – Soluble tetrameric Tyr78Phe TTR expresses a specific epitope present only in amyloid fibrils

1. INTRODUCTION

The fibrillar structure that results from the association of TTR molecules with altered conformation is thought to be the causative agent of Familial Amyloidotic Polyneuropathy. FAP is an autosomal dominant disease characterised by amyloid deposition in the peripheral nerves. Most hereditary transthyretin-associated amyloidoses are due to single aminoacid substitutions (Saraiva, 1996).

TTR is a homotetrameric protein composed of four 127 – aminoacids subunits found both in plasma and in the cerebrospinal fluid. The X-ray crystal structure shows that each subunit is assembled as a β-sheet sandwich in which one four-stranded β-sheet interacts in a face -to-face fashion with another four-stranded β-sheet, forming the hydrophobic core of the protein (Blake *et al.*, 1978b). The strands in each β-sheet are labelled A-H, according to their position in the sequence. The residues contained between the two sheets are almost exclusively hydrophobic and constitute the core of the subunit. The subunits are approximately 50% β-sheet and contain one short region of helix. The monomers are non-covalently bound into stable dimers through extensive hydrogen bonding between antiparallel strands of β-sheet. Two dimers assemble into a tetramer by opposing their inner β-sheets and interacting mainly at loop regions adjoining β-strands G and H and β-strands A and B. Some mutations in TTR increase the susceptibility to amyloid formation (Bonifácio *et al.*, 1996).

The mechanism that converts normal soluble TTR tetramers into insoluble amyloid fibrils is still unknown. In one hypothesis, the TTR tetramer dissociates into dimers or monomers, in the early stages of the amyloidogenesis cascade. These subunits form amyloidogenic intermediates that can aggregate into amyloid fibrils (Lai *et al.*, 1996). Based on experiments using antisera raised against synthetic TTR peptides, amyloid TTR and normal TTR, Gustavsson *et al.* (1994), advanced the idea that fibrillar TTR is distorted, thus exposing new epitopes.

An amyloidogenic intermediate of TTR lacking the tetrameric fold has been previously demonstrated in studies of amyloid formation *in vitro*. Thus, by generating substituted/deleted mutants for the D-strand area (53-55) of TTR, Goldsteins and co-workers (1997), isolated aggregates, which gave a typical cross-β-pattern in X-ray diffraction studies and a positive signal with Congo red or Thioflavine T. These structures were used to raise and screen monoclonal antibodies. Two Mabs were found to display affinity to exposed separate cryptic epitopes on the TTR structure [Mab 15 and Mab (56-61)] that were only expressed in amyloidogenic conformations and in amyloid fibrils (Goldsteins *et al.*, 1999).

The clinically aggressive mutant Leu55Pro TTR that induces early onset amyloidosis has an increased tendency to self-associate into amyloid-like fibrils, even under conditions where the WT protein remains stable and in a native fold. This substitution at residue 55 disrupts the hydrogen bonds between strands D and A, exposing new surfaces involved in aggregation. Along the monomer-monomer and dimer-dimer interfaces of the proposed asymmetric unit, positional differences compared with those described for the WT TTR tetramer were detected. In particular, the contacts of the α-helix and the AB loop are different, suggesting that these regions are important in amyloidogenesis. In the WT protein, the OH group of Tyr^{78} plays an important role in maintaining the tertiary structure of the AB loop (Sebastião *et al.*, 1998). In view of this, we reasoned that by substituting Tyr^{78} of the α-helix by phenylalanine, the hydrogen bonding with the AB loop would be destabilised, yielding a potential intermediate amyloidogenic structure. We report here the characteristics of the Tyr78Phe mutant, in particular its susceptibility to amyloid formation *in vitro* and its recognition by the Mab previously described as specific for amyloid fibrils.

2. MATERIALS AND METHODS

2.1. Design of domain interface variants

The computer graphics software Frodo was used to display the three-dimensional structures of WT and Leu55Pro human TTR molecules. By replacing the variant residues into the known molecular structures of the WT and Leu55Pro human TTR molecules, we assessed the changes in hydrogen bonding and/or close contacts with other main chain or side-chain atoms.

2.2. TTR *in vitro* mutagenesis and expression

The WT transthyretin cDNA cloned into the pINTR vector, preceded by a sequence encoding the ompA leader signal and flanked by *BamHI* and *PstI* sites (Furuya *et al.,* 1991), was used to produce mutant TTR molecules by site-directed mutagenesis (Quick change site directed mutagenesis kit, from Stratagene). These mutants were constructed using two mismatched primers, introducing a 1-3 base substitution in the original sequence:

Leu55Pro: 5' CTGGAGAGCCGCATGGGCTC 3'
5' GAGCCCATGCGGCTCTCCAG 3'
Ser77Tyr: 5' CCAAATTCTACTGGAAGGC 3'
5' GCCTTCCAGTAGAATTTGG 3'
Tyr78Phe: 5' CCAAATCTTTCTGGAAGGC 3'
5' GCCTTCCAGAAAGATTTGG 3'
Trp79His: 5' CCAAATCTTACAACAAGGC 3'
5' GCCTTGTTGTAAGATTTGG 3

All PCR amplified products were treated with *DpnI* to digest non-mutated parental DNA template, and 1/10 of the reaction volume containing the circular, mutated dsDNA, was used to transform *Epicurean coli* XL1-Blue supercompetent cells. Small DNA plasmid preparations were produced and tested by sequencing. Mutagenised TTR was expressed in an *Escherichia coli* expression system as described by Furuya *et al.* (1991).

2.3. Isolation and Purification of TTR variants

E. coli strain BL-21 was transformed with individual expression plasmids and the periplasmic space contents were obtained by osmotic shock. The supernatant was fractionated on DEAE-Sephadex and TTR containing peaks were dialysed O/N against water and lyophilised. Further purification was achieved by preparative gel electrophoresis, followed by a gel filtration using a 1.5x50 cm BioRad column packed with Biogel P-100 (BioRad) equilibrated with appropriate buffer (0.1 M KH_2PO_4, 0.1 M Na_2SO_4).

2.4. Gel electrophoresis

Proteins were analysed either in native or denaturing conditions. Native electrophoresis was carried out on an 8% acrylamide gels system and on a gradient system (4 - 16%). Electrophoresis under denaturing conditions was performed in SDS-PAGE gels (15% acrylamide, 0.1% SDS) with or without heat treatment of samples and with or without addition of β-mercaptoethanol (0.1 M).

2.5. Immunoprecipitation of TTR mutants

A small volume of protein G (50 μl) was mixed with 20 μg of Mab 15, for 15 minutes, at RT. After short spins at 14000 rpm, pellets were washed several times with 50 mM Tris, pH 7.5, with 0.05% Tween-20. The last wash was performed in the same buffer, without detergent. 20 μg of mutant TTR were added to the mix, and incubated for 15 minutes at RT. Spins and washes were performed as described above and the last ressuspension was done in PBS. After a short spin the pellet was dissolved in 100 μl of SDS-PAGE loading buffer (with or without β-mercaptoethanol). One tenth of this solution was loaded on 15% acrylamide denaturing gels, and these were transferred to nitrocellulose membranes. Proteins were transferred from gels into nitrocellulose membranes (Hybond ^{TM} -C pure, Amersham), using a Tris-Glycine system, for 1 hour, at 1 mA/cm^2 of membrane. After blocking and washing with PBST, the membrane was incubated with rabbit anti-TTR polyclonal antibody (DAKO), 1:1000 dilution in PBS, for 1 hour at RT. New washes were performed and a last incubation with anti-rabbit

Immunoglobulins - horseradish peroxidase conjugated (Binding Site), 1: 1000 dilution, for 1 hour at RT. TTR was visualised using the ECL method (Pierce).

2.6. T_4 Binding assay

0.5 – 2 µg of soluble TTR samples were incubated with radiolabelled T_4, for ½ hour, and the bound TTR-T_4 complex was visualised by autoradiography of native acrylamide gels, as described by Saraiva *et al.* (1988).

2.7. Size-exclusion HPLC

All TTR preparations destined for gel filtration chromatography were diluted in 20 mM sodium phosphate buffer, 150 mM sodium chloride buffer, pH 7.0. Protein concentration was determined spectrophotometrically, at 280 nm, using an extinction coefficient of 7.76×10^4 M^{-1} cm^{-1} based on a 55kDa molecular weight for TTR [A_{280} (1%) = 14.1 mg^{-1} ml cm^{-1}].

HPLC was performed on a Beckman Ultraspherogel SEC2000 column, coupled to a Gilson high precision pump P-302 and a Gilson holochrome-280 UV detector. Gel filtration chromatography buffer was used as eluent at a flow rate of 0.5 ml/min and different TTR variants were injected, each in a volume of 200 µl. Column calibration was performed using four protein standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). Before each TTR preparation was injected, the column was allowed to equilibrate with 2-5 column volumes of chromatography buffer and was cleaned with 0.5 M NaOH between injections.

2.8. Preparation of amorphous aggregates

TTR preparations were precipitated with 5% TCA in the cold, for 10 minutes and centrifuged at 14,000 rpm, for 10 minutes. Precipitated protein was washed thoroughly with PBS and ressuspended in appropriate buffer.

2.9. Preparation of amyloid fibrils:

100 to 500 μg of each mutant were incubated in 0.05 M sodium acetate/0.1 M KCl buffer on a pH scale from 2.6 to 6.8, for 48 hours, at RT, to form amyloid fibrils (Bonifácio *et al.*, 1996). Glycine-NaOH 50 mM, pH 9.0 buffer was then added to each solution and the amyloid fibrils were tested for ThT fluorescence. Excitation spectra were recorded on a Jasco FP-770 spectrofluorometer at 25 ^{0} C with 30 μM ThT (Fluka) in 50 mM Glycine-NaOH buffer, pH 9.0 in 1 ml assay volume. Excitation and emission slits were set to 5 and 10 nm, respectively. Preparations showing the characteristic excitation maximum at 440 nm upon ThT binding were used in binding experiments and the fibrils concentration was determined.

2.10. Enzyme-linked immunosorbent assay

Microtiter plates (Nunc, 96 wells) were coated with rabbit anti-TTR polyclonal antibody (DAKO) in a 1:500 dilution in coating buffer (K_2HPO_4, pH 8.0), at 37^{0}C, for 2 hours. After blocking with 5% non-fat dry milk in PBS and washing 3x with PBST and 2x with PBS, plates were incubated with TTR preparations, for 1 hour, at RT. Bound protein was probed with Mab 15 in pure unfractionated hybridoma culture supernatant or with purified monoclonal antibody (1:100 dilution in PBST) for 1 hour, at RT. Finally, after a last incubation with mouse anti-immunoglobulins-HRP conjugated (Amersham) (1:1000 dilution in PBST) the plates were developed and read at 411 nm on a Bio-Tek BL_X 800 microplate reader.

2.11. Surface Plasmon Resonance spectroscopy

Real time biospecific interaction analysis was performed using Biacore 2000 (Pharmacia Biosensor, Sweden). In brief, the principle of this approach is as follows. Surface plasmon resonance (SPR) is used to detect changes in optical properties at the surface of a sensor chip, that consists of a glass slide covered with a thin gold film bound to a carboxylated matrix of the dextran. (Löfås and Johnsson, 1990). The dextran matrix of the sensor chip is used to covalently bind one of two or several reactants (the ligand), while the others are allowed to pass over the surface (analytes). The resonance angle depends on the refractive index in the vicinity of the surface,

which changes as the concentration of the molecules on the surface is modified. The changes in the resonance signal are referred to as resonance units (RU). Monitoring the resonance angle continuously in real time generates a sensorgram, from which the association rates constant (k_a) and dissociation rate constant (k_d), as well as the affinity constant (K_A), can be determined by the Biosensor software (Figure 18).

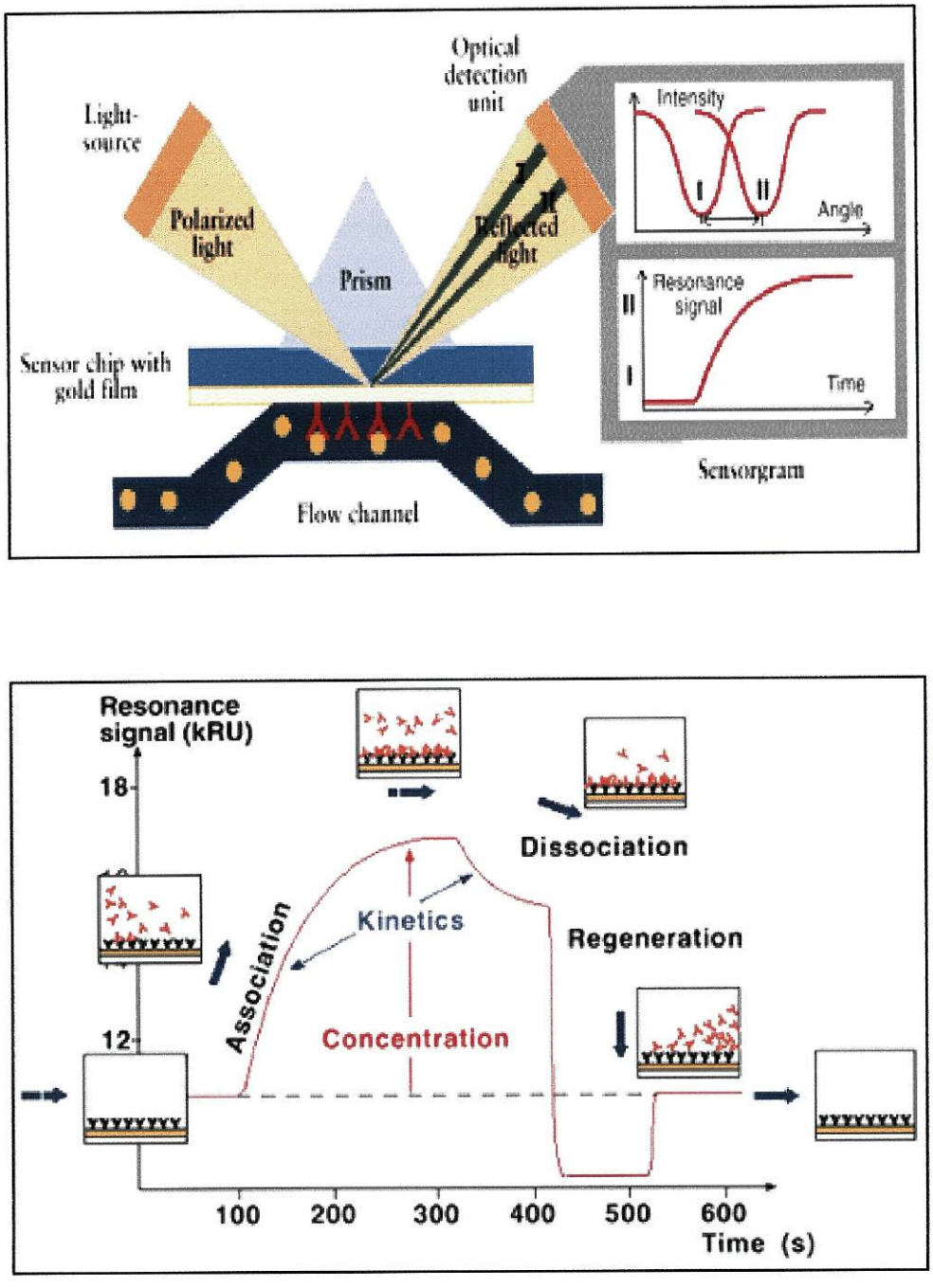


Figure 18. Surface plasmon resonance

Equipment and reagents: The BIAcore system, sensor chip CM5, surfactant P20, amine coupling kit containing N-hydroxysuccinimide (NHS), (N-ethyl-N´-(dimethyl-aminopropyl)-carbodiimide (EDC), and ethanolamine-hydrochloride, were purchased from Pharmacia Biosensor, Sweden.

Preparation of sensor chip surfaces: Mab 15 and anti-TTR polyclonal antibody (DAKO) were immobilised according to standard procedures. Antibodies were solubilised in a 10 mM Na-acetate coupling buffer, pH 5.0. To obtain an optimal capture level, the concentration was adjusted to 100 μg/ml and the time of activation by the NHS/EDC mixture, set to 4 minutes. The immobilisation procedure was carried out at a continuous flow rate of the running buffer HBS, pH 7.4 (Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM, P20 surfactant 0.05%) of 5 μl/min, whereas the real-time analysis of the specific interactions was carried out at a flow rate of 10 μl/min.

Evaluation of kinetic constants: To determine association rate (k_a) and dissociation rate (k_d) constants, BIAcore "kinetic evaluation" software was used. The affinity constant (K_A) was taken as the ratio of k_a/k_d. The software gives the amount of bound analyte (Ra) to the ligand, as well as the reaction rate (dRa/dt) at specified time intervals. R_{amax} is the maximal amount of bound analyte to the ligand, and C is the concentration of the analyte. The equation of the dR_a/dt as a function of R_a can be written as follows:

$$dR_a/dt = k_a R_{a\,max} C - R_a (k_a C + k_d)$$

To calculate k_a, parts of the dR_a/dt versus R_a curve that reflect association are chosen. The slope of dR_a/dt versus R_a curve at each concentration is plotted versus the concentrations in use, to give a straight line. This new slope gives the association rate constant and the dissociation rate constant is calculated from a specific time frame (t_1 - t_n) in the dissociation phase. The slope of the plot ln (R_{t1}/R_{tn}) versus t_n - t_1 g

3. RESULTS

3.1. Tetramer stability

We investigated the resistance to dissociation of the Tyr78Phe tetramer by native and SDS-PAGE and compared it with the WT protein. Under native conditions, with or without addition of β-mercaptoethanol, WT and Tyr78Phe proteins migrate as a tetramer (data not shown). When separated on denaturing PAGE, non-boiled samples of Tyr78Phe, with or without β-mercaptoethanol migrate as a tetramer, similarly to the WT protein. Upon boiling, both Tyr78Phe and WT TTR migrate as monomers, as shown in Figure 19. Thus, under these electrophoretic conditions, Tyr78Phe dissociates in a similar pattern as the WT protein.

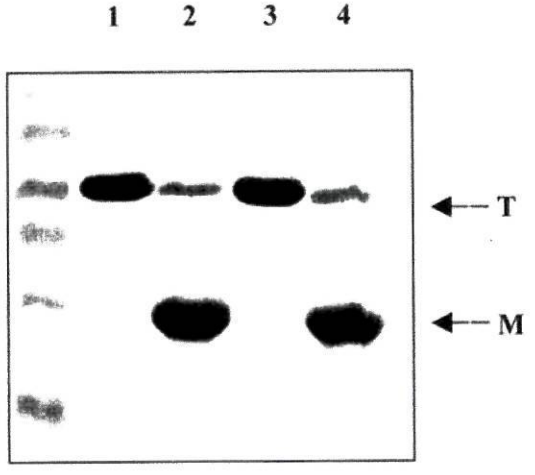


Figure 19. SDS-PAGE of recombinant WT TTR and Tyr78Phe mutant TTR.
Samples in lane 2 and 4 were boiled and those in lanes 1 and 3 were not boiled. Lanes 1 and 2 - WT TTR. Lanes 3 and 4 - Tyr78Phe mutant TTR. All samples were run in the presence of β-mercaptoethanol. T - Tetramer; M - Monomer.

The tetrameric nature of Tyr78Phe was further tested by HPLC, comparing the elution pattern of 3 μM solutions of Tyr78Phe and WT TTR. Chromatography yielded a single form, which eluted at 20 minutes. This complex corresponds to a tetrameric form of the protein, with a size of 55 kDa with a total absence of aggregates and/or monomers (data not shown).

To assess whether this mutant could bind thyroxine, a preparation of Tyr78Phe TTR was incubated with radiolabelled T_4 and its behaviour compared with the WT

protein in a human serum sample. The mutant retained the ability to bind the ligand, indicating a functional tetrameric structure.

3.2. Susceptibility to amyloid formation *in vitro*

Tyr78Phe was tested for its susceptibility to amyloid formation *in vitro*. After acidification, the tetramer dissociates into monomers and favours aggregation. Upon addition of ThT, we measured the fluorescence of protein solutions that had been pre-incubated at different pHs (2.7 - 6.2). Figure 20 shows that Tyr78Phe is as highly prone to form amyloid-like fibrils as the natural occurring mutant Leu55Pro, whereas the WT protein forms less amyloid-like fibrils and requires more acidic conditions to do so. This suggests that under acidic pH the tyr → phe substitution at position 78 favours aggregation into fibrils.

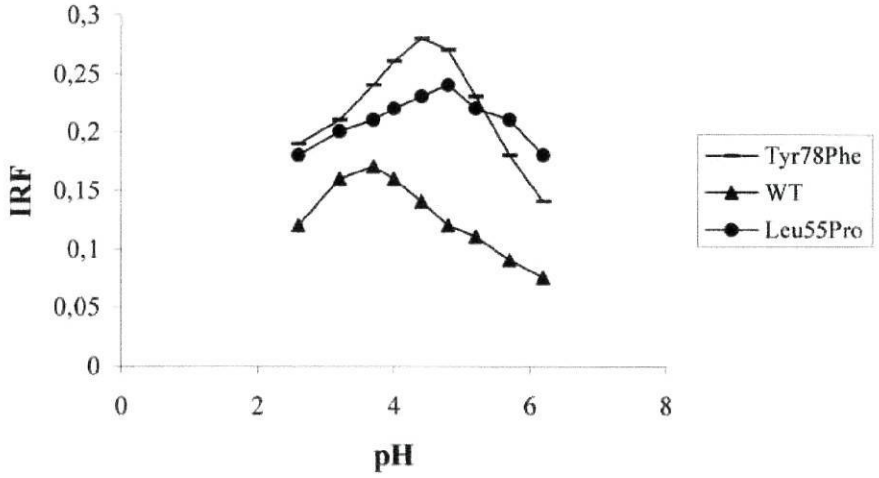


Figure 20. Graphic representation of the data obtained for amyloid formation in protein preparations of Leu55Pro, WT and Tyr78Phe TTR, upon incubation at pH range 2.7 - 6.8, and tested for ThT fluorescence (section 2.9. Materials and Methods). Each point corresponds to the maximum peak obtained at 440 nm upon ThT binding. IRF - intensity of relative fluorescence.

3.3. Determination of amyloid epitopes

The Tyr78Phe mutant was tested for Mab 15 binding by sandwich ELISA (used previously to detect fibrils and amyloidogenic folding of certain mutants, (Goldsteins *et al.*, 1999). WT, Leu55Pro, Trp79His and Ser77Tyr were also tested, but only the wells

containing either synthetic TTR fibrils or the Tyr78Phe mutant gave a positive reaction (data not shown).

To further assess the specific role of residue 78 in the expression of amyloid epitopes, we immunoprecipitated soluble preparations (pH 7.0) of Ser77Tyr, Tyr78Phe and Trp79His mutants (Figure 21) and verified that with or without reducing agent, the only immunoprecipitated product after boiling, was Tyr78Phe TTR. This experiment further confirmed the previous observations, that in fact an epitope present on the tetrameric form of the Tyr78Phe mutant is recognised by this monoclonal antibody. In particular, the location of residue 78 in the α-helix is crucial with respect tetramer "opening" and exposing a new domain recognised by the monoclonal antibody. The mutations in the two residues flanking Tyr^{78} did not cause similar alterations on the TTR molecule.

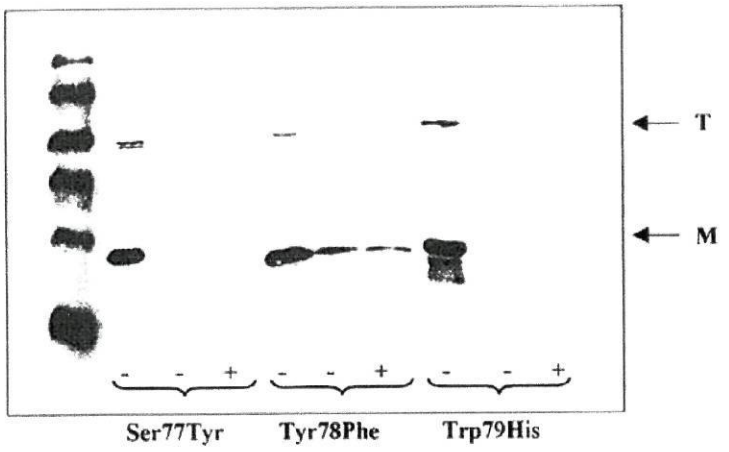


Figure 21. Immunoprecipitation of TTR mutants with Mab 15.
The three groups of lanes represent boiled samples of three different mutants: Ser77Tyr, Tyr78Phe and Trp79His, respectively. The first lane of each group represents the original protein preparations (before immunoprecipitation) and the second and third lane of each group represent immunoprecipitated samples, with (+) or without (-) addition of β-mercaptoethanol. T - Tetramer; M - Monomer.

3.4. Real time biospecific analysis and kinetic measurements

3.4.1. Characterisation of Mab 15 using SPR

Surface plasmon resonance (SPR) spectroscopy is a technique suitable for real-time studies of molecular interactions. We tested the specificity of Mab 15 in the recognition of cryptic epitopes using this technology, comparing the behaviour of this Mab with an anti-TTR polyclonal antibody.

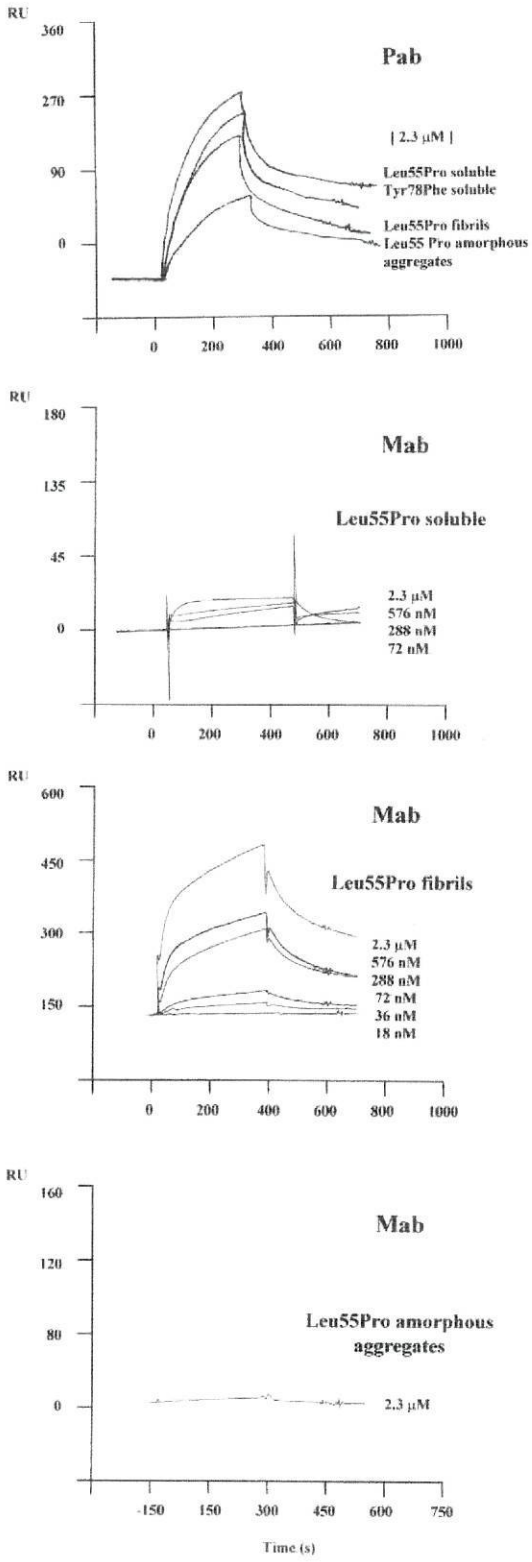


Figure 22. (legend see next page)

Figure 22. Interaction between anti-TTR polyclonal antibody (Pab) and Mab 15 coupled on a CM5 sensor chip, and different mutant TTR preparations.
Top panel: Interaction of Pab with several TTR preparations (2,3 μM); Upper-middle panel: Interaction of Mab 15 with different concentrations of soluble Leu55Pro; Lower-middle panel: Interaction of Mab 15 with different concentrations of synthetic Leu55Pro fibrils; Bottom panel: Interaction of Mab 15 with Leu55Pro amorphous aggregates. RU – resonance units.

Different TTR preparations (synthetic Leu55Pro fibrils, soluble Leu55Pro, soluble Tyr78Phe and Leu55Pro amorphous aggregates) were assayed for ThT binding and then used as analytes. TTR preparations (final concentration, 2,3 μM) were allowed to interact with the immobilised polyclonal antibody. As shown in Figure 22, top panel, all four analytes interacted with the antibody within a binding range of 70 to 140 RU.

The interactions between the four analytes and the immobilised Mab 15, in three separate experiments with protein concentrations ranging from 9 nM - 2,3 μM, were also analysed by SPR. The preparation of Leu55Pro amorphous aggregates and all soluble preparations under analysis were ThT negative. As expected, the soluble Leu55Pro mutant TTR did not bind (Figure 22, upper-middle panel) whereas evident interaction was seen for all concentrations of the synthetic Leu55Pro fibrils preparation (Figure 22, lower-middle panel). Furthermore, no binding was observed when the Leu55Pro amorphous aggregates were assayed for interaction (Figure 22, bottom panel). The behavioural differences between the polyclonal and the monoclonal antibodies clearly corroborate the specificity of Mab 15 in recognising specific epitopes that are detected only in amyloid fibrils or in structures with an amyloidogenic fold.

3.4.2. Soluble Tyr78Phe might represent an early event in *in vitro* amyloidogenesis

To confirm our previous observations suggesting that Tyr78Phe in its soluble form exhibited a fold that could be recognised by Mab 15, we studied the binding of this monoclonal antibody to the mutant TTR. We also determined the k_a and k_d for this interaction.

All preparations of soluble Tyr78Phe interacted with Mab 15 (Figure 23) and the pattern of interaction curves obtained was very similar to those of the synthetic Leu55Pro fibrils (Figure 22, lower-middle panel).

Five of the curves obtained for the Mab 15-Phe78 interaction, corresponding to analyte concentrations from 288 nM to 9 nM, were analysed using the BIAevaluation software. Subtraction of unspecific binding to the sensor chip matrix was performed using the curve obtained from an empty flow cell of the same sensor chip, previously activated and de-activated (section 2.11. Material and Methods).

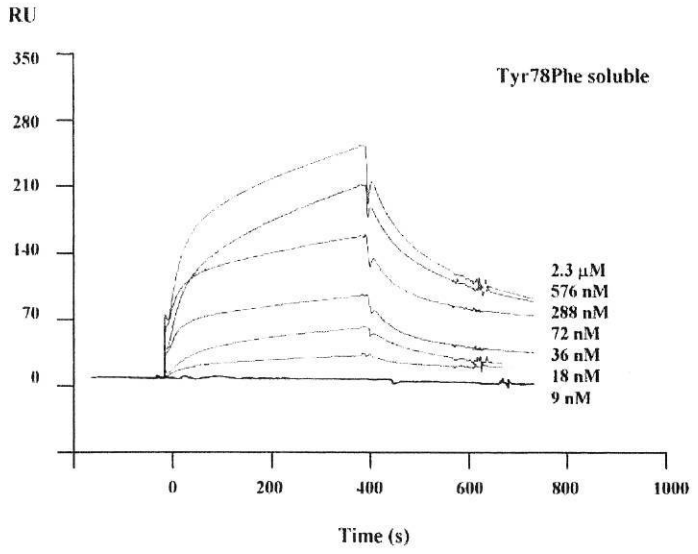


Figure 23. Interaction between Mab 15 coupled on a CM5 sensor chip, and a series of concentrations of soluble Tyr78Phe (2,3 µM – 9 nM). RU – resonance units.

The affinity of Mab 15 to soluble Tyr78Phe TTR was in the range of 10^7 M^{-1}. Table IV shows the association rate, dissociation rate and affinity constants calculated from the corresponding data points in the dR_a/dt versus R_a curves.

Table IV. Association, dissociation rate and affinity constants for the Mab 15-soluble Tyr78Phe mutant TTR interaction.

Mab 15 - Soluble Tyr78Phe interaction	
k_a (s^{-1} M^{-1})	1.59×10^5
k_d (s^{-1})	1.2×10^2
K_A (M^{-1})	1.32×10^7

Since the reliability of the constants obtained is to a large extent related to how well the model fits the experimental data, we followed the guidelines available in the Biosensor software to evaluate any discrepancies/deviations. Our results were consistent over a range of analyte concentrations and there were no significant differences between the fitted curves and the experimental data, exhibiting a noise level below 0.5 RU (data not shown). We tried to determine an affinity constant for the Mab 15-synthetic Pro55 fibrils interaction, but none of the models used produced reasonable fittings. This was probably due to the fact that several binding interfaces present in the fibrillar structure interact simultaneously, a reaction that cannot be easily analysed with simple kinetic models.

To assure that the state of aggregation of the Tyr78Phe protein preparations injected in the fluidic system had not been altered with manipulations, samples that had interacted with the Mab in the sensorchip, were recovered and analysed. All preparations were negative for ThT binding, and when submitted to size-exclusion chromatography, only a tetrameric form was eluted at 20 minutes (data not shown). Sandwich ELISA was performed on the eluted fractions of Tyr78Phe and WT preparations using both a Pab and Mab 15. The results reproduced in Figure 24 show that both antibodies recognise the peak of tetrameric Tyr78Phe TTR form present in fractions 19-22, whilst for the WT TTR, the same peak is only recognised by the polyclonal antibody. Thus, non-aggregated, tetrameric Tyr78Phe is recognised specifically by Mab 15.

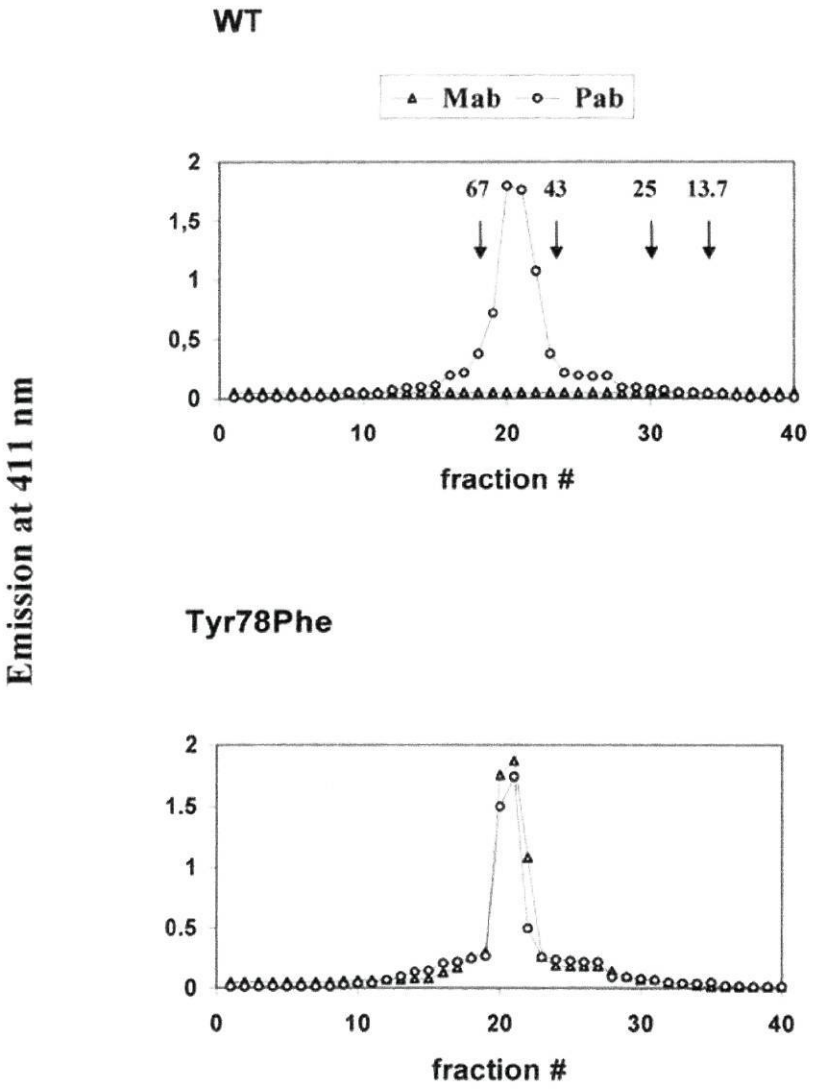


Figure 24. Immunodetection of TTR by sandwich ELISA with Mab 15 and anti-TTR polyclonal antibody (Pab). Fractions were obtained after gel chromatography of both WT and Tyr78Phe preparations that passed on the BIAcore sensorchip. The OD_{280} profile obtained for both TTR preparations (not represented) overlapped immunodetection with Pab. The protein standards used to calibrate the column were: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

4. DISCUSSION

TTR amyloid fibril development is believed to be preceded by destabilisation and partial misfolding of the native protein. So far, no unifying model for TTR amyloid fibril formation at physiological conditions has been developed. At pH 7.0 the stable TTR entity in plasma, even for highly amyloidogenic variants, is the tetramer. All current hypotheses assume that the mutations somehow affect the tetramer-dimer-monomer equilibrium by destabilising the tetramer in favour of monomeric or dimeric intermediates that have an intrinsic amyloidogenic potential. It is very unlikely that TTR amyloid fibrils are derived from tetrameric protein subunits. It is impossible to align native tetramers into the established anti-parallel β-pleated sheet model of amyloid fibrils (Inouye *et al.,* 1998). Furthermore, our data support the theory that monomers rather than dimers are the repeating structural subunits composing the amyloid-like fibrils. Dimeric TTR mutants covalently bound by disulphide bridges were unable to polymerise into amyloid-like fibrils even at pH 3.2 (Redondo *et al.,* 2000a).

Kelly *et al.* (1994) developed a model based on fibril formation from an amyloidogenic monomeric intermediate after partial acid denaturation. In *in vitro* biophysical studies, they demonstrated that TTR could self-assemble into amyloid like fibrils in an acidic environment simulating the lysosome, with the more amyloidogenic variants requiring less extreme conditions. This amyloidogenic intermediate was postulated to contain most of the native structure except for the rearrangement involving strands C and D. In this model, FAP mutations would not affect the structure of the folded state (tetramers). Instead, they would favour the denaturation pathway and/or degradation pathway(s) for TTR turnover. A weakness of this model is the assumption that amyloid fibril formation *in vivo* occurs under acidic conditions as found in the lysosome, since this would imply the formation of amyloidogenic intermediates intracellularly. This has never been observed; TTR amyloid deposits always occur extracellularly (Coimbra and Andrade, 1971).

Acid denaturation of particular TTR variants: Thr119Met, Val30Met and Leu55Pro revealed that the amyloidogenicity of the variants was correlated with the aggressivity of clinical expression (Bonifácio *et al.,* 1996). Leu55Pro has been associated with early onset and highly aggressive amyloidosis (Jacobson *et al.,* 1992) whereas Thr119Met was clinically protective in compound heterozygotic individuals for Met30/Met119 (Coelho *et al.*, 1996). Furthermore, isoelectric focusing analysis on serum of Val30Met and Thr119Met carriers, demonstrated that there was a striking difference on the resistance of TTR to dissociation into monomers (Alves *et al.*, 1997b).

While these studies present evidence that there must be intermediates in the fibrillogenesis pathway with altered conformation, information at the molecular level is necessary to fully understand the process.

X-ray crystallographic comparison studies of the WT and amyloidogenic TTR variants help explain the aggregation pathway at the molecular level. X-ray studies conducted by Sebastião *et al.* (1998) on the Leu55Pro variant, revealed conformational changes in the tetrameric structure of the protein, possibly responsible for its instability and for the exposure of new molecular surfaces involved in TTR polymerisation. The refinement of this crystal structure showed that the pro → leu substitution disrupted the hydrogen bonds between strands D and A, resulting in significantly different interface contacts from those of the WT protein. The disruption of strand D and formation of a long loop connecting strands C and E, including residues 54-56, further modified the secondary structure of Leu55Pro. This loop is involved in the crystallographic packing of the molecules, forming the outer part of the asymmetric unit, which is constituted by eight monomers. The packing interactions present in this crystal structure are significant and it is interesting to observe that one of them corresponds to the interaction of the side chain of Arg^{21} with the main chain of Gly^{83}, from the α-helix and belonging to a different monomer. Based on the novel crystallographic findings concerning the Leu55Pro structure, and exploring the new surfaces exposed in the packing contacts revealed for this crystal, we designed the mutant with a tyr → phe substitution at position 78.

When compared with the WT TTR and the Leu55Pro variant for its susceptibility to amyloid formation Tyr78Phe, revealed a similar tendency to form fibrils as the aggressive Leu55Pro variant. Tyr^{78} belongs to the α-helix region and is hydrogen bonded to Asp^{18} from the AB loop. When this interaction is disrupted it is possible that a subtle change occurs in the helix and a more drastic alteration happens on the AB loop. Thus, the substitution in Tyr78Phe results on the removal of the OH group from the tyrosine. As a consequence, the hydrogen bond between residues 78 and 18 is no longer present and we might have i) a disordered Asp^{18}, which does not form hydrogen bonds to the main chain, ii) Asp^{18} keeping the hydrogen bonds to groups NH of Val^{20} and Arg^{21}, but not tied to the body of the molecule (Figure 25b). Both situations are consistent with a modified/looser AB loop, which will affect the surface of the molecule and the dimer-dimer contacts (see Figure 25a). This modification increases the susceptibility of the molecule to *in vitro* amyloid formation. Interestingly, Tyr78Phe has recently been observed as a natural occurring mutation in an Italian patient with TTR amyloidosis (Anesi *et al.*, 2001).

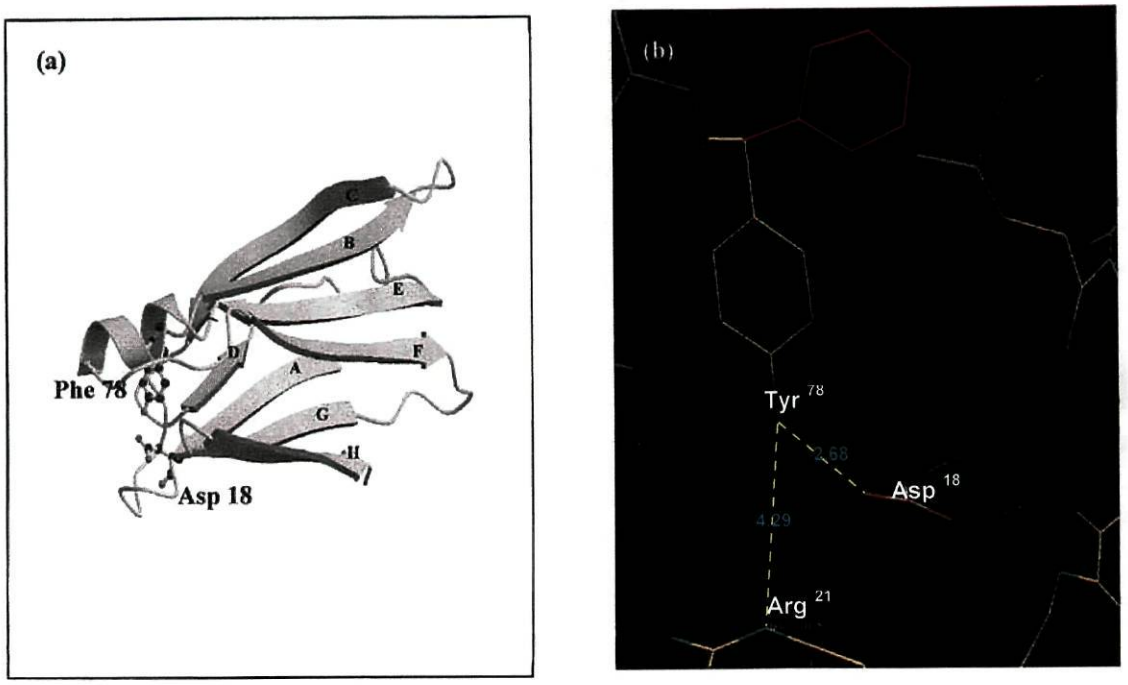


Figure 25. Ribbon representation **(a)** of the WT transthyretin monomer, showing the close proximity of residue 78 in the α-helix and residue 18, part of the AB loop. The strands composing the two sheets of the monomer are labelled A-H. The hydrogen bonds (yellow dotted lines) connecting the normal Tyr78 residue with Asp18 and Arg21 of the AB loop are represented in detail in **(b)**; the substituted Phe78 residue is represented in violet.

A peptide containing residues 71-93 of the α-helix has been studied by NMR in order to find out the effects of the amyloidogenic Ser^{84} substitution (Wilce *et al.*, 1999). It was reported that the changes introduced by the mutation are subtle and the authors foresee that the same happens to the structure of the protein. In the chicken TTR molecule, the helical structure between residues 75 and 83 is lost, although those regions differ from the human sequence only for residues 76 and 81. Even with this major conformational alteration, Tyr^{78} remains hydrogen bonded to Asp^{18} in both structures (Duan *et al.*, 1991). It is interesting to observe that Tyr^{78} and Asp^{18} are aminoacids which are conserved in all organisms for which the TTR structure is available, fact that re-enforces their important role in the stability of the TTR tetrameric structure throughout evolution (Schreiber and Richardson, 1997).

We observed that Tyr78Phe TTR exhibits similar binding properties to Mab 15 as amyloid fibrils do, whereas other soluble mutants do not bind this antibody. Mab 15 has been previously reported as being able to recognise cryptic epitopes exposed only in amyloid fibrils and in mutants with an amyloidogenic folding and thus performs a major role in recognising conformational intermediates. In addition it recognises modified circulating TTR species in the sera of FAP patients, as suggested by Palha

and co-workers (2001) after verifying reactivity of this Mab with sera of patients with different TTR mutations, but not in control healthy individuals. It was "surprising" to find a stable, tetrameric soluble mutant that also exhibits an amyloidogenic conformation. Tyr78Phe exposes cryptic sites that cannot be detected in other soluble mutants such as Leu55Pro, Ser77Tyr, and Trp79His here tested.

We believe that the amyloidogenic mutations introduce very little, yet significant, structural changes in TTR promoting the dissociation of the molecule into monomers with altered tertiary structure. The Tyr78Phe variant seems to exhibit the characteristics of an intermediate structure in the fibrillogenesis pathway. Its tetrameric structure may represent an early event in the *in vitro* amyloidogenesis cascade in which a conformational change in the TTR molecule occurs before subunit dissociation and further polymerisation into fibrils. Crystallographic data by X-ray on Tyr78Phe crystals will allow a better characterisation of the interaction of the protein with the antibody and consequently the definition of the regions that exhibit an amyloidogenic fold.

Ligands interacting with TTR modified structures resembling Tyr78Phe, may be able to arrest the amyloidogenesis cascade at an early phase avoiding the generation of monomeric species, the building blocks of amyloid fibrils.

Chapter III

Search for intermediate structures in transthyretin fibrillogenesis:

II - Peptide aptamers as molecular probes for TTR amyloidogenic epitopes

1. INTRODUCTION

In view of current structural and biochemical evidences, a potential line of therapy in FAP is the blockage of the transformation of soluble native TTR molecules into amyloidogenic intermediates. Therefore, a more detailed effort in identifying conformational amyloidogenic intermediates in the fibrillogenic cascade and the cryptic amyloidogenic sites that become exposed in those steps is fundamental.

Based on experiments using anti sera raised against synthetic TTR peptides, amyloid TTR and normal TTR, Gustavsson *et al.* (1994) advanced the idea that fibrillar TTR is distorted, thus exposing new epitopes. Goldsteins and co-workers (1999) showed that this could be tested by generating monoclonal antibodies against an amyloidogenic intermediate of TTR, lacking the tetrameric fold. Two Mabs were found to display affinity to exposed separate cryptic epitopes on the TTR structure [Mab (39-44) and Mab (56-61)] that were only expressed in amyloidogenic conformations and in amyloid fibrils. Thus these antibodies performed a major role in recognising conformational intermediates as also seen in the previous biochemical study involving an engineered TTR mutant (Tyr78Phe) and by work of Palha *et al.* (2001) after verifying reactivity of Mab (39-44) [Mab 15] with sera of patients with different TTR mutations, but not in control healthy individuals, suggesting the circulation of modified soluble TTR species in FAP patients.

To further investigate conformational amyloidogenic intermediates with exposed cryptic surfaces, we screened a 20-mer-aptamer library (2.7×10^9 transformants) developed for the "interaction trap" by Colas *et al.* (1996), using the genetic assay based on the yeast two-hybrid system described by Chen *et al.* (1996). In this system, the active site loop of *E.coli* thioredoxin (TrxA) is used as the scaffold to display conformationally constrained peptides, which can recognise distinct combinations of shape, charge and hydrophobicity.

1.1. Strategy

The yeast two-hybrid assay is based on the fact that many eukaryotic transacting transcriptional regulators are composed of physically separable, functionally independent domains: a DNA-binding domain (DNA-BD) that binds to a specific promoter sequence and an activation domain (AD) that binds to the basal transcription apparatus and directs the RNA polymerase II complex to transcribe the gene downstream of the DNA-binding site (Keegan *et al.*, 1986); Ma and Ptashne,

1987). Both domains are required to activate a gene and normally (as in the case of the native yeast Gal4 protein), the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus cannot activate the responsive genes. However, if the DNA-BD and AD can be brought into close proximity in the promoter region, the transcriptional activation function will be restored. In a two-hybrid system, two different cloning vectors are used to generate fusions of the DNA-BD and AD domains to genes encoding proteins that potentially interact with each other, and the recombinant hybrid proteins are co-expressed in yeast. An interaction between a target protein (fused to DNA-BD) and a library-encoded protein (fused to the AD) creates a novel transcriptional activator with specific DNA binding affinity (determined by the DNA binding domain). This factor then activates reporter genes having the specific DNA binding sites on their promoter regions and this makes the protein-protein interaction phenotypically detectable.

The number of positives depends of many factors: yeast strain, selection stringency, library vector, and several others. When different reporter genes are under the control of different promoters, many false positives are automatically eliminated. Yeast strains differ in the amount of leaky HIS3 expression and this background growth can be suppressed by adding 3-amino-1,2,4-triazole (3-AT) to the screening plates. If *bait* protein exhibits low level of autonomous activation, no 3-AT or low amounts of this inhibitor (5-15 mM) should be added, but too much may kill HF7c or CG-1945 strains. When using Y190 strain, 25 to 49 mM is needed to suppress leaky HIS3 expression. Also, the strength of reporter promoters may vary between strains (more or less positives). When using low-level expression vectors (like pGBT9), weak interactions might be missed but mildly toxic proteins to yeast can be detected. If high-level expression vectors are used, weak interactions can be detected but mildly toxic proteins will be lost.

The yeast strain Y190 provides a dual selection system to efficiently screen cDNA expression libraries for *bait* interactors. The strain carries two chromosomal reporter genes whose expression is regulated by GAL4: the first reporter is the *E.coli* lacZ gene which is under the control of the GAL1 promoter, and the second reporter is the selectable HIS3 gene whose regulatory sequences from the HIS3 promoter have been replaced by the $GAL1_{UAS}$. Because Y190 is deleted for GAL4 and its negative regulator GAL80, no expression of either reporters should be seen in the absence of exogenous Gal4. The very low levels of the enzyme (imidazole glycerol phosphatase (IGP) dehydratase (the HIS3 gene product) required for histidine prototrophy, make this selection very sensitive, such that proteins only weakly interacting can be selected.

Different TTR target molecules were employed as *baits* in this work, in order to identify *in vivo* interactors out of the 20-mer-peptide library, specific for soluble or aggregated TTR. Such selection ensured that the aptamers functioned *in vivo*. In this strategy we used two antibodies, a polyclonal anti-TTR and Mab 15 as immunological markers to select and differentiate yeast colonies expressing soluble TTR or containing TTR amyloidogenic aggregates. The interaction of aptamers from the library with different TTR *bait*s was next tested, and results were analysed based on the comparison of recognition patterns of those two antibodies, before and after interaction took place. Several results using this same approach demonstrated that peptide aptamers could disrupt specific protein interactions *in vivo* and thus allow their precise manipulation (Colas et al, 1996). Other authors have used two-hybrid systems to select aptamers that recognise specific proteins and allelic variants with K_Ds and half-inhibitory concentrations from $1x10^{-8}$ to $5x10^{-11}$ M (Brent *et al.*, 1997).

Y190 cells expressing WT TTR or Leu55Pro TTR fused to the GAL4 DNA-BD were transformed with an activation domain-tagged peptide aptamer library (Figure 26B). Interacting hybrids were isolated by selecting for His^+ prototrophs and subsequently screened for β-galactosidase activity. Further analysis of His^+-blue colonies revealed the existence of putative positives as well as false positives. *In vitro* studies were performed in order to validate the interactions between five selected aptamers and TTR forms as well as evaluating their specificity and effects in fibrillogenesis and competition with the two antibodies. This was tested by several approaches, including: dot-blot screening of yeast protein extracts, ELISA competition assays and BIAcore competition studies and kinetic determinations for the affinity of all interactions.

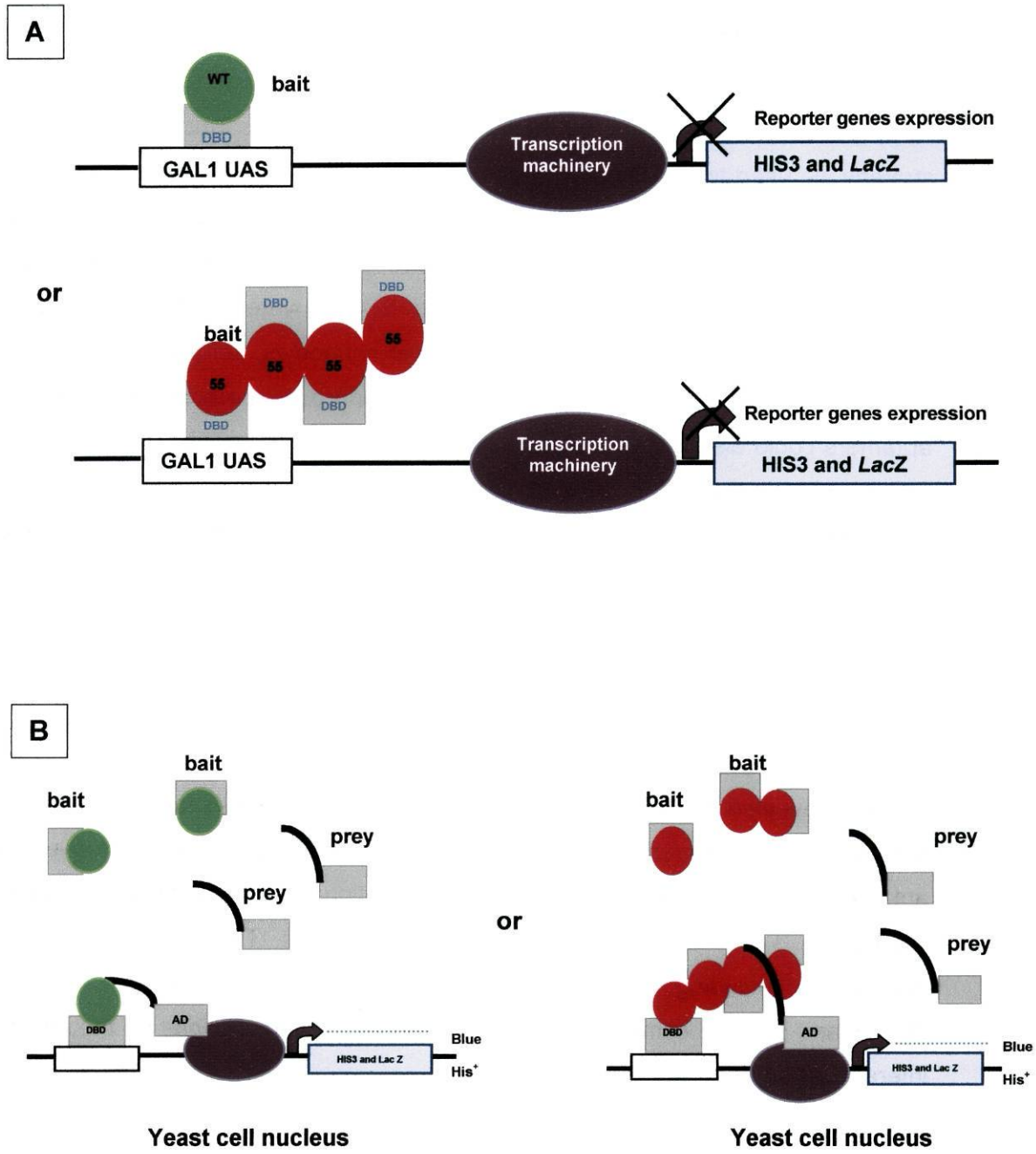


Figure 26. The Gal4-based two-hybrid system used for isolating peptide aptamers interacting with TTR *bait*s.

(A) Y190 yeast cells were initially transformed with a $GAL4_{BD}$::TTR fusion (WT or Leu55Pro) *bait*s and transformants were His^- and white owing to absence of reporter genes transcription. **(B)** Subsequent transformation with an AD-tagged peptide aptamer library allowed interacting clones to be isolated on the basis of the formation of an active transcriptional complex resulting in expression of the reporter genes. ● TTR WT; ● TTR Leu55Pro; AD - activation domain; DBD - DNA binding domain;

(·····) activation of transcription; ∪ peptide aptamer.

2. MATERIALS AND METHODS

2.1. Yeast strains

The *Saccharomyces cerevisiae* strain used in the yeast two-hybrid system was Y190 (Harper *et al.*, 1993). The genotype of this strain is MAT*a*, *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112, *gal*4Δ, *gal*80Δ, *cyh*^{r}2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-*lac*Z. This strain contains two different reporter genes, each driven by a different Gal4-dependent promoter to reduce the incidence of background. The *lac*Z reporter is regulated by the intact GAL1 promoter (including the GAL1$_{UAS}$ and GAL1 minimal promoter). The HIS3 reporter is under control of the GAL1$_{UAS}$ and a minimal promoter containing both HIS3 TATA boxes, TR and TC.

2.2. Culture media

Complete media (YPD) and minimal media (SD) were prepared as described in the "Yeast protocols handbook" (Clontech). In the 10x Dropout supplement, all the aminoacids referred to in the Handbook were included except histidine, leucine and tryptophan. These aminoacids were added separately depending on the selection medium desired. A defined minimal medium lacking one or more specific nutrients was labelled as SD-X (if a component was not indicated as missing, it was assumed to be present in the medium). For example, SD-W-L-H is a selection medium that contains all the nutrients required for growth except tryptophan, leucine and histidine.

2.3. Construction of the yeast two-hybrid TTR expression vectors

2.3.1. For TTR-TTR interactions

One of the tests used to validate positive interactions in yeast two-hybrid system, consists of switching the *baits* and *preys* in their respective plasmids, taking an insert out of a GAL4-BD vector and putting it into a GAL4-AD vector, and vice-versa. For this reason we constructed pGBT9-WT TTR and pGBT9-55 and pGADGH-WT TTR and pGADGH-55, testing the interaction patterns of each combination. The complete coding sequence of human WT TTR and the complete coding sequence of the highly

amyloidogenic human Leu55Pro TTR were subcloned in-frame into the Gal4 DNA binding domain of the pGBT9 vector (Clontech) and into the Gal4 activation domain of the pGADGH vector (Clontech). Plasmid pGBT9 is a 5.5 kb DNA-binding domain hybrid-cloning vector with TRP1 selection marker, and pGADGH, an 8.0 kb activation domain hybrid-cloning vector with LEU2 selection marker (Figure 27). Primers used for TTR cDNA amplification were: Tw1: 5'-GGAATTCGGTCCTAC GGGCACCGGTGAA-3' and Tw2: 5'-GCGTGTCGACT CATTCCTTGGGATTGGTCACG-3' for cloning in pGBT9 and Tw1: 5'-GGAATTCGGT CCTACGGGCACCGGTGAA-3' and Tw3: 5'-GGTCTCGAGTCATTCCTTGGGATTG GTGACG-3' for cloning in pGADGH. In both PCRs, pHNTR-TTR-cDNA (WT or Leu55Pro) served as template. The amplification products [including a 381 bp sequence encoding the full length TTR monomer (127 aminoacids), the STOP codon and two new restriction sites] were digested with *Eco RI* and *Sal I* and inserted in-frame into the unique restriction sites *Eco RI-Sal I* of pGBT9 or with *Eco RI* and *Xho I* to be inserted in-frame into the unique restriction sites *Eco RI-Xho I* of pGADGH. The constructs ligating in the correct orientation and containing the desired WT or mutated Leu55Pro TTR were selected and designated as pGBT9-WT and pGADGH-WT and pGBT9-55 and pGADGH-55. *E. coli* XL1-Blue supercompetent cells (Stratagene) were used for all plasmid cloning.

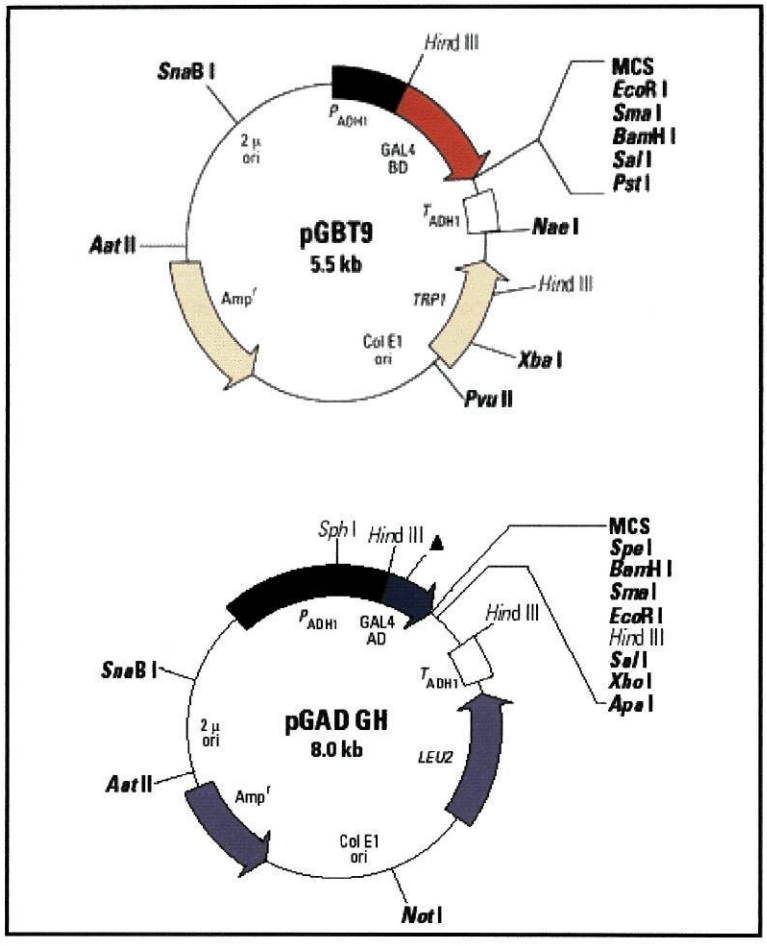


Figure 26. Shuttle and expression vectors used in two hybrid interactions.

2.3.2. For TTR-aptamer interactions

Since the random aptamer library is inserted in a plasmid with a TRP1 selection marker, and TTR *bait*s described above were cloned into pGBT9 also carrying a TRP1 marker, "new" pGBT9-BD vectors were constructed. TTR *prey*s previously cloned into the pGADGH vector, had to be extracted without the AD portion of the GAL4 gene, but with the LEU2 selection marker sequence and ligated with pGBT9-BD vector, excised on its TRP1 selection marker (Figure 28). Both plasmids were digested with *Eco RI* and *Aat II* and a fragment from pGADGH vector carrying TTR and the LEU2 selection marker sequences was inserted in-frame into the unique restriction sites *Eco RI-Aat II* of pGBT9. These "new" *bait*-containing vectors were named: pGBT9LEU-55 and pGBT9LEU-WT.

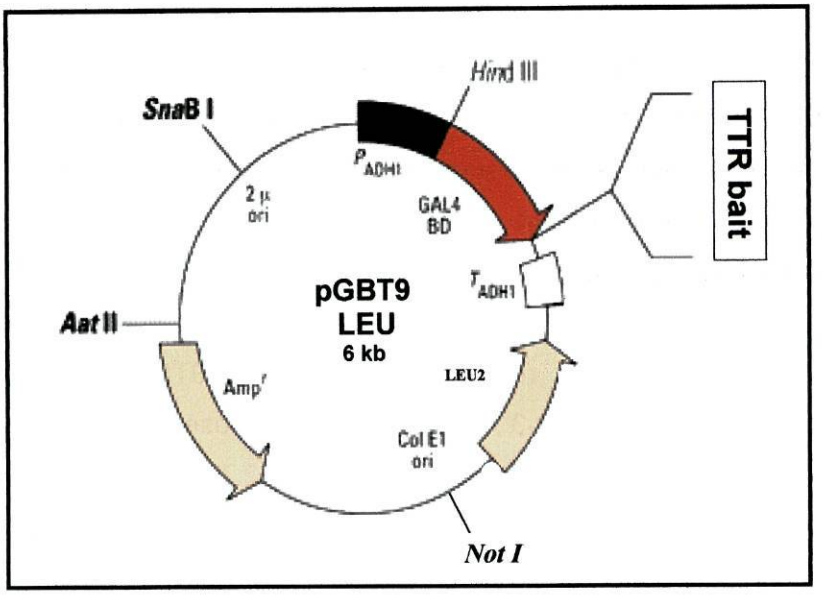
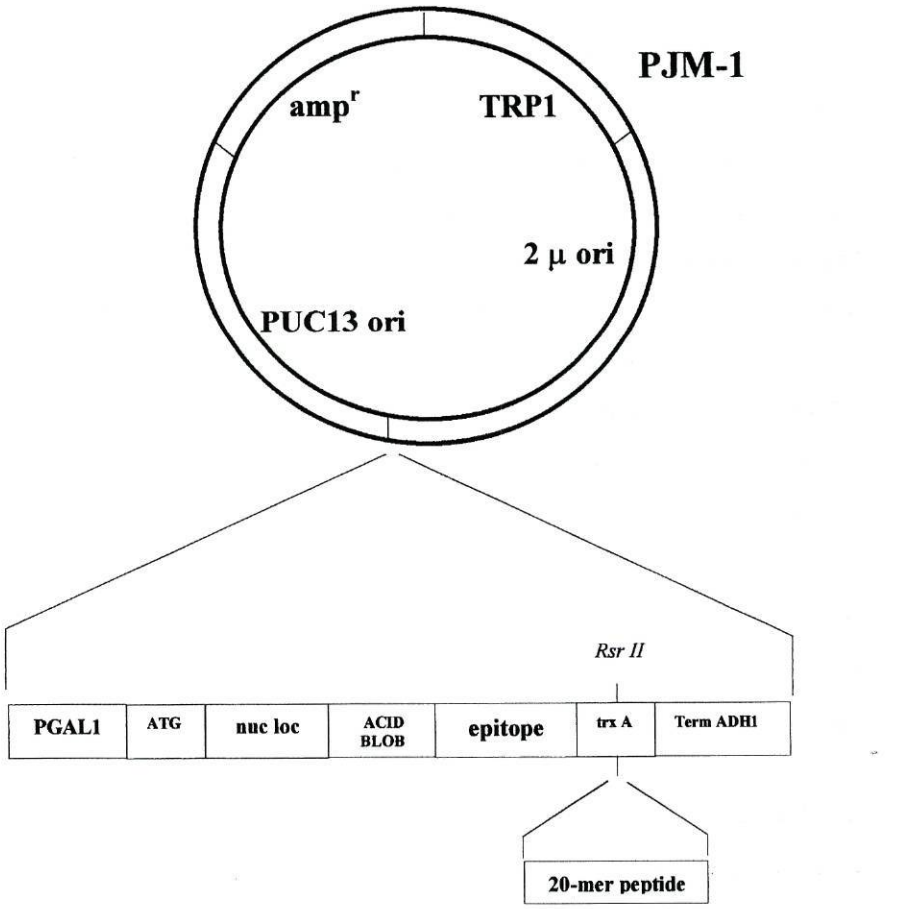


Figure 28. pGBT9LEU expression vectors used in TTR-library interactions.

2.4. Random 20-mer-aptamers library

The random aptamer library used in this study was constructed in pJM-1 plasmid by John McCoy exactly as described for the aptamer interaction library in Colas *et al.* (1996) and kindly provided by Roger Brent. pJM-1 expresses thioredoxin aptamers (Figure 29) and 20 residues (20-mer) were chosen for the length of the displayed peptides, since that was long enough to fold into many patterns of shape and charge, but sufficiently short that their encoding oligonucleotides lacked stop codons. The authors used the oligonucleotides: 5' – CAGTCAGTCAGTCAATTGAAGAAGGA GATATACATATGGGTGCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGGTTCTGAGT TCCCGGGGATCACCTTGCGGATTCAGGA-3' and 5'-ACTGACTGACTGCATATGGA ATTCAGAGGCATAATCTGGCACATCATAAGGGTAGGACCCAAAACAAAGGTCTGT TCCGCCTGAGTGACGTTCAGCACGGAACTCACCGGATGACCGCCTTTCGCAACG G-3' to generate a PCR fragment from pLEXA-B112 encoding a fusion between the SV40 nuclear localisation sequence, the B112 "acid blob", and the haemagglutinin epitope tag, flanked by *Mun I* and *Nde I* sites. TrxA from pALTRXA-781 was excised with *Nde I* and *Sal I*. Both fragments were introduced into the *Eco RI-Xho I*-cut pJG4-4 plasmid to create pJM-1. The library was made by annealing 1 nmol oligonucleotides 5'-GAGTGACTGGTCCG $(NNG/T)_{20}$ GGTCCTCAGTCAGTCAG-3' to 1 nmol 5'-CTGACTGACTGAGGACC-3', ligated into pJM-1 fused to the GAL4-AD with TRP1 as selection marker and transforming *E.coli* to get 2.7 x 10^9 transformants. Upon receipt, the random peptide library was titerred and a total of 10^7 independent clones were obtained.

a.



b.

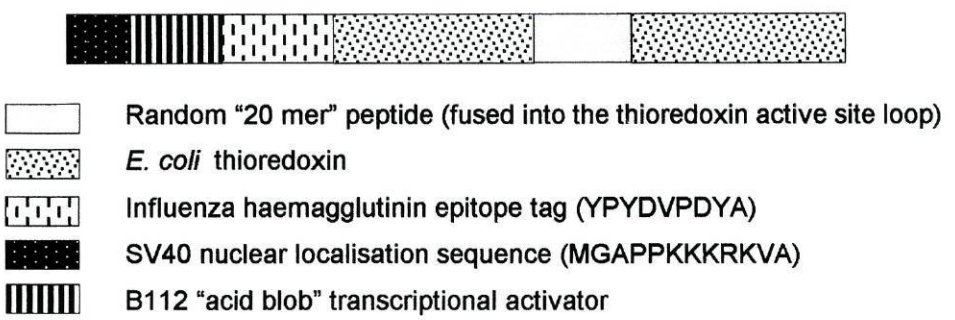


Figure 29. Peptide aptamer expression vector **(a)** and random peptide nuclear library **(b)**.

2.5. Yeast transformation

All yeast transformations were performed accordingly to the "High Efficiency Lithium Acetate Method" of Gietz and Schiestl (1995). The "1x scale up" procedure was performed as follows: Y190 strain without plasmids was grown in 10 ml of YPD medium O/N, while the pre-transformed strain was grown in the appropriate SD dropout medium to keep selective pressure on the plasmid. Overnight cultures were diluted in 5 ml YPD to an OD_{600} of 0.3 and then incubated at 28°C until the OD_{600} reached 0.6-0.8. After centrifugation at 1800 rpm for 5 minutes, cells were washed first with sterile water and then with 100mM LiAc. The following components were added to yeast pellets: 240 µl PEG 50% (w/v), 36 µl LiAc 1M, 25 µl denatured salmon sperm DNA (2 mg/ml stock solution) and 50 µl of DNA diluted in water (0.1-10 µg). Each tube was vigorously vortexed until the cell pellet was completely mixed and left incubating at 30°C, shaking, for 30 minutes. Cells were then heat-shocked at 42°C, for 20-25 minutes, inverting the tubes every five minutes. Finally, cells were centrifuged, resuspended in 1 ml of sterile water and plated onto selective agar plates.

2.6. Preparation of yeast protein extracts

Overnight cultures in SD selective medium, grown at 28°C, were used to inoculate 50 ml of SD^- at an OD_{600} of 0.3. These cultures were then grown until an OD_{600} of 0.6-0.8. Cells were centrifuged at 1800 rpm, at 4°C, for 5 minutes, washed with ice-cold water and spun again. To obtain the protein extracts, pellets were thawed on ice and resuspended in Extraction Buffer (62.5 mM Tris-HCl, pH 6.8, 5 mM PMSF, 1.8 µM leupeptin, 9 mM benzamidine). 100 µl of Extraction Buffer were used per 7.5 OD_{600} units of cells (total number of OD_{600} units is obtained by multiplying the OD_{600} of a 1 ml sample by the culture volume). To each cell suspension an equal volume of glass beads was added. Six cycles of vigorous vortexing for one minute followed by one-minute incubation on ice were performed to each sample to disrupt the yeast cells. Debris and unbroken cells were pelleted at 14,000 rpm for 10 minutes, at 4°C.

2.7. β-Galactosidase colony filter assay

The β-galactosidase assay was always performed using fresh colonies from selection plates grown at 28°C for 5 days. The transformant colonies were lifted by

placing a sterile Hybond-N filter (Amersham) over the surface of the plate. When the membrane was evenly wetted, it was transferred to a pool of liquid nitrogen and submerged during 10 seconds to allow cell permeabilisation. Then it was thawed at RT and placed, with colony side up, on a Petri dish with 3MM Whatman filter paper that had been pre-soaked in Z buffer (16.1 mg/ml $Na_2HPO_4.7H_2O$, 5.5 mg/ml $NaH_2PO_4.H_2O$, 0.75 mg/ml KCl, 0.246 mg/ml Mg_2SO_4, pH 7.0) containing 1 mg/ml X-Gal (SIGMA). Incubation was carried out up to 24 hours at 28°C, checking for the appearance of blue colour.

2.8. Library screening

Y190 was transformed to tryptophan prototrophy with pGBT9LEU-WT and pGBT9LEU-55 *bait* plasmids. Transformations were tested for the absence of both yeast cell toxicity and auto-activation of the lacZ and HIS3 reporter genes. Transformation efficiency with the library DNA was assessed before proceeding to the library screening. The *bait* strains were transformed with increasing amounts of library plasmid DNA in a standard transformation reaction (1x scale up). The amount of library DNA that gave the optimal transformation efficiency was determined to be 1 µg (yielding 1-2 x 10^5 colonies). Therefore, for the library screening, 60 µg of library DNA were used in a 60x scale up transformation (Gietz and Schiestl, 1995) to allow the screening of a total number of ~10^7 transformants. After the heat-shock, cells were resuspended in SD-W-L-H medium and plated in twelve 245 mm-Petri dishes of SD-W-L-H selective medium containing 50 mM of 3-AT to eliminate the background expression of the GAL1-HIS3 reporter. After incubation at 28°C for 5-12 days, His^+ clones were streaked on SD-W-L-H 50 mM 3-AT selective plates and grown during 5 days until a β-galactosidase filter assay was performed to detect the LacZ reporter activation. Colonies that became blue were selected for further analysis.

2.9. Generating yeast plasmid segregants

To test whether the activation of the reporter genes in the "positive" His^+-blue colonies obtained from the library screening was dependent on the presence of the *bait* plasmid, it was necessary to generate segregant strains containing only the library plasmid. Yeast cells expressing both *bait*s and candidate interacting library peptide were allowed to grow O/N in SD-W medium. This medium selects for the maintenance

of the library plasmid (which has the *TRP*1 marker) but does not select for the maintenance of the *bait* plasmid (*LEU*2 marker). Cells were then plated onto SD-W agar plates and, after 3 days at 28^{0}C, isolated yeast colonies were replica plated to plates containing either SD-W or SD-L-W. Cells that lost the *bait* only grew on SD-W. Once obtained, all segregant yeast cells were tested for their ability to interact with the *bait* to activate the Y190 reporter genes.

2.10. Plasmid isolation from yeast

Yeast colonies were grown O/N, at 28^{0}C in the appropriate SD$^-$ selective liquid medium. The cell pellet from 2 ml of an O/N culture was resuspended in 50 µl of the following buffer: 100mM Tris, pH 7.5, 10 mM EDTA, pH 8.0 and 100 mM β-mercaptoethanol. After 30 minutes stirring at room temperature, cells were spun again, at 4000rpm, for one minute. Pellets were resuspended in 40 µl of a second buffer containing: 100mM Tris, pH 7.5 and 10 mM of EDTA, pH 8.0. 50U of lyticase (Roche) were added and cells were left incubating at 37^{0}C during 45 minutes to 1 hour, with vigorous shacking. The resulting spheroplasts were lysed in 1/10 of the total volume of SDS 10% and were incubated at 65^{0}C, for 10 minutes. Then, Potassium acetate (5M) was added (1/3 of the total volume) and 30 minutes incubation on ice and another centrifugation, at 14,000rpm for 20 minutes, followed. The supernatant was collected and DNA was precipitated with 0.8 volumes of isopropanol. After a new spin at 14,000 rpm, for 10 minutes, the DNA was washed with 100% ethanol and centrifuged at 14,000, for 2 minutes. The dried DNA pellet was resuspended in 50 µl of sterile water and 10 µl were used to transform 100 µl of XL-1 Blue supercompetent *E.coli* cells (Stratagene), following the manufacturer's instructions.

2.11. *In vitro* binding assays

2.11.1. Immuno-screening for TTR "in situ"

Plates with colonies grown for 5 days in appropriate selective medium were covered with nitrocellulose filter membrane (Hybond-C extra, Amersham); the filter was allowed to be in contact with yeast colonies for 2 minutes, carefully lifted and placed colony side up onto fresh media plates and incubated O/N, at 28^{0}C. Filters were then placed onto 3MM Whatman paper [previously soaked in 4 ml SCE (1 M sorbitol, 100

mM sodium citrate, 60 mM EDTA), 30 µl DTT (2 M) and 560 U lyticase], in a Petri dish. Plates were covered and incubated O/N at 28°C. To obtain lysis and protein fixation onto the membrane, filters were placed on 3MM Whatman paper saturated in the following solutions: 10% SDS (5 minutes), 0.5 M NaOH (10 minutes) and 200 mM Tris, pH 7.5 (5 minutes). This last step was repeated 3 times, with fresh solution. Filters were then moved onto damp 3MM Whatman paper (dH_2O) and incubated in a chloroform vapour chamber for 60 minutes, after which filters were moved again onto 3 MM Whatman paper saturated with colony denaturing solution (20 mM Tris-HCl, pH 7.9, 6 M urea, 0.5 M NaCl) and incubated for 15 minutes at RT. Non-specific binding was blocked by immersing the filters in blocking solution (5% dried milk in PBST), at RT, for 1 hour. Filters were incubated with primary antibodies (either 1:500 dilution of rabbit anti-human TTR Pab (DAKO) or 1:20 dilution of Mab 15 (culture supernatant) and then with secondary antibodies [1:5000 dilution of goat anti-rabbit-HRP conjugated (Pierce) or 1:5000 of goat anti-mouse-HRP conjugated (Pierce), respectively], for one-hour periods, and washes were performed twice with PBST, between incubations. Development was achieved using either the ECL method (Pierce) or incubating the filters in a developing solution containing 20% Chloronaphthol (5 mg/ml stock solution) in PBS and 0.05% H_2O_2. Plate replicas were made and membranes were developed by different methods. Alternatively, the same filter was initially incubated with Mab 15 and with the respective secondary antibody and developed using chloronaphthol, and after stripping for complete removal of primary and secondary antibodies, the filter was reprobed with the Pab and respective secondary antibody, using the ECL method. Stripping started by submerging the filters in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubating at 50°C for 30 minutes with agitation. The filters were then washed twice for 10 minutes in PBST at RT using large volumes of wash buffer and then blocked in 5% non-fat dried milk in PBST for 1 hour at RT. Colonies exhibiting different recognition patterns (with Pab and Mab 15) were selected for further studies.

2.11.2. Dot-blots for detecting TTR in yeast extracts

50 –200 µl of yeast protein extracts were immobilised onto a nitrocellulose membrane (Hybond-C pure, Amersham). Soluble recombinant TTR and TTR fibrils obtained by acidification, used as negative and positive controls (for Mab 15 interaction), were also applied. The membrane was then blocked with 5% powdered milk, at RT, for 1 hour, washed with PBST and incubated with antibodies as described

above. Soluble or aggregated fusion proteins present in protein extracts were visualised using the ECL method (Pierce) or with chloronaphthol.

2.12. Enzyme-linked Immunosorbent Assay (ELISA)

The wells of 96 well-microtiter plates were coated O/N at 4°C, with TTR at 1μg/ml in coating buffer (K_2HPO_4, pH 8.0). Unbound proteins were washed from the wells and non-specific binding sites were blocked by incubation with 10% non-fat dry milk in PBST, for an hour at RT. Direct binding assays were performed adding increasing concentrations of peptides previously incubated with anti-human TTR Pab (DAKO) or Mab 15, for 1 hour at RT to immobilised TTR coated plates in PBS and incubating for an extra hour. Wells were washed with PBS and PBST (3x each) and bound antibodies were detected by incubating the plates with appropriate polyclonal antibodies, conjugated with horseradish peroxidase (HRP) (anti-rabbit and anti-mouse, respectively), and then washed with PBS and PBST as before. The reaction was developed using ABTS [2, 2'-azino-bis (3-ethylbenzthiazidine-6-sulfonic) acid] at 1 mg/ml in 0.1 M sodium citrate, pH 4.5 and the change in colour was determined at 405 nm on a Bio-Tek BL_X-800 microplate reader.

2.13. Assay for Inhibition of fibrillogenesis

Samples of WT and Leu55Pro TTR (200 μg), pre-dialysed against water, pH 7.0, were incubated at 37°C in the absence or presence of a selected peptide. At different time points, 100 μl aliquots were withdrawn and added to 900 μl of a solution containing 30 μM ThT (Fluka) and Glycine-NaOH buffer 50 mM, pH 9.0. To measure amyloid formation by ThT binding and characteristic excitation maximum at 440 nm. Rates of fibrillogenesis were calculated for each of the various experimental conditions and expressed as a percentage of the maximum rate of fibril formation in the absence of peptide. The final concentrations of TTR and peptide were 0.5 μM and 2 μM, respectively. Excitation spectra were recorded on a Jasco FP-770 spectrofluorometer at 25°C, with excitation and emission slits set to 5 and 10 nm, respectively.

2.14. SPR kinetic determination

Surface plasmon resonance (SPR) is used to detect changes in optical properties at the surface of a sensor chip, that consists of a glass slide covered with a thin gold film bound to a carboxylated matrix of the dextran (Löfås and Johnsson, 1990). The dextran matrix of the sensor chip is used to covalently bind one of two or several reactants (the ligand), while the others are allowed to pass over the surface (analytes). The resonance angle depends on the refractive index in the vicinity of the surface, which changes as the concentration of the molecules on the surface is modified. The changes in the resonance signal are referred to as resonance units (RU). Monitoring the resonance angle continuously in real time generates a sensorgram, from which the association rates constant (k_a) and dissociation rate constant (k_d), as well as the affinity constant (K_A), can be determined by the Biosensor software.

To determine association rate (k_a) and dissociation rate (k_d) constants, BIAcore "kinetic evaluation" software was used. The affinity constant (K_A) was taken as the ratio of k_a/k_d. The software gives the amount of bound analyte (Ra) to the ligand, as well as the reaction rate (dRa/dt) at specified time intervals. R_{amax} is the maximal amount of bound analyte to the ligand, and C is the concentration of the analyte. The equation of the dR_a/dt as a function of R_a can be written as follows:

$$dR_a/dt = k_a R_{a\,max} C - R_a (k_a C + k_d)$$

To calculate k_a, parts of the dR_a/dt versus R_a curve that reflect association are chosen. The slope of dR_a/dt versus R_a curve at each concentration is plotted versus the concentrations in use, to give a straight line. This new slope gives the association rate constant and the dissociation rate constant is calculated from a specific time frame (t_1 - t_n) in the dissociation phase. The slope of the plot ln (R_{t1}/R_{tn}) versus t_n - t_1 g. These kinetic parameters between TTR mutants and interacting aptamers were measured by SPR using a BIAcore 2000 (Pharmacia Biosensor, Sweden).

Briefly, purified TTR (WT, Leu55Pro soluble and fibrillar and Tyr78Phe soluble) was diluted to 0.5 mg/ml in HBS buffer [10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and 3.4 mM EDTA] and was covalently attached to the carboxymethyl dextran-modified gold surface of CM5 sensor-chips (BIAcore) previously activated with N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl carbodiimide according to the manufacturer's instructions. To obtain an optimal capture level, the concentration was adjusted to 50 μg/ml and the time of activation by the NHS/EDC mixture, set to 4 minutes. The immobilisation procedure was carried out at a continuous flow rate of the running buffer HBS of 5 μl/min, whereas the real-time analysis of the specific interactions was carried out at a flow rate of 10 μl/min, at 25^{0}C.

All analyte solutions were initially dialysed/diluted in HBS buffer and then injected over the test surfaces at a flow rate of 5 μl/min. Regeneration of the sensor-chips with a short pulse of 200 mM Glycine-HCl, pH 2.0 did not result in a loss of subsequent signal. The amount of analytes (aptamers/antibodies) bound to immobilised TTR was monitored by measuring the variation of the SPR angle as a function of time. Results were expressed in resonance units, the arbitrary unit specific for the BIAcore instrument (1000 RU correspond to approximately 1 ng of bound protein/mm^2). The association and dissociation rate constants, were determined separately from individual association and dissociation phases and the overall affinity constant K_D, was derived from k_d/k_a, using BIAevaluation 3.0 software.

2.15. Bioinformatic analysis

Comparison between aminoacid sequences of TTR and/or aptamers was performed using DIALIGN2 and ClustalW, for multiple sequence alignment and ProtScale, for hydropathic profiling.

3. RESULTS

3.1. Assessing TTR *baits* for yeast cell toxicity, self-transcriptional activities and direct interactions with TTR *preys*

The first step in the two-hybrid approach for screening of TTR-TTR interactions was the construction of the hybrid plasmids (section 2.3.1. Materials and Methods). Plasmid pGBT9, which encodes the DNA-binding domain of the GAL4 protein ($Gal4_{BD}$), has a *TRP*1 gene that allows the yeast cells to grow in the absence of tryptophan and was used as expression vector for the $Gal4_{BD}$ hybrid proteins (*baits*). PGADGH, which encodes the transcriptional activation domain of Gal4 ($Gal4_{AD}$), has a *LEU*2 gene that confers leucine prototrophy to yeast and was the expression vector used to generate $Gal4_{AD}$ fusion proteins (*preys*). The entire coding regions of WT TTR and Leu55Pro TTR were fused in-frame with the $Gal4_{BD}$ sequence of the expression vector pGBT9 to generate the hybrid proteins $Gal4_{BD}$: TTRWT and $Gal4_{BD}$:TTR55; the same procedure was followed for pGADGH to generate the hybrid proteins $Gal4_{AD}$::TTRWT and $Gal4_{AD}$::TTR55.

Table V. Hybrid expression vectors constructed in this study and used to transform Y190 yeast strain. Each plasmid shown in the left column encodes for the hybrid protein shown in the right column.

Expression vector	Hybrid protein
pGBT9	$Gal4_{BD}$
pGBT9-TTRWT	$Gal4_{BD}$::TTRWT
pGBT9-TTR55	$Gal4_{BD}$::TTR55
pGADGH	$Gal4_{AD}$
pGADGH-TTRWT	$Gal4_{AD}$::TTRWT
pGADGH-TTR55	$Gal4_{AD}$::TTR55

The expression of all hybrid constructs (see Table V) was confirmed by Western blot analysis using a polyclonal antibody against human TTR (Figure 29 b). Immunoblot analysis of proteins extracted from yeast transformed with WT and Leu55Pro TTR sequences, revealed TTR-immunoreactive bands migrating at about 30 kDa, the size of the expected fusion proteins (Figure 30 a). In addition, other TTR-immunoreactive bands migrating faster than the fusion protein (in the case of the WT fusion proteins), or slower than the fusion proteins, (for the Leu55Pro fusion TTRs) were also observed.

The first presumably representing degradation products of the fusion proteins and the second case represents aggregation. Preparations of Leu55Pro TTR, when containing aggregates and run on SDS gels, typically exhibit a smeared pattern of high molecular weight fragments above the size of TTR dimer.

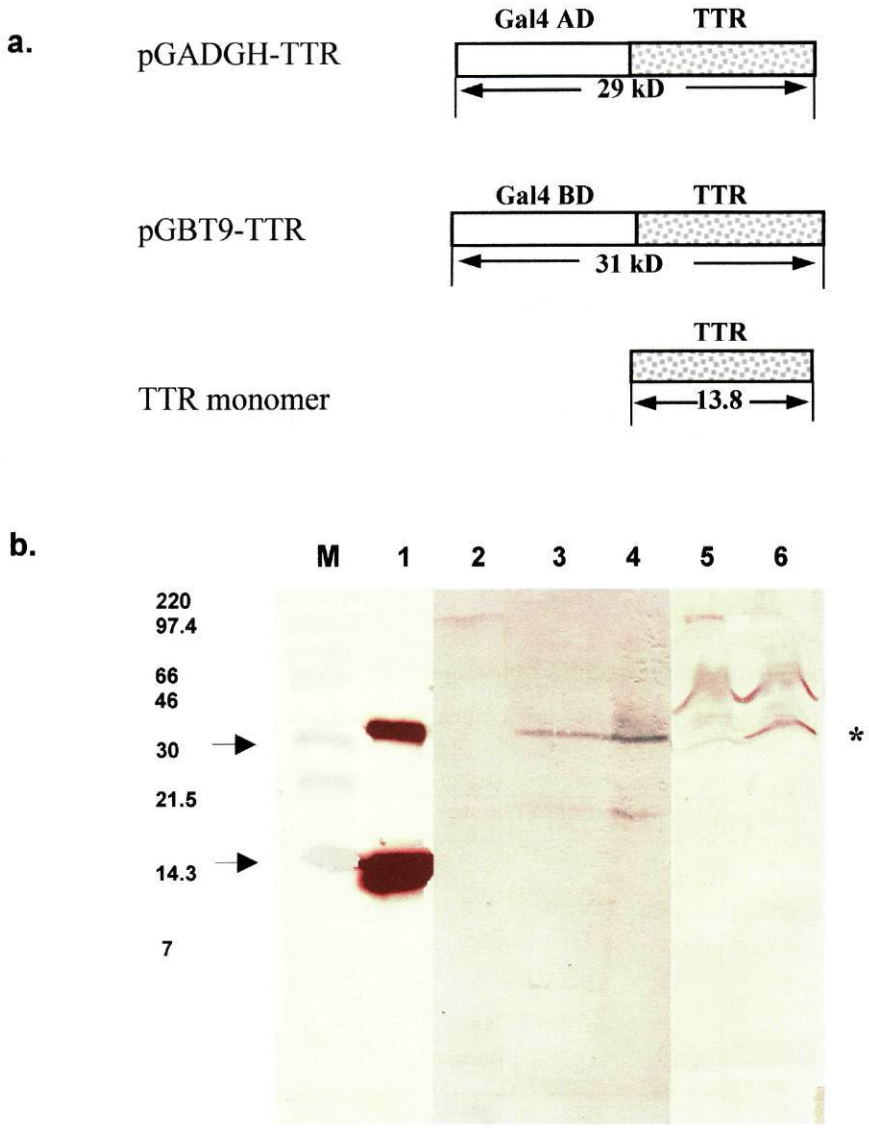


Figure 30. **(a)** Schematic representation of TTR fusion proteins. Expected sizes of fusion proteins and TTR monomer are indicated. (**b**) TTR fusion proteins demonstrated by Western blotting. Protein molecular markers are indicated on the left, in kDa (M). Purified recombinant TTR (lane 1) and proteins extracted from yeast (lanes 2 - 6), were separated by 12% SDS-PAGE and revealed with rabbit α-human prealbumin antibody. (*) Indicates the major TTR-immunoreactive bands migrating with size of the expected fusion proteins. Lane 2: non-transformed yeast; lane 3: pGBT9-WT; lane 4: pGADGH-WT; lane 5: pGADGH-55; lane 6: pGBT9-55.

Some hybrid proteins that inhibit the growth of yeast cells may not be suitable for yeast two-hybrid interactions. In particular, the *bait* $GAL4_{BD}$-55 could become toxic, if aggregation occurred inside the yeast cells. However, comparing the growth of WT and 55 *bait*s, colonies formed had a similar development (~1.5 mm diameter colonies) in both cases. These measurements, for (55/WT alone growing in SD-W medium or WT-55; WT-WT; 55-55; 55-WT interactions growing in SD-L-T-H medium) indicated that none of the hybrid proteins is toxic to yeast cells.

The hybrid constructs were also checked for their autonomous activation of the Y190 strain reporter genes $GAL1_{UAS}$ -HIS3 and GAL1-LacZ. In order to be suitable for yeast two-hybrid experiments, the hybrids cannot activate the reporter genes by themselves. Autonomous activation of reporter transcription is often due to the fact that some proteins, with no apparent function in transcription, may contain domains that cause some level of self-activation when fused to a DNA-BD domain; self-activation can also be due to the non-specific binding of AD-fusion proteins to promoters bound at the promoters. Hybrid proteins that do not activate any transcription of the reporter $GAL1_{UAS}$ -HIS3, do not allow the growth of yeast on selective medium SD-H containing a specific concentration of 3-AT. 3-AT is a chemical inhibitor of the IGP dehydratase (the HIS3 gene product) that can be included into SD-H selective media to eliminate the HIS prototrophy resultant from the residual expression of the HIS promoter (Kishore and Shah, 1988; Durfee *et al.*, 1993). The ability of the hybrid constructs to self-activate the HIS3 reporter, was assessed by transforming yeast with each hybrid vector and screening them for 5 days in selective media: SD-W-H (10 mM 3-AT → 75 mM 3-AT) or SD-L-H (10 mM 3-AT → 75 mM 3-AT), depending on whether $GAL4_{BD}$ (pGBT9-TTR) or $GAL4_{AD}$ (pGADGH-TTR) fusions were being tested. The constructs pGBT9-WT and pGBT9-55 were leading to activation of the HIS3 reporter, however this His auxotrophy was restored with 50 mM 3-AT included in the medium. The same proteins, fused to the $GAL4_{AD}$ did not exhibit auto-activation. β-galactosidase filter assays of the colonies grown in the selective plates SD- (W/L)-H revealed that no hybrid led to transcription of the lacZ reporter. To be considered positive, two-hybrid interactions with the GAL4-BD-wt/55 fusion proteins would have to originate blue colonies able to grow in SD-W-L-H + 50 mM 3-AT.

Yeast that contained either empty $GAL4_{AD}$ (∅), empty $GAL4_{BD}$ vectors (∅), or both fusion proteins were transformed with plasmids encoding the $Gal4_{AD}$:TTR or $Gal4_{BD}$: TTR. While growth was detected on plates lacking Leu and Trp (Fig 31, plate 1, colonies 1, 2, 3), growth was visible on selective media only if both fusion plasmids were present (Fig 31, plate 2, colony 3), confirming that activation of the reporter genes resulted from at least transient interaction between TTR subunits of different plasmids.

Also shown in Fig 31, yeast transformed with pGBT9-WT and pGADGH-WT or pGBT9-55 and pGADGH-55 demonstrated intense blue colour in the filter β-galactosidase assays. Confirming further the specificity of the assay, no colour was observed when either pGBT9-TTR was co-transformed with pGADGH vector or pGADGH-TTR was co-transformed with pGBT9 vector. SD-L-W medium will select for the plasmids and SD-L-W-H medium will select for the plasmids as well as for the hybrid proteins encoded by them. It takes some time for the HIS3 peptide to accumulate in the medium, as a result of two-hybrid interaction.

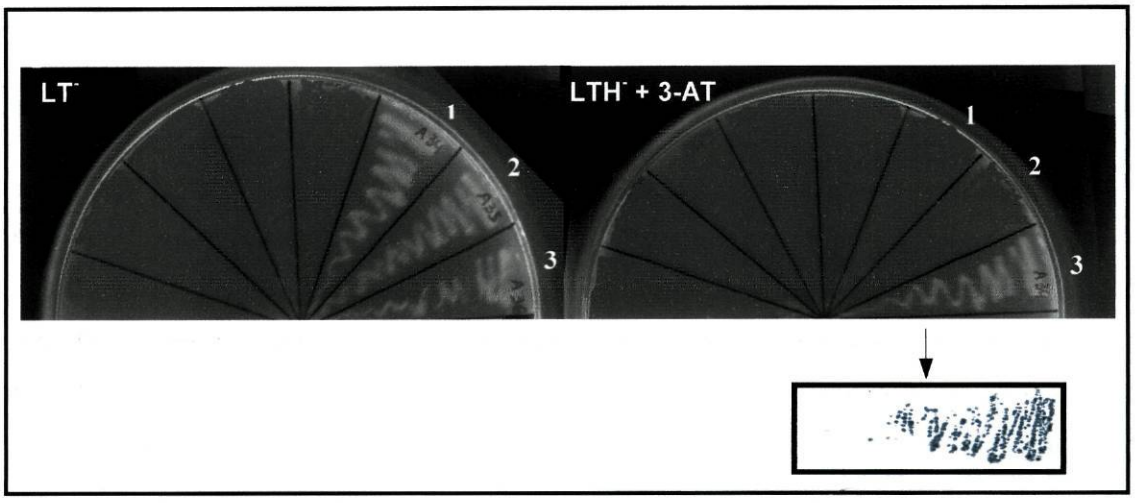


Figure 31. Yeast two-hybrid screen. Yeast strain Y190 was transformed with either the $GAL4_{BD}$::TTR fusion plus empty $GAL4_{AD}$ plasmids (colony 1), $GAL4_{AD}$::TTR fusion plus empty $GAL4_{BD}$ plasmids (colony 2), or $GAL4_{BD}$ ::TTR plus $GAL4_{AD}$::TTR (colony 3) and then streaked onto an LT^- plate (plate 1- left) or a selective plate LTH^- supplemented with 3-AT (plate 2 - right). While all clones grew under non.-selective conditions (plate 1), only yeast which had been transformed with both TTR fusions grew under the selective conditions (plate 2, colony 3). The β-galactosidase assay for this colony is also shown. These colonies correspond to GAL4-WT TTR fusion proteins interaction, but GAL4-55 TTR fusion proteins exhibited the same behaviour.

We have demonstrated that TTR fusion proteins are capable of homomeric interactions *in vivo*. The intense blue colonies indicated that when two monomers are co-expressed as GAL4 fusion proteins, they bring the GAL4 DNA-binding and GAL4 activation domains into proximity sufficient to activate a gene under GAL4 control. Intermolecular binding between WT and Leu55Pro TTR monomers was next attempted by the following double transformations: pGBT9-WT / pGADGH-55 and pGBT9-55 / pGADGH-WT. These two interactions produced a weaker blue colour in β-galactosidase assays (figure 32), compared to pGBT9-WT / pGADGH-WT or pGBT9-55 / pGADGH-55 interactions (positive controls).

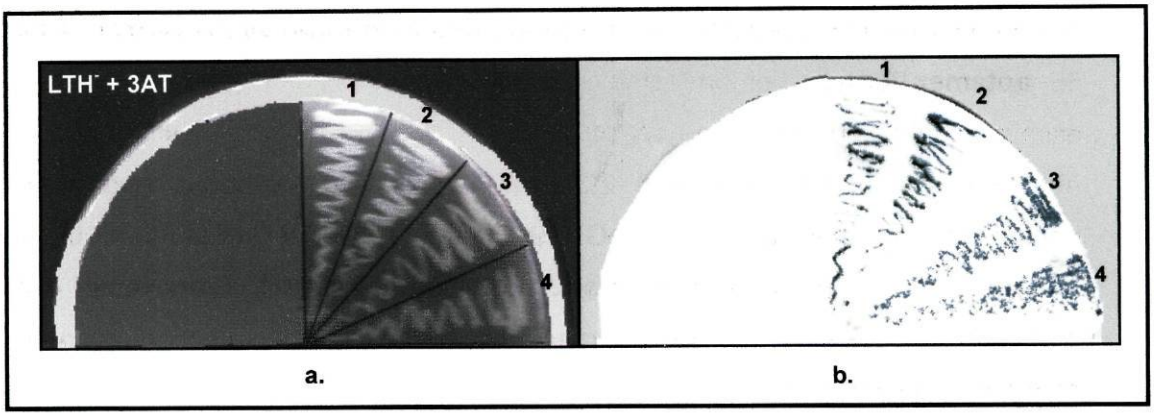


Figure 32. Yeast two-hybrid screen of intermolecular binding between WT and Leu55Pro TTR monomers. Cells were plated onto selective medium lacking leucine, tryptophan, histidine and supplemented with 50 mM 3-aminotriazole (a) and tested for β-galactosidase activity by using X-Gal on a filter lift assay (b). Y190 cells were transformed with the following: colony 1: pGBT9-WT / pGADGH-WT; colony 2: pGBT9-55 / pGADGH-55 colony 3: pGBT9-WT / pGADGH-55; colony 4: pGBT9-55 / pGADGH-WT.

The Leu55Pro TTR mutant was expected to aggregate inside the yeast cells, enabling this system to be used as an *in vivo* amyloid formation assay. Immunoscreenings for TTR "in situ" (section 2.11.1. Materials and methods) were performed in yeast colonies grown in appropriate selective medium for pGBT9-55 and pGBT9-WT (SD-W) and transferred to filter membranes, using Mab 15 as an amyloid specific marker and a polyclonal antibody anti-human TTR. This allowed us to distinguish between yeast colonies that presented TTR aggregation and/or TTR soluble forms. Dot blot assays were next performed using protein extracts of differently reactive yeast colonies (Figure 33) and we concluded that these methods would allow us to select different immunoreactive TTR *bait*s to interact with the peptide aptamer library.

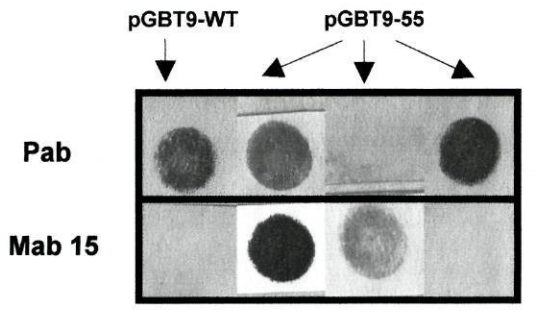


Figure 33. Immunoreactive assay for yeast TTR. Protein extracts of colonies transformed with either pGBT9-WT or pGBT9-55 were applied onto a nitrocellulose membrane and screened for reactivity with Pab and Mab 15. Yeast cells transformed with pGBT9-WT contained soluble TTR, therefore positive for Pab only. In contrast, cells transformed with pGBT9-55 produced three different types of immuno-reactivity patterns with the antibodies used in the screen, indicating that three different forms of TTR were present. The same amount of extracted proteins was used in each well and membranes were exposed to either the pab (top panel) or Mab 15 (bottom panel).

3.2 Assessing TTR *baits* for direct interactions with putative *preys* from a peptide aptamer library

As mentioned in section 2.3.2. (Materials and Methods), two "new" *bait* containing vectors were created: pGBT9LEU-WT and pGBT9LEU-55, to be able to interact with the $GAL4_{AD}$ peptide library, since both the pJM-1 library vector and "old" pGBT9-*bait* containing vectors carried the same TRP1 selection marker. The pGBT9LEU plasmid encodes $GAL4_{BD}$ and has a LEU2 gene that allows the yeast cells to grow in the absence of leucine. The entire coding regions of WT TTR and Leu55Pro TTR were fused in-frame with the $GAL4_{BD}$ sequence of this vector to generate the hybrid proteins $GAL4LEU_{BD}$:TTRWT and $GAL4LEU_{BD}$:TTR55.

These hybrid constructs were also checked for their autonomous activation of the Y190 strain reporter genes $GAL1_{UAS}$-HIS3 and GAL1-LacZ, and similarly to pGBT9 vectors, they led to activation of the HIS3 reporter but this His auxotrophy was restored with 50 mM 3-AT included in the medium. Yeast colonies were grown in appropriate selective medium for pGBT9LEU-55 and pGBT9LEU-WT (SD-L) and transferred to filter membranes, to perform the immunoscreenings using Mab 15 and the Pab as described above. We selected three different *bait*s, which by themselves, growing in selective medium, produced the differential immuno-recognition pattern represented in Table VI.

Table VI. Immunoreactive pattern of three TTR *baits* (built for library screens) using a polyclonal antibody (Pab) and Mab 15 as amyloid marker.

baits	**Pab**	**Mab 15**
pGBT9LEU55 – "**Type I aggregates**"	-	+
pGBT9LEU55 – "**Type II aggregates**"	+	+
pGBT9LEUWT – "**soluble**"	+	-

We were able to find two types of colonies: some that did not react with Mab 15, referred to as "soluble" and others that exhibited a strong reactivity, indicating the presence of amyloid aggregates on the protein extracts of those colonies (Figure 34). Furthermore, within those colonies whose protein extracts were positive for Mab 15 recognition, some did not react with the polyclonal antibody, referred to as "type I aggregates", and others were positive for Pab recognition, referred to as "type II

aggregates", indicating that two different types of aggregates could be present in those cells. Our results indicated that not only monomer-monomer interactions took place in this system, but also the formation of monomeric TTR intermediates, functioning as amyloid precursors for fibril formation in the yeast cell. Screening of several pGBT9LEU-WT yeast colonies never produced different patterns of antibody recognition. All colonies were positive for Pab and negative for Mab 15.

Three colonies were selected out of the ones that exhibited stronger signals, two for the different types of aggregates of pGBT9LEU-55 and one for pGBT9LEU-WT soluble, to screen for peptide aptamers that would be able to interact with WT TTR (soluble) and aggregate type I and type II *bait*s. For this purpose Y190 strain containing *bait*s: soluble/(type I)/(type II), was transformed with plasmid DNA of a peptide aptamer $GAL4_{AD}$-fusion expression library (see section 2.8. Materials and Methods).

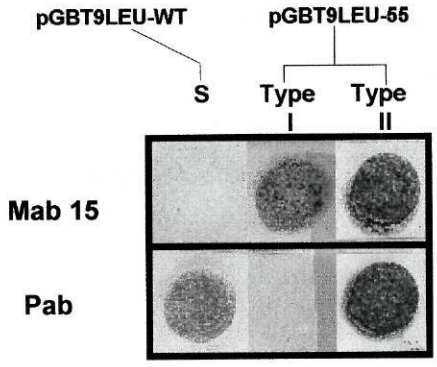


Figure 34. Immunoreactive assay for yeast pGBT9LEU-TTR fusion proteins.
Protein extracts of pGBT9LEU-TTR were applied onto a nitrocellulose membrane, screened for reactivity with Pab and Mab 15 and then selected for screening the aptamer library. S –"soluble"-WT *bait*; Type I- "Type I aggregates"- 55 *bait*; Type II -"Type II aggregates" - 55 *bait*.

Since neither *bait*s were toxic to yeast or self-activating for the reporter genes growing in medium supplemented with 50 mM 3-AT, sequential transformation, rather than co-transformation with the library DNA was performed, allowing high transformation efficiency.

A total of 6 x 10^6 transformants were placed under selection; this number was estimated by plating an aliquot of the transformation on medium selecting only for the presence of the plasmids (SD-W-L). 431/451/469 his^+ colonies were collected for each interaction (soluble / type I agg / type II agg) respectively, and streaked onto fresh SD-W-L-H 50 mM 3-AT selective plates (the streaking procedure allows segregation of multiple library plasmids sometimes present within the same colony). After growing for

5 days, the streaked colonies were screened for their ability to produce β-galactosidase activity using a filter lift assay. Exactly 45/58/48 colonies became blue in this assay for soluble / type I agg / type II agg, respectively. In general, larger his^+ colonies were more likely to be blue (showing up on the 5^{th}-9^{th} day of growth) than the growing colonies that appeared first (3^{rd} day). After repeated streaks in SD-W-L-H + 50 mM 3-AT, β-galactosidase activity was consistently detected for 29/32/27 of these colonies for soluble / type I agg / type II agg, respectively. These numbers of colonies were considered positives in this initial screen and were used in additional studies.

3.3. *In vitro* screening for TTR aggregation after interaction with peptide aptamers

At the end of the peptide library screen it was expected that transformants that expressed peptide aptamers interacting specifically with mutant aggregated TTR and not with the WT soluble TTR and vice-versa, would be isolated. 6 $x10^6$ clones were screened to reveal approximately 30 interactors (for each *bait*) able to grow on SD-W-L-H medium and able to induce β-galactosidase activity. Using the same amyloid marker (Mab 15) and the polyclonal antibody (Pab) as before (for *bait* selection), protein extracts were obtained from the positive interaction colonies and tested for immuno-reactivity. The reactions obtained are summarised in Table VII, for each TTR-*bait* used.

Aptamers were organised into groups according to the different recognition patterns obtained: **1)** Interactors with soluble TTR *bait* that caused no change in the antibody recognition pattern; this situation can be due to: **(i)** the aptamers did not interfere with the Pab recognition of the soluble protein; **(ii)** these short peptides did not alter the protein in a way that would favour β-sheet amyloidogenic formation, or a TTR intermediate amyloidogenic structure, that would be recognised by Mab 15.

2) Interactors with the "Type I aggregates" *bait* that produced different effects on antibody reactivity: **(i)** Some produced no effect in the recognition pattern, and so *bait*s that were positive for Mab 15 interaction and negative for Pab, were not affected by the interaction with these aptamers; **(ii)** another group appeared to be capable of reverting the aggregation pattern of the *bait* (since both antibodies exhibited different behaviour): the *bait* now being positive for Pab and negative for Mab 15 interaction, suggesting that there might be a blocking of amyloidogenic epitopes and maybe a modification in the amyloidogenic structure formation, since the Pab can now recognise this *bait*; **(iii)** finally, a third group of aptamers changed the recognition pattern of the *bait* for the Pab

only, and one of the interpretations of this result could be that the surface of the aggregates changes and "frees" other surfaces available for Pab recognition.

3) Interactors with the "Type II aggregates" *bait*, that altered Ab recognition, can also be organised into three different groups: **(i)** some produced no effect in the recognition pattern, and so *bait*s that were positive for both Mab 15 and Pab interaction before library screening, were not affected by these aptamers; **(ii)** a second group of aptamers seemed to revert the recognition pattern of Mab 15, whilst not affecting the Pab reaction, suggesting that there might be blocking/modification of amyloidogenic epitopes; **(iii)** the third and last group, in contrast, did not affect Mab 15 recognition but might have interfered with Pab recognition of the *bait*, since Pab no longer reacted with these type of aggregates after aptamer binding.

Table VII. Twenty aptamers interactants with three different *bait*s. The ones highlighted in bold were selected for synthesis and further characterisation.

Baits	*Interactant Ab*	*Colony type Before / after*	*Comment*	*Preys*
Type I aggregates	Pab	- / -	No change	**C45**
	Mab 15	+ / +		20B
				62B
				61C
	Pab	- / +	Changes type of aggregation	**67**
	Mab 15	+ / +		20E
	Pab	- / +	Reverts aggregates	**C26**
	Mab 15	+ / -		
Type II aggregates	Pab	+ / +	No change	7p115
	Mab 15	+ / +		27
				18B
	Pab	+ / +	Reverts aggregates	**40W**
	Mab 15	+ / -		40PB
	Pab	+ / -	Changes type of aggregation	47B
	Mab 15	+ / +		
soluble	Pab	+ / +	No change	**29B**
				58P
				74P
	Mab 15	- / -		29C87
				58W
				A19B16
				24W

3.4. Identification and selection of aptamers

Twenty interacting peptides, out of all the groups described above were selected for sequencing (7/7/6, for baits: soluble / type I agg / type II agg, respectively) and the results are shown on Table VIII and on Figure 35. Only one of the aptamers was found to be repeated twice, but specific for the same *bait*.

Table VIII. Variable regions of aptamers that interact with WT TTR (soluble) and Leu55Pro TTR (Type I and Type II aggregates). The sequence of these variable regions is shown in bold, flanked by 5 aminoacids on each side from the thioredoxin platform. X – represents aminoacids that could not be identified upon repeated sequencing.

Aptamer	Variable region
20B	EWCGP**QVVHGAXAXYPFGVTEEAAX**GPCKM
C45	EWCGP***KYNNGQDFATSEQQGSCGTG***GPCKM
62B	EWCGP**TAVGLVYXALVFTSKPSAPC**GPCKM
61C	EWCGP**FSSFXLRRLRLSWCCVPMGV**GPCKM
20E	EWCGP**QVVHGAXAXYPFGVTEEAAE**GPCKM
67	EWCGP***LLTITMALGWRLRSQTGCRQ***GPCKM
C26	EWCGP***XCLCLKLVMLNENRELLYDTQ***GPCKM
7p 115	EWCGP**VPYSLXAGRVIRLQKVRTYA**GPCKM
27	EWCGP**XGXMANCWSVWGHCRTTGRX**GPCKM
18B	EWCGP**XGGPXXXLESWLXNRXRTIF**GPCKM
40W	EWCGP***GLATWAQMPGYVWFSYSLL***GGPCKM
40PB	EWCGP**GLATWAQMPGYVWFSYSLLG**GPCKM
47B	EWCGP**WGNMTCRSSVESLLESRRXD**GPCKM
58W	EWCGP**GXIXPXVXGGLGCGFLMFDL**GPCKM
A19B 16	EWCGP**XVXYXGSLLMYTVLGLVFRX**GPCKM
24W	EWCGP**RMSSGVLSCCFCDLVPSVCC**GPCKM
29B	EWCGP***XMKAPMWHSFASMFWGRGDS***GPCKM
58P	EWCGP**WWLXGXVHDNXKWPVCLCWX**GPCKM
74P	EWCGP**XXKGXRDGKTAMSFDSLXGP**GPCKM
29C 87	EWCGP**LMKAXMWHSFASMFXGRGDX**GPCKM

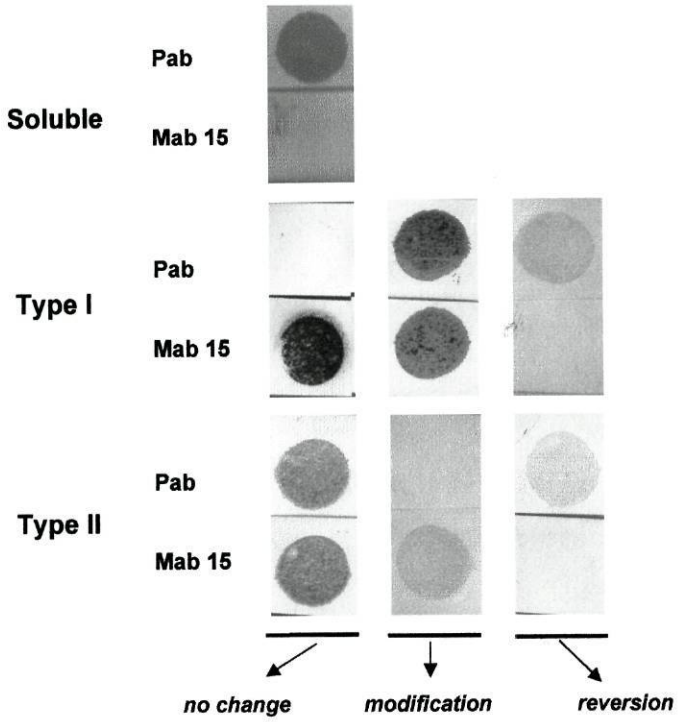


Figure 35. Immunoreactive assays for yeast pGBT9LEU-TTR – library interaction.
Protein extracts of yeast colonies corresponding to true interactions between TTR *baits* and *prey* aptamers were applied onto a nitrocellulose membrane and screened for reactivity with Pab and Mab 15 to assess the differences in recognition patterns of these Abs, due to aptamer binding to the three TTR *baits* (Soluble – WT; Type I – aggregates Type I; Type II – aggregates Type II.

To test further whether the his$^+$-blue phenotype of the colonies isolated in the original screen was reproducible and dependent on the interaction with the pGBT9LEU hybrid proteins (soluble / type I agg / type II agg), yeast segregants containing only the library plasmid were generated (section 2.10. Materials and Methods). Positive colonies were grown in SD-W in order to lose the *bait* naturally, due to the absence of selective pressure for maintenance of the *bait* plasmid. *Prey* segregants were obtained for all positives except for three interactors of the soluble *bait* (26/32/27). They were isolated from the positive yeast colonies and used to transform *E.coli*. The *prey* plasmid was then purified from bacteria and used to re-transform yeast. Ability to self-activate Y190 reporter genes was next analysed and none was found to induce either HIS3 or LacZ by themselves. All *bait*s were transformed with test fusions (soluble / type I agg / type II agg) to verify whether the his$^+$-blue phenotype could only be restored in the presence of pGBT9LEU specific *bait*s. Of these, (25/27/25) were found to interact specifically with TTR-*bait*s soluble / type I agg / type II agg, respectively, and only true positives followed for additional tests.

Multiple alignment methods were assayed in order to find conserved short sequences/domains amongst the aptamers. The hydropathy profile of each aptamer

was also analysed and five aptamers were selected to be synthesised (the most representative ones from five groups out of seven) (In bold and italic -Table VIII). The fact that different aptamers showed selectivity for different TTR forms indicated that these aptamers recognised different epitopes conserved among different forms of TTR (soluble/fibrillar) and since all five peptides are charged it can be suggested that some of their interactions with TTR could be ionic.

Of the five selected aptamers, one was intended to be used as a control, binding only to WT soluble TTR (29B); another aptamer (40W) bound only to Leu55Pro Type II aggregates and the final three binding to Leu55Pro Type I aggregates (67, 26C and C45). There were other aptamers binding to Leu55Pro Type II aggregates that were not selected at this phase, because this type of aggregate might not be so "interesting" as type I, since before interaction with aptamers, colonies of yeast transformed with this *bait* are reactive for both Abs.

3.5. Quantitative analysis of interactions between TTR *baits* and peptide aptamers

SPR was used to quantify the interactions between immobilised TTR and five selected aptamers isolated from the screened interaction library. The association and dissociation of TTR peptides was monitored at a buffer flow rate of 10 μl/min. Some peptides showed some non-specific binding, which could be subtracted from the true interaction response by using a control sensor chip with an activated and blocked surface (section 2.14 Materials and Methods). Using SPR, we first analysed the kinetic properties of complex formation between soluble and fibrillar TTR and different aptamers. Four different TTR proteins were immobilised on the sensor chip surface: WT, Leu55Pro soluble, Leu55Pro fibrillar and Tyr78Phe soluble. The latter was included in this study to test whether any of the aptamers interacting with type I and type II aggregates would interact with this tetrameric amyloidogenic intermediate form of TTR. Complex formation between TTR and aptamers was then monitored.

Injection of aptamers as analytes to sensorchips containing soluble or fibrillar forms of TTR, produced a typical SPR binding signal and the binding of all five aptamers to different TTR forms was dose-dependent (Figure 36 D-H). The sensorgrams obtained match the specificity of aptamers for soluble or aggregated TTR, previously obtained in the two-hybrid system (Figure 36 A-C), confirming that aptamer 29B was specific for TTR soluble forms and that 26C, C45, C26 and 40W interacted with aggregated TTR only.

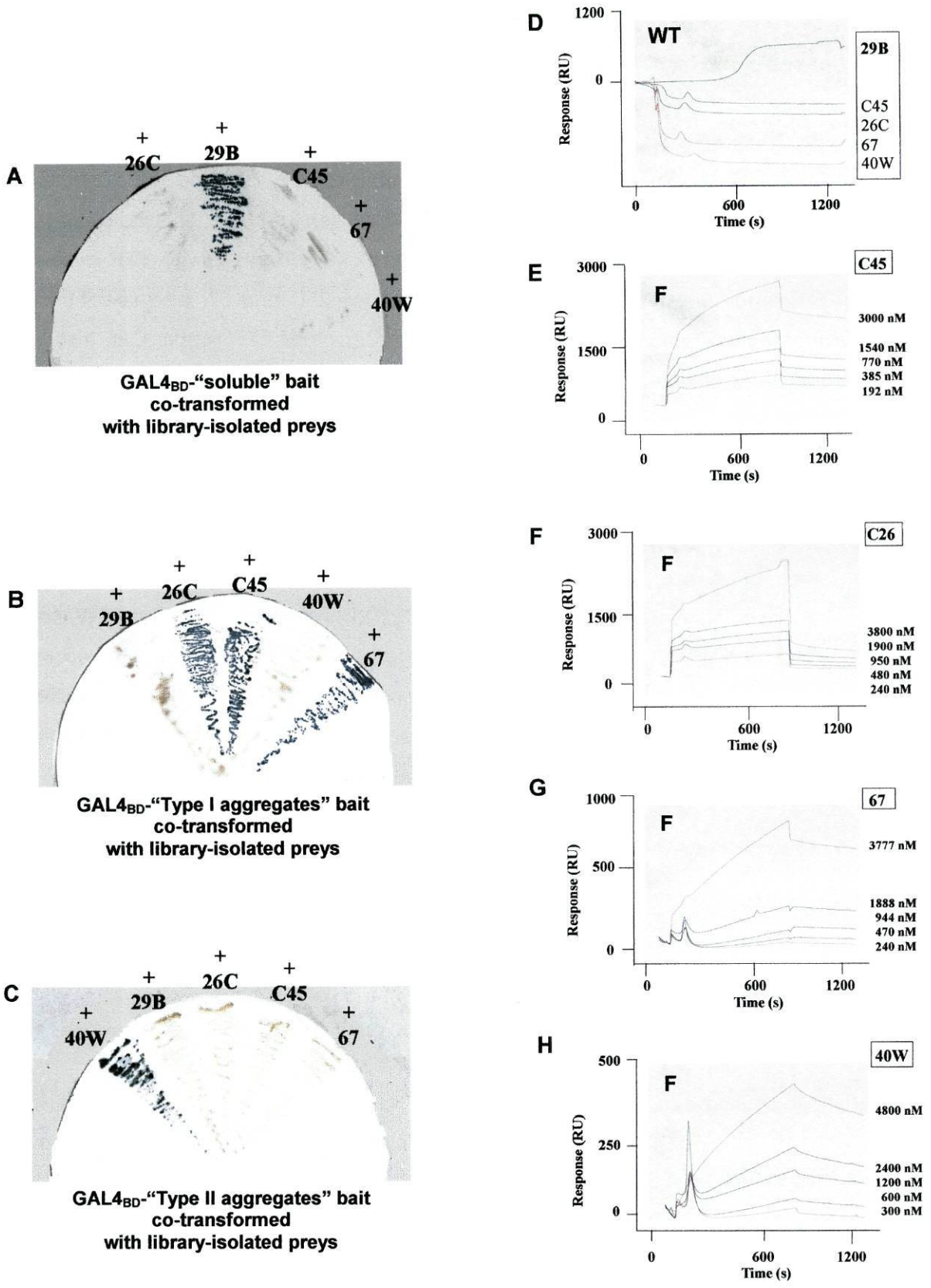


Figure 36. Specificity of aptamers for different TTR forms. Evidence obtained from yeast two-hybrid (A-C) and SPR analysis (D-H). In the two-hybrid system, "soluble", "type I agg" and "type II agg" fused to the $GAL4_{BD}$ were tested for specificity of interactions with the $GAL4_{AD}$ fused *preys* (previously isolated from the peptide aptamer library). Activation of the β-galactosidase reporter gene is shown by blue staining. F (Leu55Pro TTR fibrils), WT (soluble WtTTTR) coupled on CM5 sensor chip.

The k_d of these specific interactions was determined from the dissociation phase of the binding of aptamers to immobilised TTR. Similarly, k_a was evaluated from the association phase and finally the apparent K_D of the binding was calculated (Table IX). The two peptides isolated from the library screen, specifically interacting with the soluble *bait* and "type I aggregates" of Leu55Pro *bait*, 29B and C45 respectively, did not bind to any other TTR forms coupled on the sensor-chips. This indicates that these two aptamers are quite specific for epitopes present only in WT TTR molecules (for 29B) and fibrillar structures (for C45). Peptides 26C and 67, also specific for "type I aggregates"-Leu55Pro *bait*, showed affinity for other TTR forms. C26 also bound to Tyr78Phe soluble TTR, though with an affinity 7x lower, indicating that this peptide might recognise some amyloidogenic structures partially present in intermediate amyloid structures like Tyr78Phe. As for aptamer 67, though exhibiting strong affinity for Leu55Pro fibrils and a 3x lower affinity for Tyr78Phe, might be recognising an epitope more specific for the Leu55Pro mutation itself, than for an amyloidogenic fold, since it has even stronger affinity for the Leu55Pro soluble form (~6x higher than for Leu55Pro fibrils and 20x higher than for Tyr78Phe). Finally, aptamer 40W, specifically interacting with "type II aggregates" of the Leu55Pro-*bait*, again like C45 must bind strongly to an epitope specific for amyloid aggregates. As seen in Table IX, the interactions between Val30Met soluble and peptide 67, and Leu55Pro soluble and peptide 40W, are too weak to be considered specific binding.

Table IX. Rate constants for binding of TTR to aptamers. TTR was covalently immobilised on a BIAcore CM5 sensor-chip. The analytes were injected at 25^{0}C and the binding was followed over time by the change in RU. The k_a and k_d were determined from the association and dissociation phases, respectively, with five different concentrations of aptamers. Apparent K_D corresponds to k_a/ k_d ratio. **Values considered not statistically significant, because RU values for the different analyte concentrations tested were below limit for specific binding, therefore the binding affinities are extremely low.**

	Wt soluble				Val30Met soluble				Leu55Pro soluble				Tyr78Phe soluble				Leu55Pro fibrils			
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	K_D (nM)
29B	**3.57x 10^3**	**7.18x 10^{-4}**	**4.98x 10^6**	201																
67					**76.1**	**8.45x 10^{-5}**	**9.01x 10^5**	1110	**7.75x 10^2**	**9.23x 10^{-6}**	**8.4x 10^7**	11.9	**9.96x 10^3**	**2.55x 10^{-3}**	**3.91x 10^6**	256	**4.68x 10^4**	**3.79x 10^{-3}**	**1.23x 10^7**	81
26C													**4.2x 10^2**	**2.01x 10^{-4}**	**2.09x 10^6**	479	**1.5x 10^2**	**9.97x 10^{-6}**	**1.5x 10^7**	66.6
40W									**0.338**	**9.94x 10^{-6}**	**3.4x 10^4**	29400					**3.6x 10^5**	**1.24x 10^{-2}**	**2.89x 10^7**	34.6
C45																	**1.7x 10^3**	**4.94x 10^{-5}**	**3.44x 10^7**	29

3.6. Competition studies

Interaction of TTR and the five peptides was further analysed by ELISA. TTR WT and TTR Leu55Pro (fibrillar form) were immobilised on microtiter wells, followed by incubation with varied concentrations of the five peptides previously incubated with Pab or Mab 15. Bound antibodies were quantified using either goat anti-mouse-IgG-HRP conjugated or goat anti-rabbit-IgG-HRP conjugated (as secondary antibodies). Two of the peptides competing to interact with fibrillar Leu55Pro TTR might interfere with Ab recognition (26C-Mab15 and C45-Pab), suggesting an interference with this physical interaction. The results obtained for competition of other peptides with both Abs indicated no interference in the Ab-Leu55Pro TTR fibril complex formation (Figure 37).

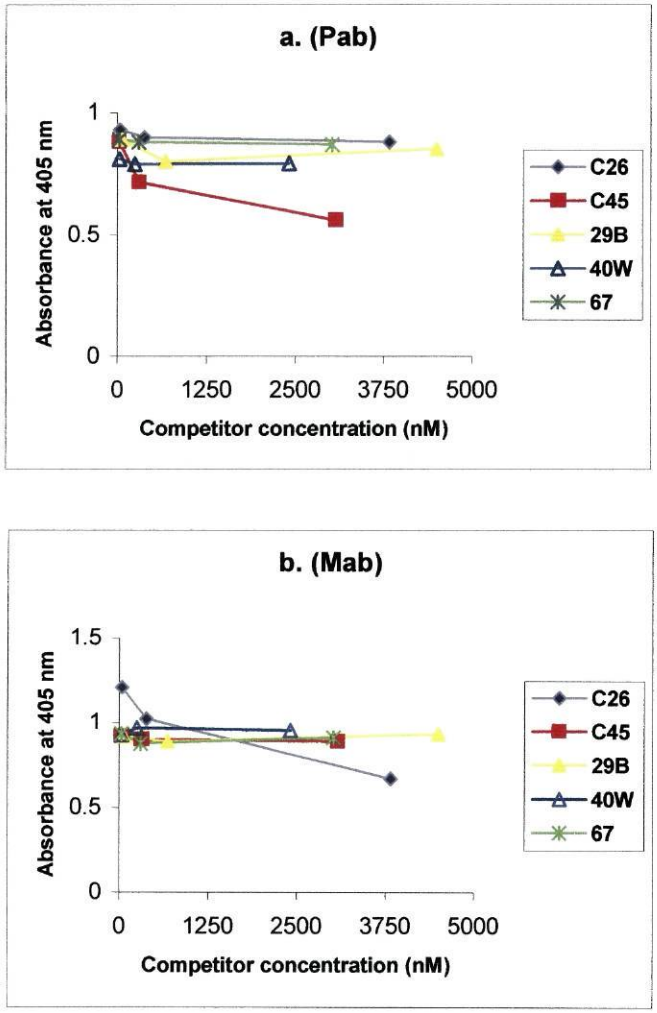


Figure 37. Competitive ELISA analysis of the ability of peptide aptamers to prevent binding between Pab or Mab 15 with Leu55Pro TTR fibrils (**a.** and **b.**, respectively). Both Pab and Mab 15 were previously incubated with the indicated concentrations of each aptamer, for one hour at RT and then transferred to a microtiter plate that had been coated with 1 µg of Leu55Pro TTR fibrils.

For interactions of Mab 15/Pab with WT, Leu55Pro soluble and Tyr78Phe soluble, the results were not always reproducible for the concentrations tested (data not shown).

In order to test whether some of these peptides could interfere with antibody/TTR complex formation, and so confirm these preliminary ELISA results, interactions were next monitored in real-time, using SPR technology. Soluble peptide aptamers were co-injected with antibodies as competitors for the antibody/TTR complex formation. The relative binding rate was calculated by dividing the binding rate in the presence of the competitor by that in the absence of the competitor. The average concentration of immobilised TTR in the flow cell was 1.5 μM; the antibody concentration was constant (~0.2 μM) in every incubation with several concentrations of aptamers (1-40 μM). IC_{50} values for all these interactions (Table X) correspond to the concentration of peptide that reduces by 50% the formation of complex TTR-Ab.

Table X. IC_{50} values for the inhibitory effect of peptides on Antibodies-TTR interaction.

■ Inhibitory effect on antibody binding to TTR

□ No interference with antibody binding to TTR

peptides	Pab	Mab 15
C45	Leu55Pro fibrils IC_{50} = 1.7 μM	
26C	Tyr78Phe soluble IC_{50} = 0.8 μM	Leu55Pro fibrils IC_{50} = 15.7 μM
40W	Leu55Pro fibrils IC_{50} = 3.7 μM	Leu55Pro fibrils IC_{50} = 8.5 μM
67	Leu55Pro fibrils IC_{50} = 6.5 μM	Tyr78Phe soluble IC_{50} = 5 μM
29B		

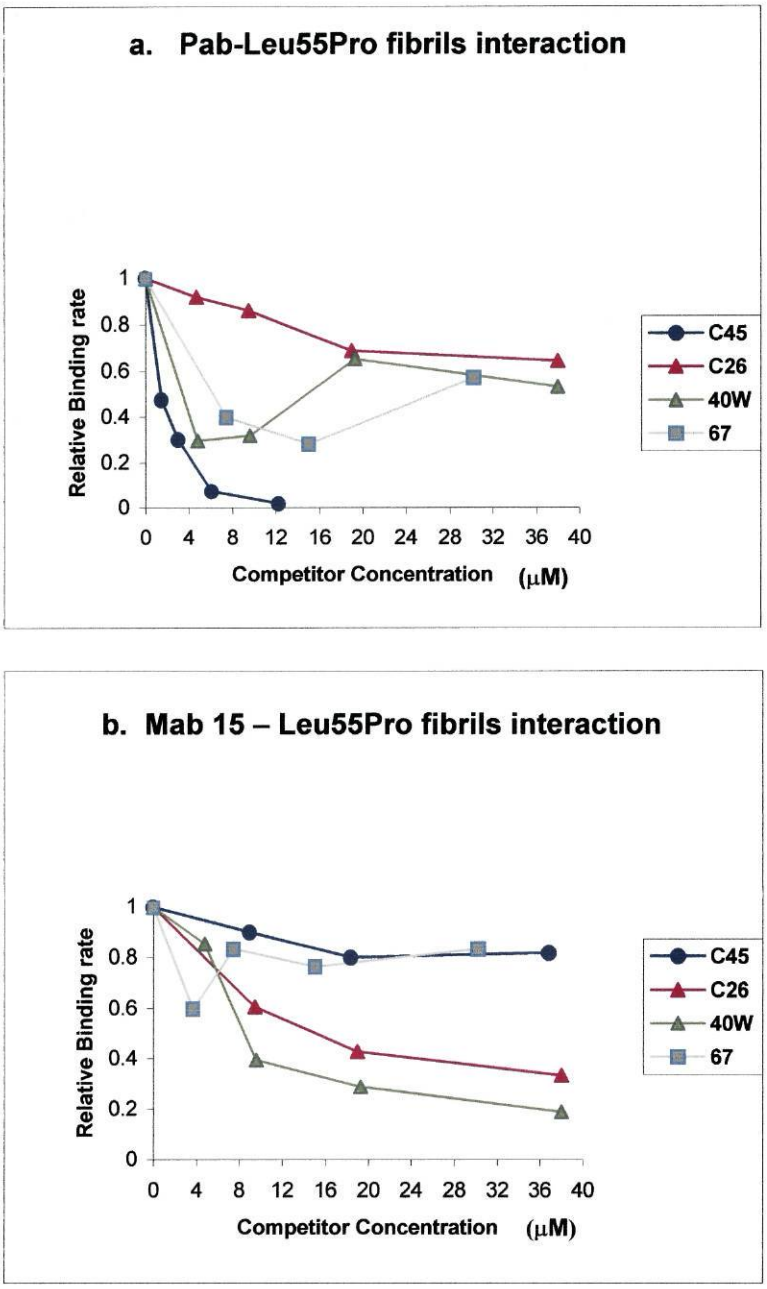


Figure 38. Competitive inhibition of the interaction between Mab 15/Pab with immobilised Leu55Pro TTR fibrils by peptide aptamers.

Figure 38a, shows the results of the inhibition of complex formation between the Pab with Leu55Pro fibrils. Peptides C45 and 67 compete with Pab for fibril interaction, though no obvious effect was seen for Mab 15-Leu55Pro fibril interaction. This interference with Pab recognition is quite strong for C45 and not so evident for peptide 67, since concentrations as high as 30 μM are no more effective than 6.5 μM (determined IC_{50} value).

As for aptamers 40W and 26C, though isolated from the peptide library using different "types of Leu55Pro aggregates", both compete with Mab 15 binding to Leu55Pro fibrils (Figure 38b). Since aptamer 40W binds to fibrils twice as strongly as to 26C, the IC_{50} for inhibiting Mab 15-Leu55Pro fibril interaction is nearly half of the concentration needed for aptamer 26C to have the same effect.

As referred to above, two of the peptides selected from the 20-mer-aptamer library, seen to interact specifically with "type I aggregates" Leu55Pro-*bait*, also showed affinity for soluble Tyr78Phe TTR mutant, i.e. 67 and C26.

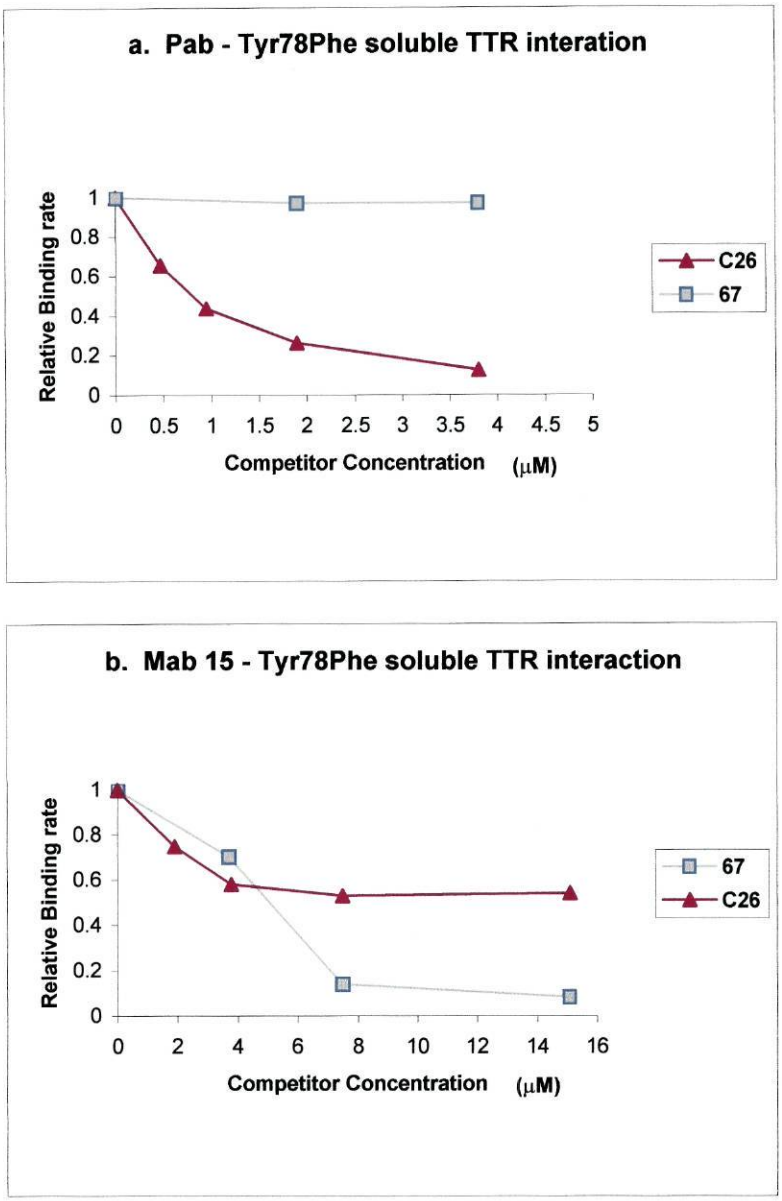


Figure 39. Competitive inhibition of the interaction between Mab 15/Pab with immobilised Tyr78Phe soluble TTR by peptide aptamers.

The results of the inhibition of peptide aptamers in Mab 15-Tyr78Phe versus Pab-Tyr78Phe complex formation are shown in Figure 39 a. and b. Peptide 67, binding twice as strongly to Tyr78Phe as C26, interferes with Mab 15 binding to this intermediate amyloidogenic tetrameric mutant with an IC_{50} of 5 μM, whereas C26 inhibits Mab 15 binding but an IC_{50} value is not reached (for concentrations up to 16 μM). As for the interference with the Pab-Tyr78Phe interaction, complex formation is inhibited by peptide C26 with a very low IC_{50} value of 0.8 μM while peptide 67 does not interfere at all.

Peptide 67, specific for "type I aggregates" Leu55Pro-*bait* interaction, surprisingly exhibited a very high affinity also for the soluble form of this TTR mutant, immobilised on the sensor-chip. Analysing Figure 40, there was no effect on Pab binding to Leu55Pro soluble TTR, despite a K_D of 11nM for 67-Leu55Pro soluble TTR interaction, which indicates that the binding surfaces for the aptamer and for Pab are not the same. Since Mab 15 does not react with Leu55Pro soluble TTR, it was not possible to check the effect of the aptamer on that interaction.

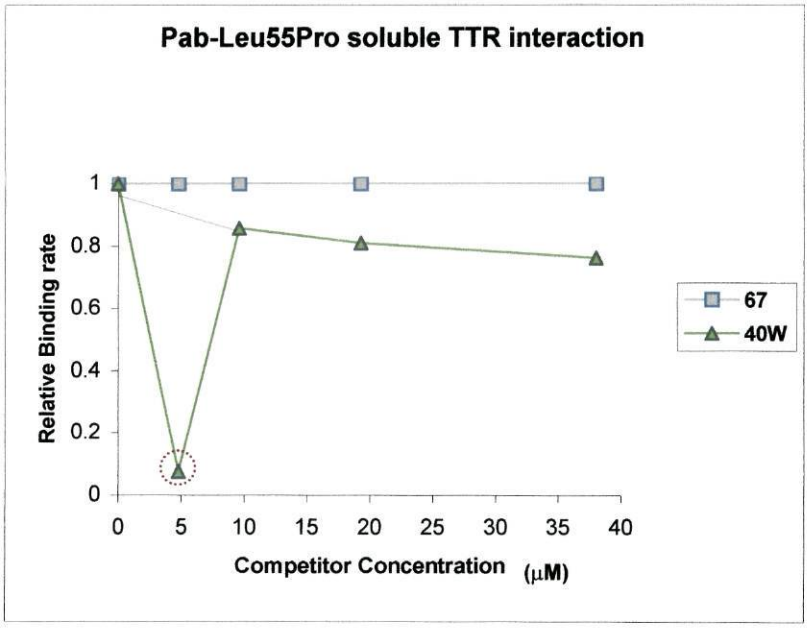


Figure 40. Competitive inhibition of the interaction between Pab with immobilised Leu55Pro soluble TTR by peptide aptamers. The point represent by an ◌ was considered an artefact.

The previous two-hybrid observations of modified TTR aggregation patterns upon interaction with aptamers can now be interpreted under the light of the results obtained from the competition of those aptamers with the antibodies for different TTR

forms (Table XI). The cause for different colony phenotypes (native or aggregated TTR) upon interaction with aptamers can be a conformational change modifying the surfaces recognised by the Abs or a competition effect of the aptamers binding to those same surfaces, masking the presence of those areas.

Table XI. Relationship of two-hybrid and SPR results concerning the immuno-screening of colonies before and after TTR interaction with aptamers and TTR interaction with antibodies after aptamer injection, respectively.

	Leu55Pro aggregates		**Tyr78Phe**
	Two-hybrid results	***SPR results***	***SPR results***
C45	No changes in aggregation type I phenotype	Competes with Pab	
67	Changes in aggregation type I phenotype	Competes with Pab	Competes with Mab 15
C26	Reversion of aggregates type I towards native phenotype	Competes with Mab 15	Competes with Mab 15 Competes with Pab
40W	Reversion of aggregates type II towards native phenotype	Competes with Mab 15	

1) Peptide C45 specifically interacts with fibrillar forms of TTR and interferes with the Pab-Leu55Pro fibrils interaction, causing no effect on Mab 15 binding, suggesting that the interaction of the peptide with TTR aggregates does not induce any conformational changes and that its binding surface includes part of the epitopes recognised by the Pab.

2) Peptide 67 leads to a conformational change of aggregated forms recognising an epitope different from Mab 15 since there is only competition with the Pab. It also competes for a region close to the Mab 15 binding site in TTR intermediate amyloidogenic soluble forms (Tyr78Phe).

3) Peptides C26 and 40W both lead to changes of colony phenotype from aggregates towards a native fold. This can be due to an induction of conformational

change or to competition with Mab 15. Since 40W competes with Mab 15 it might just be binding at the same site as this Mab on the fibrils, whilst C26 produces evident conformational change as Pab reactivity towards yeast TTR changed too.

3.7. Inhibition studies of peptide interference on TTR fibrillogenesis

To further investigate the nature of the interaction between TTR Leu55Pro fibrils and peptide C45, we tested whether complex formation between the two interactants could interfere in the growth of fibrils. For this purpose, soluble Leu55Pro TTR was incubated in the absence or presence of C45 peptide (2 μM) at 37^{0}C and the ability of C45 to inhibit fibril formation was analysed at different points as indicated in Figure 41. There is a clear reduction in ThT fluorescence between Leu55Pro TTR solutions incubated with C45, compared with those that did not contact with the aptamer, from day 5 onwards. After five days at 37^{0}C, some fibril formation occurred, but the peptide did not seem to interfere with that process, probably because it has no or little affinity for soluble Leu55Pro. Upon binding to TTR (around day 10), peptide C45 significantly reduced the progression of amyloid formation, and that difference is more evident at day 15 (~40% reduction). Although the difference between 15 and 20 days is not so marked, it is apparent that though there is still some amyloid formation, part of the fibril population is still blocked. This allows us to suggest that indeed C45 is a peptide with anti-amyloidogenic activities and that although Leu55Pro TTR can still form fibrils in its presence, it does so at a slower rate or in smaller proportions. To test whether the effect seen was on quenching of binding of ThT to fibrils, fibrils of Leu55Pro TTR were measured for ThT fluorescence/binding before and after incubation with C45, for an hour, at 37^{0}C. There was no secondary effect or interference of peptide in ThT binding, for the same surfaces. WT TTR (included as negative control) was neither affected nor produced fibrils, at 37^{0}C, with or without the peptide.

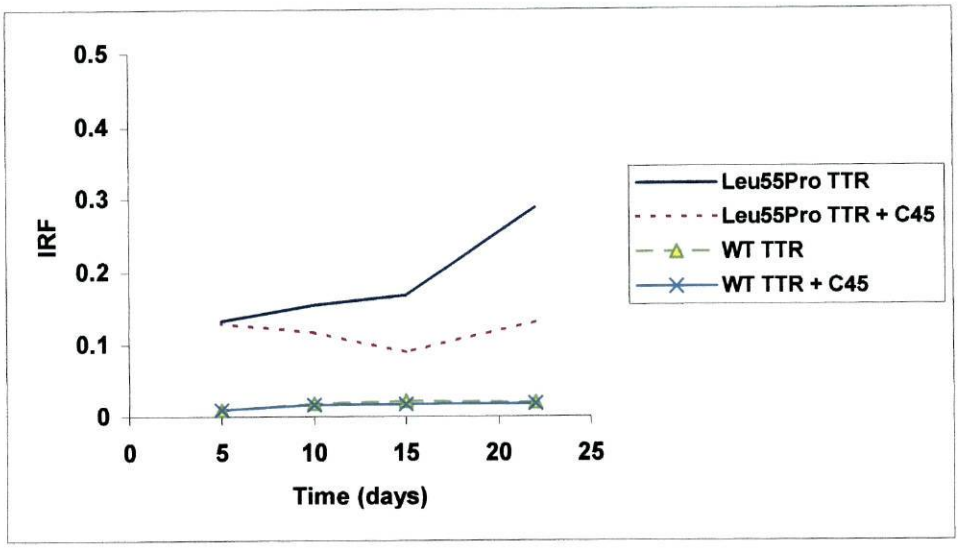


Figure 41. Amyloid formation with protein preparations of WT and Leu55Pro TTR, in the presence or absence of aptamer C45 and tested by ThT fluorescence. Each point corresponds to the maximum peak obtained at 440 nm upon ThT binding. IRF, intensity of relative fluorescence.

4. DISCUSSION

The process that leads to the deposition of soluble TTR into an amyloid fibril is still unknown, though it was hypothesised that an amyloidogenic intermediate should be initially formed and prompt the protein to deposit. Whether this modified amyloidogenic subunit results from a destabilisation of the TTR tetramer leading to dissociation into monomers or from a proteolytic event is not clear (Damas and Saraiva, 2000). The modifications introduced by the amyloidogenic mutations contribute to a destabilisation of the protein quaternary structure, leading to an increase in the number of available units with an amyloidogenic structure, which is responsible for amyloid formation. Inouye *et al.* (1998) proposed a model for the TTR protofibril based on an interpretation of a set of reflections to a 29 Å repeat unit, which was considered a true repeat, corresponding to a TTR monomer, containing two four-stranded β-sheets. The simple axial stacking of TTR monomers envisioned by these authors produces a β-sheet helix due to the native twisting of β-strands in the TTR monomer.

Preventing, altering, or reversing TTR fibril formation may have therapeutic value. Tools of biochemistry, biophysics and molecular biology have been utilised to determine the structure of TTR, to elucidate the folding pathways of the molecule, to determine how different conformers participate in the self-assembly of TTR into fibrils, and to model intermediate and mature fibril assemblies. A therapeutic strategy for amyloid prevention is thus to administer ligands to inhibit fibril formation, but several possible mechanisms of inhibition must be considered. (i) The ligand may bind specifically and with high affinity to the native protein to form a more stabilised soluble protein than the improperly folded intermediates; (ii) a target drug may preferentially bind to the aggregation-prone intermediate or unfolded protein and thermodynamically "freeze" the species to prevent fibril formation; or (iii) ligand binding to nascent fibrils to form blocked aggregates, which are incapable of further growth and would remain undetected by ThT. Stabilisation of native TTR would lead to decreased concentration of the partially unfolded intermediate and might therefore inhibit the formation of fibrils.

Interfering with amyloid fibril formation by small organic molecules is a pharmacological therapy already proven to work for systemic amyloidoses such as AL (immunoglobulin light chain) and AA (serum amyloid A protein) [Kisilevski *et al.* (1995) and Tagliavini *et al.* (1997)]. For Alzheimer's disease (AD), peptides [Tjernberg *et al.* (1996)] and Mabs [Solomon *et al.* (1996)] have also been proposed as potential therapeutic agents, although it is unclear how blood-brain barrier penetration and cellular penetration would be achieved with these large, polar molecules. Several

groups have described efforts to define the structural basis for the fibrillogenic nature of Aβ and defined approaches to develop inhibitors of amyloid formation. While larger peptides can have inhibitory effects on fibrillogenesis, such materials are less appealing as leads for drugs development than smaller hydrophobic molecules. It might also be possible that inhibitors can display alternative effects through binding differentially to various forms of amyloid (there might be a delay in polymerisation for instance, but still allowing a normal rate of polymerisation once initiated) (Naiki and Nakakuki, 1996). Using thus artificial agents to break interactions might be useful and have effective therapeutic effects. A popular strategy is the creation of libraries of nucleic acids or peptides, to select individual small molecules that recognise one or the other surface of a target molecule, and block protein-protein interactions involving that surface.

Two groups have demonstrated that two-hybrid methods could be used to isolate peptides that bind specific proteins, and in one case such peptides have been shown to break interactions. Yang *et al.* (1995) made a library that expressed random 16-aminoacid peptides fused to the GAL4 activation region and screened it for peptides that interacted with a human retinoblastoma gene product, pRb, *bait*. The experiments demonstrated that along with phage display and other combinatorial peptide methods (Smith *et al.*, 1993), two-hybrid methods could be used to select unconstrained peptides that define consensus-binding sequences formed in nature.

Somewhat different experiments were performed by Colas *et al.* (1996), starting from another perspective: that antibodies could recognise most combinations of shape, charge and hydrophobicity and that they did so by displaying conformationally constrained peptide loops of variable sequence. They wished to isolate synthetic peptide agents that could bind to most faces of cellular proteins and specifically disrupt particular cellular protein-protein interactions. To this end, in this work, they used two-hybrid methods to isolate, from a library of conformationally constrained 20-mers displayed by *E. coli* TrxA, variable region sequences that recognised cyclin-dependent kinases, and these TrxA-variable region chimeras were named peptide aptamers. Aptamers cross-reacted with cyclin-dependent kinases of related sequence, indicating that these proteins recognised conserved antigenic regions (epitopes) on the related proteins. As measured by SPR experiments, binding was strong, with Kds in the nanomolar range. These results showed that peptide aptamers could be constructed that were somewhat like antibodies, in that members of collections of them would recognise many different protein surfaces; only unlike antibodies, peptide aptamers were designed to work inside cells, providing reagents to probe protein function *in vivo*.

The same authors showed afterwards (Colas *et al.*, 2000) that peptide aptamers could disrupt specific protein interactions *in vivo* allowing their precise manipulation and suggesting the possibility to select aptamers that distinguished among allelic variants of proteins. The generation and use of new peptide aptamer derivatives should facilitate high-resolution study of regulatory pathways and could possibly inspire new therapeutic strategies.

Hughes *et al.* (1996) assayed the study of the interaction of Aβ monomers *in vivo*, also using a two-hybrid system, demonstrating that two monomers of Aβ were capable of interacting in the eukaryotic cell. However, this system needed further testing with methods known to accelerate or inhibit the monomer-monomer interaction in fibrillogenesis.

A number of pharmaceutical and biotechnology companies are now employing two-hybrid methods to search for small molecules that interrupt particular protein interactions and advances in peptidomimetic chemistry will allow an alternative to screening for these small molecules. Determination of the structures of peptide aptamers variable regions bound to their targets may provide sufficient information to allow synthesis of non-peptide interaction disrupting molecules. Thus, several studies have established the use of aptamers as inhibitors of protein contacts aiming to aid the dissection of the networks of protein interactions. Libraries of unconstrained peptides contain sequences capable or recognising targets *in vitro* and in yeast (unlike the sequences reported here, such peptides often bear similarity to natural interactors). In contrast to unconstrained libraries, constrained peptide libraries are less conformationally diverse, but this lack of conformationally diversity should lower the entropic cost if binding causes the loop to adopt a single conformation.

In our work, short peptides interacting with soluble and fibrillar TTR have been identified after employing a yeast two-hybrid system to screen a random 20-mer-peptide library, fused to the activation domain of the GAL4 gene. This interaction library was introduced into a selection strain containing either the WT *bait* or the Leu55Pro mutant *bait*s, fused to the DNA binding domain of the GAL4 gene. BIAcore detection supported the specificity of interactions between peptide aptamers and TTR and new interactions were found for some of the peptides, namely towards Tyr78Phe soluble TTR and the soluble form of Leu55Pro TTR mutants immobilised on sensor-chips. Of all five aptamers examined in detail, due to its specificity to the fibrillar form of Leu55Pro TTR, C45 was subjected to inhibition assays of *in vitro* fibrillogenesis.

Aptamer C45 probably binds to a site in the monomer-monomer interface of the growing fibril. Until the fifth day of incubation (when fibrils or pre-fibrils already co-exist with soluble protein) there is no inhibitory action, compared to what is seen at 15 or 22

days of incubation. This aptamer has no inhibitory effect on Mab 15 binding to Leu55Pro fibrils, indicating that this Mab must bind to exposed epitopes on the fibril and not in between subunits. This C45 action would reduce fibril growth rather than inhibit fibril growth/formation, since ThT binding still occurs, though to a lesser extent. Therefore, C45 does not prevent β-sheet formation or fibril initiation, rather appears to bind to the growing TTR fibrils and disrupts its elongation processes. It has been suggested that inhibition of fibril growth processes may be a more feasible therapeutic target that inhibition of fibril initiation (Esler *et al.*, 1996). Additional studies must be undertaken in order to elucidate the mechanisms of inhibition and the specificity of binding for Leu55Pro fibrils or for other types of aggregation.

The aim of this study was also to identify cryptic regions present only on fibrils or amyloidogenic structures and this was partially indicated by the results obtained with some of the aptamers. In figure 42 we represent a scheme of the amyloidogenic pathway. The tetrameric form of TTR must undergo sequential conformational modifications that ultimately lead to its dissociation into non-native monomers, which finally associate into fibrillar amyloidogenic structures. Along with these tetrameric conformational changes, new areas become exposed and available for interaction with molecules. These molecules can be specific for the following forms: native tetramer (WT); partially modified tetramer (Leu55Pro); highly modified tetramer (Tyr78Phe); modified monomer or fibrils.

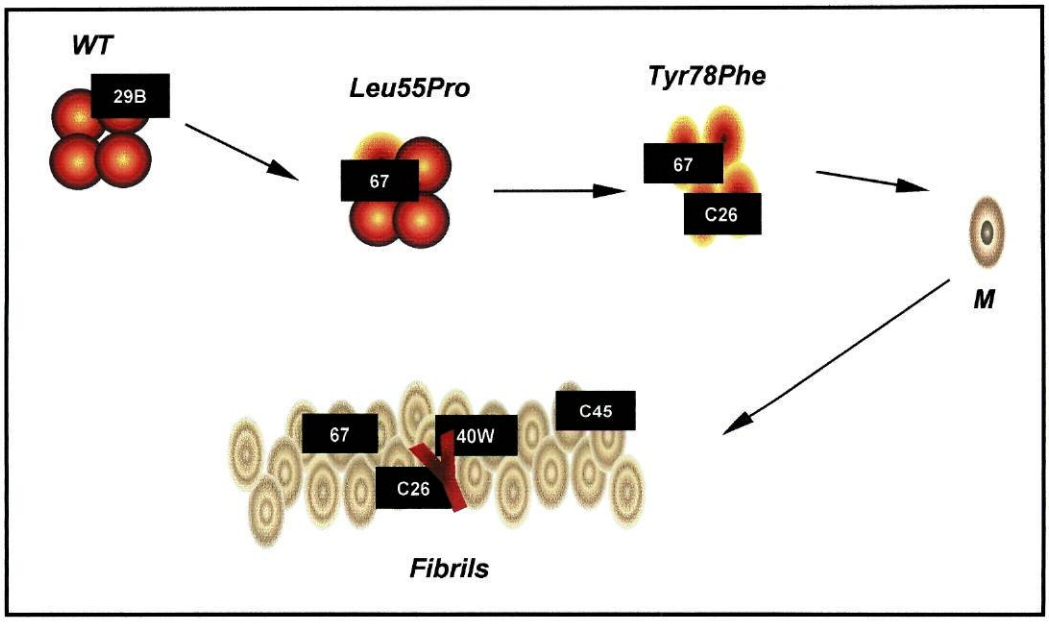


Figure 42. Epitope recognition by aptamers in different forms of TTR fibrillogenesis pathway. ( ) Mab15; WT - WT TTR tetramer; Leu55Pro – tetrameric soluble form of TTR amyloidogenic mutant Leu55Pro; Tyr78Phe – tetrameric soluble form of Tyr78Phe TTR; *M* – modified TTR monomer.

We propose that some antigenic cryptic regions are conserved between intermediate soluble forms of TTR and TTR fibrils, whilst others are specific for initial intermediate TTR forms, and revealed only in early intermediate stages. The five peptides studied in this work were able to recognise and bind to three different types of epitopes conserved or lost along the amyloidogenic process.

The first epitope was suggested by the only one of the five aptamers, which interacts with the WT protein. This indicates that none of the modified surfaces recognised by other aptamers are present in the native tetrameric structure and that this epitope is characteristic of native TTR and is lost in subsequent intermediate structures. Using multiple alignment methods, comparing TTR WT primary sequence with the primary sequence of aptamer 29B, we could predict that in fact this peptide exhibits a higher similarity towards a specific area of the TTR sequence. Though this analysis is not so informative, since peptide structure in solution will adopt different conformations, the region of TTR exhibiting the closest match (chemistry of amino-acid structure and hydrophobicity) includes residues 40-46, part of strand C. This observation is in agreement with the hypothesis that this region is modified in TTR fibrillar structures.

Two of the other epitopes identified are fibrillar epitopes only. The first is recognised by aptamer C45 and is located in an area different from the one recognised by Mab 15. Since we also observed inhibition of Pab recognition of TTR fibrils upon binding of peptide C45, we can suggest that this aptamer might bind to an area that includes one of the Pab's epitopes, usually hidden in the native tetrameric TTR structure. Gustavsson *et al.* (1994) conducted studies to determine whether the TTR molecule assumed an altered configuration in the amyloid fibril, thus exposing different antigenic epitopes. They proposed that an abnormal interaction between monomers might take place in the amyloid structure, based on the finding that region 115-124 of TTR, corresponding primarily to the H strand, was exposed, and could be recognised by an anti-peptide polyclonal antibody. The second fibril-specific epitope, recognised by aptamer 40W is located in an area possibly shared with Mab 15.

The binding of aptamers C26 to Tyr78Phe and of 67 to Tyr78Phe and soluble Leu55Pro expanded our view of the amyloidogenic cascade. It became more clear that there were two types of conformational modifications: i) regions recognised by the two aptamers in tetrameric soluble forms of TTR corresponding to early amyloidogenic modifications that would be gradually " substituted" by others, and ii) intermediate amyloidogenic modifications that could be conserved in the fibril. In fact, C26 recognises an epitope in the same region as Mab 15, conserved in Tyr78Phe and Leu55Pro fibrils that could include residues 39-44. Peptide 67 recognises a different

cryptic region, "non-conserved" between soluble Leu55Pro and fibrils, that seems to undergo transformations along different intermediate TTR forms of the amyloidogenic cascade. This aptamer competes with Mab 15 for binding to Tyr78Phe, though the cryptic region that it recognises in the Leu55Pro fibrils is distinct from this interacting area.

These evidences allow us to propose three candidate regions for amyloidogenic cryptic epitopes. One of these epitopes is probably located in an area involved in TTR destabilisation previously reported to be recognised by Mab 15, the C strand-CD loop area, as suggested by the results with aptamers 40W, C26 and possibly 29B. We also found two new epitopes, one specifically exposed in fibrils, and another undergoing conformational changes between TTR soluble forms and fibrils, revealed by aptamers C45 and 67, respectively.

The usefulness of molecules as lead substances in the development of anti-amyloid drugs was always compromised by their lack of specificity [as was the case of cyclodextrins and Congo red, Camilleri *et al.* (1994) and Lorenzo *et al.* (1994), respectively]. Based on C45 primary structure, shorter peptides could be produced and successively selected or even the synthesis of non-peptide homologues of C45 may turn out to be useful as a pharmacological tool for the detailed characterisation of FAP amyloidogenic processes. Also, the effect of C45 and C26 could be investigated for pre-amyloidogenic intermediate structures like Tyr78Phe TTR tetramer, aiming at retarding the progression of fibril formation as early as possible. A yet more detailed knowledge of the interface regions of fibril sub-unit interactions as well as cryptic epitopes only exposed in amyloidogenic intermediates is necessary, so that these future therapeutic strategies involving disrupting/specific binding molecules, can be accomplished successfully.

GENERAL DISCUSSION

The ability to form amyloid seems to be an inherent property of TTR. In the tissues of patients affected with FAP, the building up of extracellular insoluble aggregates, which might be promoted by mutations altering how TTR folds initially or destabilise its final structure, can eventually prove fatal. The specificity of protein aggregation depends on interactions between particular aminoacid in the protein molecules. Several crystal determinations of TTR variants showed that the mutations do not alter significantly the normal folding of the protein. However, they do seem to destabilise the protein's structure, facilitating the formation of partially folded intermediates that readily aggregate with one another, forming amyloid fibrils.

The studies aiming at the characterisation of five constructed TTR mutants, regarding tetrameric stability and sensitivity towards *in vitro* amyloid formation, revealed important surfaces in the TTR molecule that might be involved in aggregation. We generated two "destabilised mutants", Asp18Asn and Leu110Ala and performed some biochemical studies that allowed us to conclude that these two mutant structures exhibited altered surfaces that contributed strongly to aggregation. On one hand, Asp18Asn was an unstable tetramer due to the interruption of the close contacts between aminoacid 18 of the A strand and aminoacids 20 and 21 in the region between strands A and B of TTR; on the other hand, Leu110Ala, showed a high susceptibility to dissociate into monomers, and the substitution of aminoacid 110 in the G strand affected the strength of dimeric contacts in the tetramer. Therefore, the importance of areas like the AB loop and the contacts between dimeric interfaces, i.e. strands G and H, are primordial for tetrameric stability.

We next tried to elucidate the nature of the amyloidogenic building blocks, supporting the hypothesis that monomers rather than tetramers or dimers were the sub-units of amyloid fibrils. In opposition to the mutants described above, all three engineered "stabilised mutants", were incapable of forming amyloid *in vitro*, due to disulphide bonding between extra cysteines introduced by directed mutagenesis. Though several evidences support the fact that indeed monomeric species compose the amyloid fibrils, Serag and colleagues, recently (2001), based on a semblance of continuous intermolecular β-sheets, suggested that some aspects of the natural dimeric association could be retained in the fibrillar structure, proposing that the mechanism of fibrillogenesis would involve an oligomeric intermediate, either a dimeric building block or a multiple of such. They used thiol to cross-link several engineered TTR mutants, in which they introduced cysteines, forming dimers. According to their results, the dimeric species were competent to form fibrils and they stated that formation of free monomers was not an obligate step in fibrillogenesis. However, and

when they confront their results with our data where two engineered disulphide-bonded dimers did not form fibrils (Ser117Cys and Glu92Cys), they suggest that some conformational changes might have occurred, making it impossible for these dimers to form fibrils. This in fact, could have happened with their mutants, since the authors present no data concerning the binding of T_4/RBP, to the tetrameric forms of these molecules, which would prove that the native structure of TTR was preserved in these dimers. As shown in our work, these two "stabilised mutants", bound T_4, constituting functional tetramers, and further dissociating into stable dimers upon pH 3.0. The dimeric structure was thus conserved at very low pH and no amyloid fibrils were formed *in vitro* from these dimeric species

Also, the work of Olofsson *et al.* (2001), showed that two aminoacid substitutions in the hydrophobic A-strand of TTR were highly unfavourable for the native tetrameric conformation and that the resulting unstable molecule was prone to form amyloid even at physiological pH and ionic strength. The discovery that a stable dimeric species could be trapped at low temperature was found by these authors to be in agreement with Serag and co-workers (2001), suggesting that the native dimeric interactions are preserved within the amyloid fibril. However it is possible that the formation of a stable tetramer did not occur in the first place, since no binding of T_4 or RBP could be detected for this mutant.

A partial unfolding and conformational fluctuations of a marginally stable TTR non-native monomer might play a key role in the fibrillogenesis process, as suggested in a model recently proposed by Quintas *et al.* (2001). These authors proposed that TTR variants with the least thermodynamically stable non-native monomer produce the largest number and amount of partially unfolded monomeric species. These monomeric species, in turn, can self-assemble into high molecular mass aggregates, at pH 7.0 and near physiological ionic strengths, model consistent with extracellular amyloid deposit formation. Our data, showing that dimers resulting from disulphide-linked monomers could not form amyloid *in vitro*, as could not monomers with increased stability (due to intra disulphide bonding in the double mutant Val30Cys/Leu55Cys), support this model, since under reducing conditions, all "stabilised mutants" readily formed ThT positive fibrils. A rearrangement of strands C and D might be part of the modifications that occur in a native tetrameric TTR structure, which lead to dissociation into partially unfolded or unstable monomers involved in the formation of amyloid fibrils.

In addition to the identity of the building block composing amyloid fibrils, the identification of amyloidogenic intermediates in the amyloidogenesis cascade and the epitopes that become exposed in the phases of this process, were also a concern in this project.

The hypothesis that the exposed cryptic epitopes in the two amyloidogenic mutants created by Goldsteins *et al.* (1997), could be part of an intermediate amyloidogenic structure, lead, two years later, to the production and characterisation of Mabs against these mutants. The fact that these Mabs could distinguish sera from carriers of amyloidogenic mutations (Palha *et al.* 2001) supports the notion that an amyloidogenic intermediate exists and circulates, and at some point deposits in tissues as amyloid. Our finding that an amyloidogenic engineered mutant, in its soluble tetrameric form, was recognised by an amyloid-specific Mab, lead us to propose Tyr78Phe TTR as an early amyloidogenic intermediate structure. The tetrameric structure of Tyr78Phe TTR might be altered due to the loosening of the AB loops of the tetramer, causing a conformational change in the molecule, exposing a new cryptic epitope, before subunit dissociation and further polymerisation into fibrils. However, further assessment of the crystal structure of the mutant is necessary to elucidate the surfaces interacting with the Mab. We have already obtained crystals of the mutant and the refinement of the structure is currently being performed.

Upon aggregation, TTR partially unfolded monomeric species may reacquire β-structure stabilised by intermolecular interactions, maintaining cryptic epitopes revealed in early intermediate unfolding steps. Thus, prior to dissociation into monomers, early tetrameric intermediate forms may exist in the fibrillogenesis pathway, exhibiting cryptic epitopes kept or not in the final amyloid structure. To further investigate TTR conformational intermediates with exposed cryptic epitopes that might reveal amyloidogenic surfaces, we performed a genetic screening using peptide aptamers, 20 aminoacid long, from a 20-mer-aptamer library, in an adapted yeast two-hybrid system. Using as bait the highly aggressive mutant Leu55Pro TTR, we screened a library of putative interacting aptamers aiming at identifying regions in the TTR molecule being important for binding during polymerisation and/or others, exposed before polymerisation in intermediate forms, and still conserved in fibrils.

Several studies have established the use of aptamers as inhibitors of protein contacts aiming to aid the dissection of the networks of protein interactions and the design of potent therapeutic agents. Colas *et al.* (2000) shown that peptide aptamers could disrupt specific protein interactions *in vivo* allowing their precise manipulation and suggested that the ability to select aptamers that distinguish among allelic variants of proteins should allow selective modification of the activities of individual alleles. Whereas the yeast two-hybrid system used in this study is useful in studying intermolecular interactions, the intramolecular interactions may not by fully captured in

a fusion-protein context. However, though the TTR molecules were expressed as fusion proteins inside the yeast cells, the results obtained in SPR analyses, using recombinant soluble and fibrillar TTR support the interactions observed *in vivo*. Using a polyclonal antibody and an amyloid specific monoclonal antibody we succeeded in differentiating soluble from aggregated TTR forms through the comparison of their recognition patterns when analysing yeast colonies.

The identification of cryptic regions present only in fibrils or amyloidogenic structures seems to have been indicated by the results obtained with the five selected aptamers in this study. Our results support the idea of a sequential series of conformational modifications, starting with a native tetrameric soluble form of TTR that gradually suffers amyloidogenic changes, evolving from a partially modified structure to highly modified tetrameric arrangements and culminating in its dissociation into disarranged monomers, which finally associate into fibrils. The identification of new cryptic epitopes will reveal transfer motions occurring in the intermediate amyloidogenic forms of TTR as seems to be indicated by the area recognised by aptamer 67, present in soluble and fibrillar Leu55Pro TTR but undetected in Tyr78Phe, suggesting a movement in this region that in some TTR forms may be "invisible". Other epitopes seem to be specific of the growing fibril, as the one recognised by aptamer C45.

We explored further the specificity of aptamer C45 to the fibrillar form of Leu55Pro TTR by testing its ability to inhibit *in vitro* amyloid formation. The apparent secondary structure of amyloid did not seem to be altered, rather, this aptamer might cause changes in aggregation kinetics and probably disaggregates some of the growing fibrils. Evidence for these effects includes changes in fibril morphology/growth as seen in electron microscopy (Cardoso *et al*, manuscript in press) and a reduction in ThT fluorescence, of samples of Leu55Pro TTR incubated with peptide C45, after 5 days. We believe that the novel surfaces generated as a result of the adoption of β-pleated sheet structures during polymerisation serve as binding sites for C45, since this peptide does not have affinity for any other forms of TTR.

The cytotoxic effects of different stages of TTR fibrillogenesis were investigated both *in vivo* and *in vitro* by Sousa *et al.* (2001), assessing nerves from FAP patients in different stages of disease progression and studying caspase-3 activation on a Schwannoma cell line. Immunohistochemistry of TTR deposition in nerves showed that small toxic nonfibrillar aggregates occur locally before amyloid formation, and the activation of caspase-3 in cultured cells further demonstrated the cytotoxicity of prefibrillar TTR structures. The results obtained with aptamer C45 results might contribute to the idea that complete disruption of amyloid fibril is not necessary for

elimination of toxicity. However, future experiments including the examination of the ability of this aptamer in modulating the cellular toxicity of Leu55Pro TTR fibrils in cultured cells, would be necessary. Also, further structural studies are needed to determine and elucidate the mechanism of inhibition of fibril growth (attempts of co-crystallising C45 with soluble Leu55Pro TTR are underway). More effective inhibitors, for example, shorter peptide sequences, based on C45, could function as amyloid disrupting agents.

In order to increase success in the therapeutic challenge of FAP, identification of putative precursor folds, intermediate amyloidogenic structures and key interacting surfaces within the fibrils, is necessary. There is often a positive correlation between severity of the disease and the extent of fibril formation and the slow and thermodynamically unfavourable interactions between individual monomers may be a rate-limiting step in aggregation. Since candidate substances may act at different stages of the polymerisation process, a thorough understanding of what occurs along the assembly pathway from TTR monomers to mature polymorphic amyloid fibrils is required. Detailed structural analysis of the development of tetrameric intermediate TTR forms into monomeric non-native species and further association into protofibrils is now a major focus of interest, aiming at developing newer inhibitory compounds of the polymerisation pathway.

PART III

APPENDIX

APPENDIX

ABBREVIATIONS

α-2M, α2-macroglobulin

β-gal, β-galactosidase

3-AT, 3-amino-1,2,4-triazole

3D, Three-dimensional

Aβ, amyloid β-peptide or β-peptide amyloidosis

AA, amyloid A associated amyloidosis

AANF, ANF amyloidosis

ABTS, 3-ethylbenzethiazoline-6-sulfonic acid

Acalc, Calc amyloidosis

AD, Alzheimer's disease

AD-FAD, familial AD

Agel, gelsolin related amyloidosis

AH, heavy immunoglobulin chains associated amyloidosis

AIAPP, amyloid polypeptide derived amyloidosis

Ains, insulin related amyloidosis

AL, light chain associated amyloidosis

Alac, lactoferrin associated amyloidosis

Amp^r, ampicilin resistant

ANF, atrial natriuretic factor

Apo AI, apolipoprotein AI

Apo E, apolipoprotein E

Apo E4, apoE ε4 allele

APP, amyloid precursor protein

Apro, prolactin related amyloidosis

APrP, prion protein diseases

ATP, adenosine triphosphate

AβPP, amyloid β protein precursor

BIA, biomolecular interaction analysis

bp, base pairs

BSA, bovine serum albumin

Calc, calcitonin

cDNA, complementary DNA

CNS, central nervous system

CRBP, cellular retinol binding protein

CSF, cerebrospinal fluid

C-terminal, carboxy-terminal

DAB, 3,3'-diaminobenzidine

DMSO, dimethylsulphoxide

DNA, desoxyribonucleic acid

Dnase, desoxyribonuclease

dsDNA, double-stranded DNA

DTT, dithiothreitol

E.coli, *Escherichia coli*

ECL, enhanced chemiluminescent (detecting reagent)

EDC, (N-ethyl - N'- (dimethyl-aminopropyl)-carbodiimide

EDTA, ethylmethylsulphonate

ELISA, enzyme linked immunoreactive assay

EM, electron microscopy

ER, endoplasmic reticulum

FAC, familial amyloidotic cardiomyopathy

FAP, familial amyloidotic polyneuropathy

FCS, fetal calf serum

FMF, familial Mediterranean fever

GAG, glycosaminoglycan

Gal4 AD, Gal4 activation domain

Gal4 BD, Gal4 DNA binding domain

HBS, Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM, P20 surfactant 0.05%

HCHWA, hereditary cerebral haemmorrhage with amyloidosis

HDL, high-density lipoprotein

HepG2, human hepatoma cells

HLA, human leukocyte antigen

HNF-1, HNF-3, HNF-4, hepatocyte nuclear factors 1, 3 and 4, respectively

HPLC, high performance liquid chromatography

HRP, horseradish peroxidase

HSPG, heparan sulphate proteoglycans

IAPP, islet amyloid polypeptide

IDOX, 4'-deoxy-4'-iododoxorubicin

IEF, isoelectric focusing

IGP dehydratase, imidazole glycerol phosphate dehydratase

IL-6, interleukine-6

IPTG, isopropyl-β-D-thiogalactopyranoside

Kb, kilobase(s)

kDa, kilodalton

LDL, low density lipoproteins

LDLr, low-density lipoprotein receptor family

LiAc, lithium acetate

Mab15, monoclonal antibody recognising residues 39-44 of transthyretin

Mabs, monoclonal antibodies

mRNA, messenger RNA

MT, methallothionein mouse promoter

NHS, N-hydroxysuccinimide

NMR, nuclear magnetic resonance

N-terminal, amino-terminal

O/N, overnight

ORF, open reading frame

Pab, polyclonal antibody

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate-buffered saline

PBST, phosphate-buffered saline with 0,05% Tween-20

PCR, polymerase chain reaction

PMSF, phenylmethylsulphonyl fluoride

pSK, pBluescript vector

RAP, receptor-associated protein

RBP, retinol binding protein

RNA, ribonucleic acid

RNAse, ribonuclease

rpm, rotations per minute

RT, room temepertaure

RU, resonance units

S-S, disulphide bridge

S. *cerevisiae*, *Saccharomyces cerevisiae*

SAA, serum amyloid A

SAP, serum amyloid P component

SD, yeast minimal medium

SDS, sodium dodecyl sulphate

SD-X, minimal medium lacking nutrient X

SPR, surface plasmon resonance

SSA, systemic senile amyloidosis

T_3, triiodothyroxine

T_4, thyroxine

TBG, thyroxine binding globulin

TCA, trichloroacetic acid

ThT, thioflavine T

Tris, tris(hydroxymethyl-aminomethane)

TTR Val30Cys/Leu55Cys, valine to cysteine and leucine to cysteine exhanges

TTR Xxx00Yyy, aa Xxx to Yyy exhange at position 00 of TTR

TTR, transthyretin

ttr, transthyretin gene

WT, wild-type

X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Y190, *S. cerevisiae* strain used in the two hybrid system

YPD, yeast complete media

β_2M, β_2-microglobulin

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