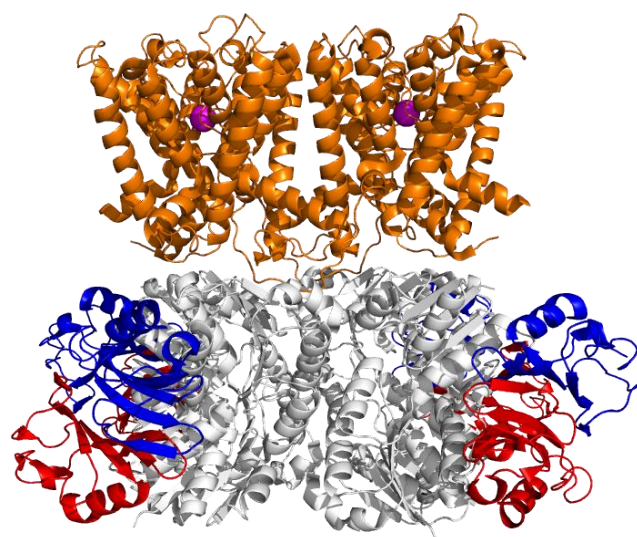




Biochemical studies of a membrane protein: The KtrAB ion transporter

Celso Moura Teixeira Duarte

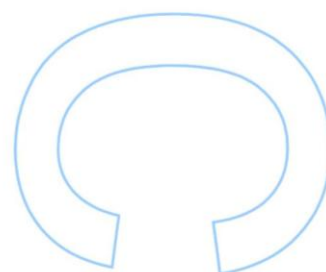
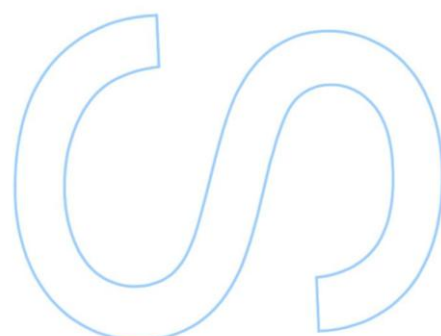
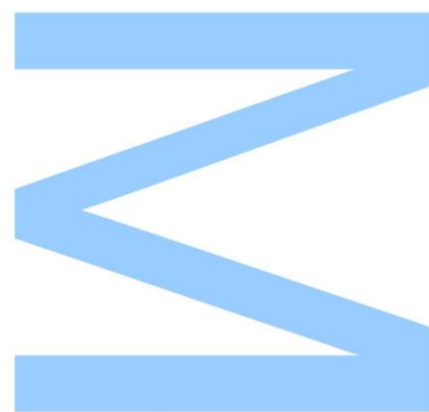
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João Morais Cabral, Investigador Principal, IBMC



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Abstract

KtrAB is a potassium transporter, present widely among prokaryotes, which is essential for the adaptation to osmotic stress and resistance to high salinity, among other properties. This transporter is composed of two polypeptides: a membrane protein, KtrB, which facilitates ion passage, and a cytosolic protein, KtrA, that has regulatory functions and is an RCK domain (regulates conductance of potassium).

Unlike the very well known potassium channels, the mechanisms by which this transporter regulates and enables ion passage are not well established and so, it is critical to obtain high resolution structures that can help understand these aspects.

The aim of this project was to obtain a KtrA mutant that makes it more stable and that can help improve the diffraction quality of protein crystals resulting in higher resolution structures. In order to do so, several residues in the C-terminal domain of KtrA were mutated, both in the core and at the surface of the domain, introducing amino acids that could potentially stabilize the protein. The mutants that were tolerated were purified and tested in a thermal shift assay, which evaluated the melting temperature of each mutant. All mutants tested resulted in a stabilization of the protein structure and were combined to further improve stability. Some of the properties of final combination mutant, named KtrA CysLHDN, were evaluated. In particular, it was verified that the mutant assembles with the KtrB membrane protein and that it forms protein crystals that diffract better than the wild-type protein. As a result, a crystal structure of KtrA CysLHDN bound to ATP was obtained at 2.8 Å.

The recent discovery of a new ligand that binds to the C-terminal domain of KtrA, c-di-AMP, led to a new aim in the project. This ligand was tested with the native KtrA and some mutants in thermal shift and ITC assays. It was possible to show that the ligand not only binds to KtrA, but also stabilizes the protein. This stabilization occurs when KtrA is bound to ADP but not when KtrA is bound to ATP. The ITC allowed the determination of a dissociation constant of 12 µM and a stoichiometry of 1 c-di-AMP molecule per KtrA dimer. ITC measurements confirmed the thermal shift results and c-di-AMP appears to not bind to the KtrA-ATP complex.

Key-words: K⁺ transporter; KtrAB; KtrA; c-di-AMP; crystal structures; RCK domain; protein stability.

Resumo

O KtrAB é um transportador de potássio, amplamente presente em procariotas, que é essencial para a adaptação ao stress osmótico e para a resistência a salinidade elevada, entre outras propriedades. Este transportador é composto por dois polipeptídeos: uma proteína de membrana, KtrB, que facilita a passagem de iões, e uma proteína citosólica, KtrA, que tem funções reguladoras e é um domínio RCK (regula a condutância de potássio).

Contrariamente aos conhecidos canais de potássio, os mecanismos através dos quais este transportador regula e permite a passagem de iões ainda não estão bem definidos e portanto, é crucial obter estruturas com resolução elevada que permitam entender estes aspectos.

O objectivo deste projecto foi obter um mutante de KtrA que a torne mais estável e ajude a melhorar a qualidade de difracção dos cristais proteicos de modo a obter estruturas com resolução mais elevada. Neste sentido, foram mutados resíduos no domínio C-terminal de KtrA, tanto no núcleo como à superfície, introduzindo aminoácidos que poderiam eventualmente estabilizar a proteína. Os mutantes tolerados foram purificados e testados num ensaio de variação térmica que avaliou a temperatura de desnaturação de cada mutante. Todos os mutantes testados contribuíram para uma estabilização da estrutura proteica e foram combinados de forma a aumentar ainda mais a estabilidade. Algumas propriedades do mutante resultante da combinação final, denominado KtrA CysLHDN, foram avaliadas. Particularmente verificou-se que o mutante se liga à proteína de membrana KtrB e forma cristais proteicos que difractam melhor do que a proteína nativa. Consequentemente, uma estrutura cristalográfica de KtrA CysLHDN ligada a ATP foi obtida a 2.8 Å.

A descoberta recente de um novo ligando que se liga ao domínio C-terminal de KtrA, c-di-AMP, levou a um novo objectivo no projecto. Este ligando foi testado com KtrA nativa e alguns mutantes em ensaios de variação térmica e ITC. Foi possível demonstrar que este ligando não só se liga a KtrA, como também estabiliza a proteína. Esta estabilização ocorre quando KtrA está ligada a ADP mas não quando KtrA está ligada a ATP. O ITC permitiu a determinação de uma constante de dissociação de 12 µM e uma estequiometria de 1 molécula de c-di-AMP por dímero de KtrA. O ITC confirmou os resultados da variação térmica e c-di-AMP parece não se ligar ao complexo KtrA-ATP.

Palavras-chave: Transportador de K⁺; KtrAB; KtrA; c-di-AMP; estruturas cristalográficas; domínio RCK; estabilidade proteica.

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Abbreviations

ADA-	N-(2-Acetamide) iminodiacetic acid
ADP-	Adenosine diphosphate
AMS-	Ammonium sulphate
ATP-	Adenosine triphosphate
ATPase-	Adenosine triphosphatase
Ca ²⁺ -	Calcium ion
cAMP-	Cyclic adenosine monophosphate
CAPSO-	3-(Cyclohexylamine)-2-hydroxy-1-propanesulfonic acid
c-di-AMP-	Cyclic diadenosine monophosphate
c-di-GMP-	Cyclic diguanosine monophosphate
c-di-IMP-	Cyclic diinosine monophosphate
Cymal-6-	6-cyclohexyl-1-hexyl-β-D-maltoside
Da-	Dalton
DAC-	Diadenylyl cyclase
DDM-	n-dodecyl-β-D-maltoside
DMSO-	Dimethylsulfoxide
DNA-	Desoxyribonucleic acid
DNase-	Deoxyribonuclease
DOPC-	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphatidylcholine
ds-	Double strand
DTT-	1,4- Dithio-DL-threitol
GTP-	Guanosine triphosphate
H ⁺ -	Hydrogen ion
HEPES-	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HCl-	Hydrochloric acid
HILIDE-	High concentrations of lipid and detergent
IFN-β-	Interferon beta
IPTG-	Isopropyl-1-thio-β-D-galactopyranoside
ITC-	Isothermal titration calorimetry
K ⁺ -	Potassium ion
KCl-	Potassium chloride
K _d -	Dissociation constant
KTN-	K ⁺ transport, nucleotide-binding
LB-	Lysogeny broth

MES- 2-(N-morpholine) ethanesulfonic acid
 MgSO₄- Magnesium sulphate
 mM- Milimolar
 MPD- 2-Methyl-2,4-pentanediol
 MPM- Transmembrane helix, pore, transmembrane helix
 Na⁺- Sodium ion
 NaCl- Sodium chloride
 NAD⁺- Nicotinamide adenine dinucleotide, oxidized
 NADH- Nicotinamide adenine dinucleotide, reduced
 nM- Nanomolar
 NTP- Nucleoside triphosphate
 OD- Optical density
 pApA- 5'-Phosphoadenylyl-(3',5') adenosine
 PCR- Polymerase chain reaction
 PDE- Phosphodiesterase
 PEG- Polyethylene glycol
 PMSF- Phenylmethanesulfonyl fluoride
 RCK- Regulating the conductance of K⁺
 RFU- Relative Fluorescence Units
 rpm- Revolutions per minute
 rRNA- Ribosomal ribonucleic acid
 RT-PCR- Real-time polymerase chain reaction
 SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis
 SER- Surface entropy reduction
 TCEP- Tris-(2-Carboxyethyl) phosphine
 T_m- Melting temperature
 T_M- Transmembrane segment
 Tris- Tris(hydroxymethyl) aminomethane
 µL- Microliter
 µM- Micromolar
 Å- Ångström

Introduction

Physiological role of the potassium (K^+) ion

Potassium (K^+) is the major inorganic cation present in the cell cytoplasm of both prokaryotic and eukaryotic cells, being an essential element to all living organisms [1]. In bacteria, K^+ concentration in the cytosol ranges between 300 and 500 mM [2], and the accumulation of this particular ion is attributed to its compatibility with water and proteins [1].

K^+ contributes to the electrical neutralization of anionic groups in macromolecules, while also playing a determinant role in several biological processes, such as osmoregulation [2], maintaining intracellular pH [3], and establishing the transmembrane electrical potential [4].

In regard to osmoregulation, K^+ levels inside the cell are determined by the osmolarity of the external medium, rising with high osmolarity, and decreasing when the osmolarity lowers [2, 5], thereby maintaining the turgor pressure stable. Also, the accumulation of potassium in situations of osmotic stress leads to the expression and stimulation of specific proteins like ProU [6] and BetP [7], transport systems for betaine, a compound that acts as an osmoprotectant.

On the other hand, solute transport and cell motility in prokaryotes are driven by a transmembrane electrochemical potential of protons known as the protonmotive force. This force has two components: the membrane potential and the pH gradient [3]. The potassium ion flux regulates these two components, since it has been shown that K^+ uptake leads to a depolarization of the membrane (reduction of a membrane potential to a less negative value due to an increase in positive charges inside the cell) [4], which in turn translates in an increase of the internal pH of the bacteria, achieved by partial exchange of K^+ by H^+ [8]. Potassium ions also seem to play a role in protein synthesis by binding close to the active centre of the 23S-rRNA from the ribosome, although it is not yet clear if this binding is essential for the catalytic function of the ribosome [9].

In order to regulate these vital potassium fluxes and adapt rapidly to changes in the environment, prokaryotes use several channels, transporters and pumps since the cell membrane is virtually impermeable to K^+ .

K⁺ uptake systems

Potassium uptake in animal cells is regulated mainly by the Na⁺/K⁺-ATPase [10]. However, in bacteria, fungi and plants it is mediated largely by two specific families of transporters, namely Trk/Ktr/HKT and HAK/Kup/KT, that don't exist in animal cells [1]. The Trk/Ktr/HKT family is present almost ubiquitously in plants, fungi and bacteria and is comprised by a large variety of transporters including H⁺-K⁺ symporters, Na⁺-K⁺ symporters and Na⁺ transporters. On the other hand, the HAK/Kup/KT family is present in organisms adapted to low potassium conditions [11] and is composed mainly of K⁺ transporters [1], however this family won't be discussed further here.

The Trk/Ktr/HKT superfamily is divided into different groups: the Transport of K⁺ in fungi and bacteria (Trk), the K⁺ transporter in bacteria (Ktr) and the High-affinity K⁺ transporter in plants (HKT).

Importantly, this superfamily is evolutionary related to small two transmembrane potassium channels such as the KcsA and MthK potassium channels [12].

Trk and Ktr systems in bacteria

Trk and Ktr are multimeric complexes formed by an ion-conducting transmembrane subunit, which can be TrkH, G or I (in Trk systems) and KtrB or D (in Ktr systems), and a regulatory subunit: TrkA or E and KtrA, C or E respectively [1].

The Trk subfamily has been extensively studied in *Escherichia coli*, since this organism contains not only the ion conducting TrkH subunit, but also the TrkG subunit in the K12 strain [13]. These two subunits have been shown to establish multimeric functional transporters in association with the regulatory cytoplasmatic subunit TrkA [14]. Several studies have also demonstrated that TrkA is essential for high-level Trk activity since mutating the *trkA* gene abolishes K⁺ transport [15].

TrkA subunits contain two similar domains that are RCK domains. RCK (regulating the conductance of K⁺) domains modulate the function of some eukaryotic and prokaryotic potassium channels and transporters that are essential to regulation of K⁺ uptake in the cell. These domains can be classified into two types based on their cognate ligands: one type consists in the bacterial RCK domains that possess a conserved GXGXXG motif (Rossman fold) that binds to nucleotides, and is sometimes known as a KTN (K⁺ transport, nucleotide-binding) domain [16]. The other is composed of the bacterial and mammalian RCK domains that lack this motif and bind ions such as Ca²⁺ [17, 18].

Each of the TrkH domains has a nucleotide-binding site that has been shown to bind NAD^+/NADH . It has been proposed that this allows the transporter to be responsive to the redox state of the cell [19]. One other regulating subunit identified in *E.coli* is TrkE that, unlike TrkA, confers ATP-responsiveness to the transporter when in complex with TrkG or H creating an ATP-dependent stimulation of K^+ transport [20].

Overall the Trk system in *E.coli* is a constitutive, low-affinity K^+ transport system, regulated by the protonmotive force and ATP. It displays an estimated affinity of 1mM (K_M) for K^+ and has been proposed to perform symport of 1 H^+ with 1 K^+ , with ATP acting as a regulator [21]. This symport with H^+ is particularly interesting when it comes to halophilic bacteria such as *Halomonas elongata* that are thought to rely on Trk systems, mainly TrkI and TrkA, for potassium transport in order to avoid Na^+ intake in saline environments and therefore diminish salt stress [22].

The Ktr subfamily has been well characterized in the cyanobacterium *Synechocystis* and in *Bacillus subtilis*. In *Synechocystis*, there is only one KtrB ion-conducting subunit, previously known as NtpJ [23]. This subunit forms a complex with two regulatory subunits, KtrA and KtrE, that is essential for potassium uptake and adaptation to hyperosmotic shock in this bacteria [24]. KtrA is similar to TrkA from the Trk system but has only one RCK domain. On the other hand KtrE shows similarities to glycosyl transferases.

In *B.subtilis*, there are two Ktr transport systems, KtrAB and KtrCD [25]. These systems are required for adaptation to hyperosmolarity. They differ in affinity for potassium, KtrAB has a K_d for K^+ of 1 mM as opposed to the 10 mM in KtrCD. KtrB has been shown to form homodimers [26] that assemble with an octameric ring comprised of four KtrA dimers to form a functional complex. KtrA binds ATP, displaying eight binding sites in the octameric ring [27]. Binding of these ligands results in conformational changes of the complex KtrAB that regulate potassium uptake.

Structural organization of Trk and Ktr systems

Typically, the transmembrane subunit of Ktr in bacteria is composed of four M₁PM₂ (transmembrane helix, pore, transmembrane helix) repeats, whereas the Trk transmembrane subunit has two additional transmembrane segments (TM₁ and TM₂) before the first M₁PM₂ repeat as shown in Figure 1. In each repeat, M₁ and M₂ are helices connected by the P loop, a protein region, which is composed of a pore-helix spanning part of the membrane, followed by an extended loop [28]. These MPM domains are thought to have evolved from a common potassium channel ancestor with a MPM protomer through a process of gene duplication and fusion [1]. Since K⁺ channels have the same subunit fold and are tetramers, it has been proposed that the structures of Trk/Ktr/HKT membrane proteins resemble those of K⁺ channels [29].

Several functional studies supported this proposal, namely by showing that a mutation in a conserved glycine residue in the P loop changes the ion selectivity of the Trk/Ktr/HKT proteins in bacteria [30]. However, unlike K⁺ channels, where the P segment contains a very conserved signature sequence (TVGYG) that forms the selectivity filter responsible for the coordination with the potassium ion, in Trk/Ktr/HKT proteins the equivalent sequence is not so strictly conserved.

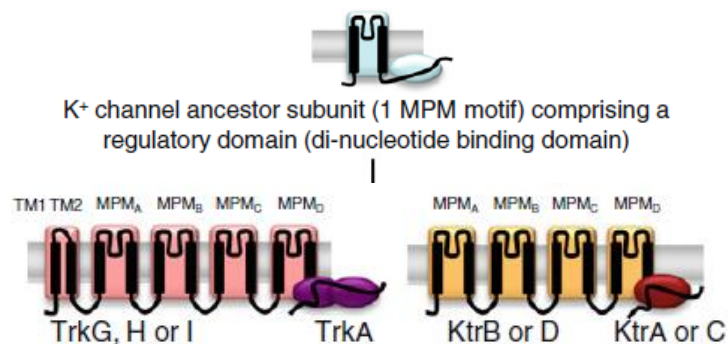


Fig.1- Scheme depicting the structural architectures of Trk and Ktr proteins (bottom) and of their putative potassium channel ancestor (top). Adapted from Corratge-Faillie 2010.

TrkAH structure

Recently, the structures of TrkH alone and of TrkH in complex with TrkA have been solved [16, 31]. TrkH was shown to be a dimer, in which each subunit is composed of five regions (D0 to D4) and contains one K^+ ion. D0 corresponds to the TM_1 - TM_2 segment previously discussed and D1 to D4 are MPM repeats. The dimer interface between the two subunits is formed by helices from both D3 and D4.

D1 to D4 assemble in a pseudo-fourfold symmetry axis that creates an ion permeation pathway with an hourglass shape. This ion permeation pathway is contained in each TrkH subunit and has two important features: a selectivity filter and an intramembrane loop. The selectivity filter is part of the pore loop; it is encompassed by the four pore helices, in a similar arrangement as seen for K^+ channels, and includes a conserved glycine residue as expected (see Figure 2). However, the selectivity filter of TrkH has a wider opening on the extracellular side, compared to those of K^+ channels, and a shorter constricted region for potassium coordination [16].

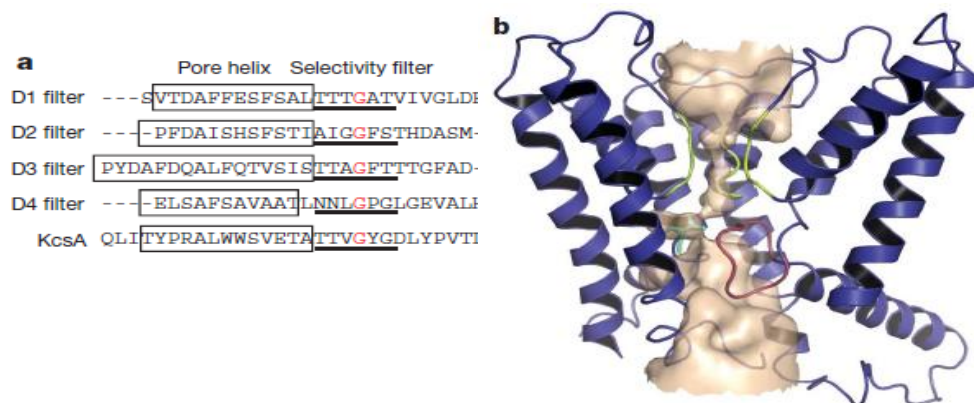


Fig. 2 - TrkH pore. a) sequence alignment of the pore helix (box) and selectivity filter (underlined) of each TrkH repeat and of the K^+ channel KcsA, showing the conserved glycine in red. b) Surface representation of the ion permeation pathway hourglass shape in one TrkH subunit: the selectivity filter is colored yellow and the intramembrane loop colored magenta. Adapted from Cao 2011.

The intramembrane loop acts as a barrier for K^+ permeation and is positioned close to the selectivity filter. This barrier is formed by two elements: Arginine 468 from the second transmembrane segment of D4, conserved in all bacterial Trk/Ktr/HKT proteins, and an intramembrane circular loop that is present in TrkH, TrkG and KtrB and is rich in glycine and other small residues such as serine and alanine, which are well conserved across different sequences (see Figure 2) [16]. These two elements form a narrow constriction that needs to widen in order to allow K^+ passage, possibly acting as a gate, a feature that hasn't been observed in the structure of any potassium channels to date.

TrkH assembles in a complex with TrkA, so that a TrkH dimer interacts with a ring formed by four TrkA protomers. Each TrkH monomer interacts with TrkA through a cytosolic loop in D3 that is directly connected to the intramembrane loop [31]. This interaction is another difference when compared to K^+ channels that are regulated by RCK domains, since the RCK domain is encoded in the same polypeptide so that the membrane protein and the regulatory ring in the cytoplasm are covalently bound.

The TrkA protomer contains two tandem RCK domains (RCK1 and RCK2), and the ring has a total of eight RCK domains with two GXGXXG nucleotide-binding site motifs (Rossman fold) in each RCK domain. The tetramer of TrkA protomers shows two different subunit interfaces: one between the N-terminal lobes of RCK1 (N1-N1) and another between the N-terminal lobes of RCK2 (N2-N2) as shown in Figure 3. This organization is different from the one observed for K^+ channels such as BK and MthK, where the gating ring contains four identical N1-N2 interfaces.

The structure obtained by the same authors for TrkA alone in solution shows a substantial conformational change compared to the complex structure. In this second structure, the N2-N2 interfaces rotate and slide past each other, converting TrkA from a planar to a twisted conformation. This conformational change may be regulated by nucleotide-binding and act as a mechanism for activating the TrkH-TrkA complex. Cao and colleagues propose a model in which binding of ADP to TrkA results in a closed state corresponding to the planar structure obtained for the TrkH-TrkA complex, where the ion permeation pathway is blocked by the intramembrane loop. Upon exchange of ADP for ATP, the gating ring would adopt a twisted conformation where the tetramer could lead to movement of the loop out of the pore, freeing the ion permeation pathway and opening the channel [31]. This model is supported by studies that show that ion flux through the complex is upregulated by ATP and downregulated by ADP [31], but higher resolution structures of the complex are still needed in order to confirm the hypothesis.

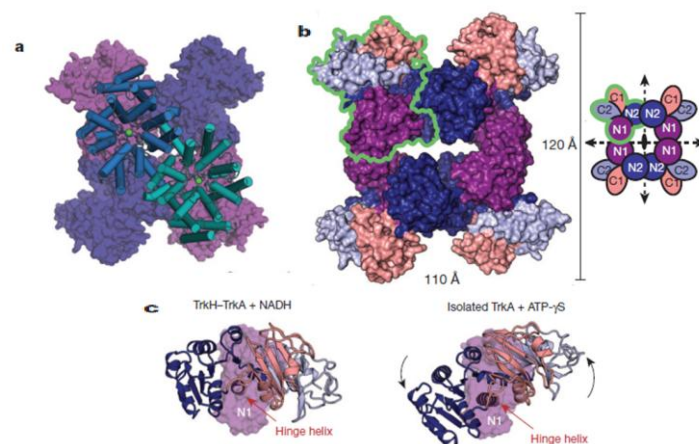


Fig.3 - Structure of the TrkH-TrkA complex and TrkA alone. a) Structure of the complex formed by the TrkH dimer (light blue and teal) and the TrkA tetramer (dark blue and purple). b) Structure of the TrkA ring with the N1-N2 protomer highlighted. c) Structure representation of TrkA in complex with TrkH and alone, showing the 2 different conformations: planar on the left and twisted on the right. Adapted from Cao 2013.

KtrAB Structure

KtrAB is a novel high-affinity Na⁺-dependent K⁺ uptake system found in *Vibrio alginolyticus*. This constitutively expressed system was identified by Nakamura and colleagues as the product of two adjacent genes that encode for: KtrB, previously known as NtpJ, of predicted molecular mass of 45,623 Da, and KtrA, of predicted molecular mass of 23,804 Da [23].

The system is formed by the assembly of a complex between a homodimer of the membrane protein KtrB and an octamer of the cytosolic protein KtrA (see Figure 4) [27]. In *Staphylococcus aureus*, KtrAB has already been shown to play a determinant role in the regulation of several biological processes such as pH regulation, growth in low K⁺ conditions, resistance to osmotic stress, regulation of the membrane potential and antimicrobial resistance [32].

The crystal structure of KtrAB with KtrA bound to ATP was recently solved at 3.5 Å [33], showing the expected homodimeric KtrB bound to the cytoplasmic octameric ring of KtrA. As previously stated, KtrB is an integral membrane protein composed by four repeats of the M₁PM₂ structural motif (D1 to D4), lacking the N-terminal TM₁-TM₂ helices present in Trk. The P loop region includes the pore (P) helix and the selectivity filter sequence. Just like in TrkH, below the selectivity filter there is an intramembrane loop in repeat D3 composed mainly of glycine, alanine and serine residues [33]. Together with a highly conserved arginine residue (R417), this loop forms a structure that blocks access between the selectivity filter and the cytoplasmic pore, most likely acting as a gate [34].

The major difference between KtrB and TrkH lies in the C-terminus. TrkH has a shorter C-terminus that does not establish any contacts whereas KtrB has a long C-terminus that runs from one subunit to the other, forming a lateral contact with KtrA and entering the pore of the neighbouring subunit, finishing just below the intramembrane loop [33]. Also, in KtrB a highly conserved C-terminal glycine (G445) is positioned close to the intramembrane loop so that its C-terminal carboxylate group interacts with the side chain of a lysine that is part of the intramembrane loop and is conserved in both KtrB (K315) and TrkH (K357). This C-terminal glycine does not exist in Trk proteins, but appears to be replaced by a glutamate (E470) in TrkH that also interacts with the lysine (K357). The glycine carboxylate by itself does not appear to have a substantial effect in the regulation of the transporter has shown by truncation studies [33]. However, removal of the last four residues of the C-terminus of KtrB results in a non-functional transporter, since KtrB loses its ability to form a complex with KtrA [33]; these residues also appear to be important for the stability of the KtrB dimer [26].

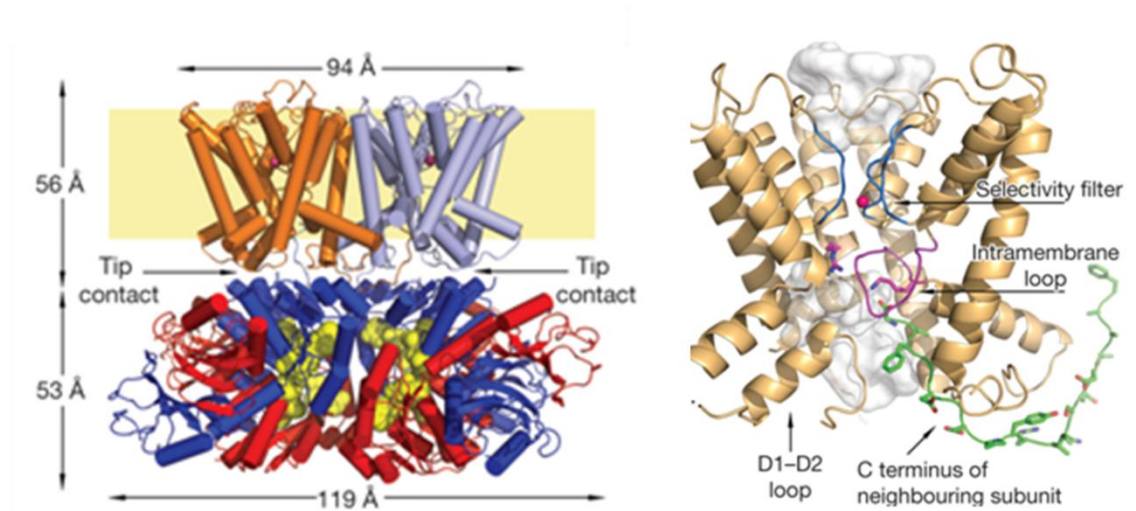


Fig.4 - KtrAB transporter. Cartoon of the KtrAB transporter with KtrB subunits shown in orange and light blue; KtrA subunits are in dark blue and red; ATP is shown as yellow spheres and K^+ as magenta spheres; cell membrane is shown as a pale yellow rectangle (left). Cartoon of one subunit of KtrB showing the pore and pointing out the selectivity filter and the intramembrane loop with K^+ as a magenta sphere (right). Adapted from Vieira-Pires 2013.

The KtrB dimer sits directly on the face of the KtrA ring, establishing two types of contacts with it through cytoplasmic loops: a lateral contact and a tip contact. The lateral contact is established by a section of the KtrB C-terminus which runs along the homodimer interacting with the neighbouring KtrB subunit and the KtrA ring. The tip contact occurs at the apexes of the cytoplasmic face of KtrB where cytoplasmic loops connecting D1 to D2 interact with two opposing KtrA subunits as illustrated in Figure 5.

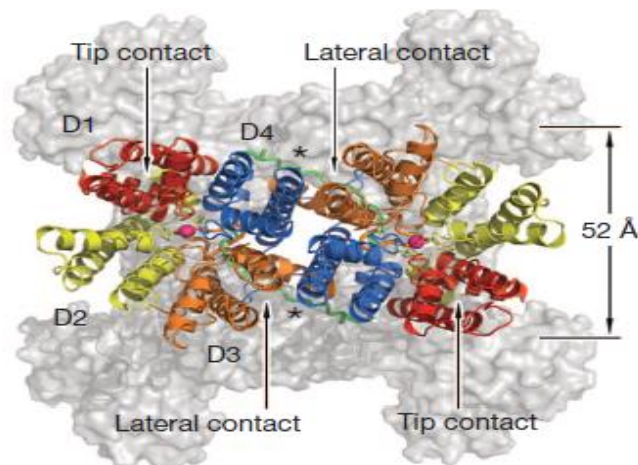


Fig.5 - Top view of KtrAB with cartoon representation KtrB domains: D1 (red), D2 (yellow), D3 (orange) and D4 (blue). KtrA octameric ring is shown as a grey surface and the lateral and tip contacts are pointed out.

KtrA Structure

A C-terminal truncated KtrA crystal structure was first solved bound to NADH by Roosild and colleagues in 2002 at 2.85 Å resolution [19]. This structure showed a homodimer assembly with NADH bound to the N-terminal and it was proposed that four RCK domains (a dimer of dimers) would bind to KtrB and regulate the transporter. However, later on a structure at 2.20 Å resolution was determined by Albright and colleagues [27], displaying the homodimers organized in an octameric ring with a diamond shape conformation, similar to the one observed for the MthK potassium channel. The NADH ligands are bound to the N-terminal facing the hole of the ring whereas the C-terminals of the dimers are facing outward. Moreover, it was shown that the KtrA truncate could also bind ATP and ADP, even more tightly than NADH or NAD⁺, raising the possibility that these nucleotides have a functional role in the regulation of KtrAB.

Crystal structures of the truncated KtrA obtained in the presence of the different ligands revealed that the octameric ring is capable of adopting three different conformations: a diamond, previously seen with NADH and also seen for ATP and ADP; a square when bound to ATP and NADH and a rectangle, observed with NADH, as illustrated in Figure 6. This finding is interesting in the measure that RCK domains regulate ion transporters by changing conformation upon ligand binding and, in light of these results, the same conformation can be achieved by different ligands. However, this might be explained by a different behaviour of the ring in the absence of KtrB or by the lack of the C-terminus.

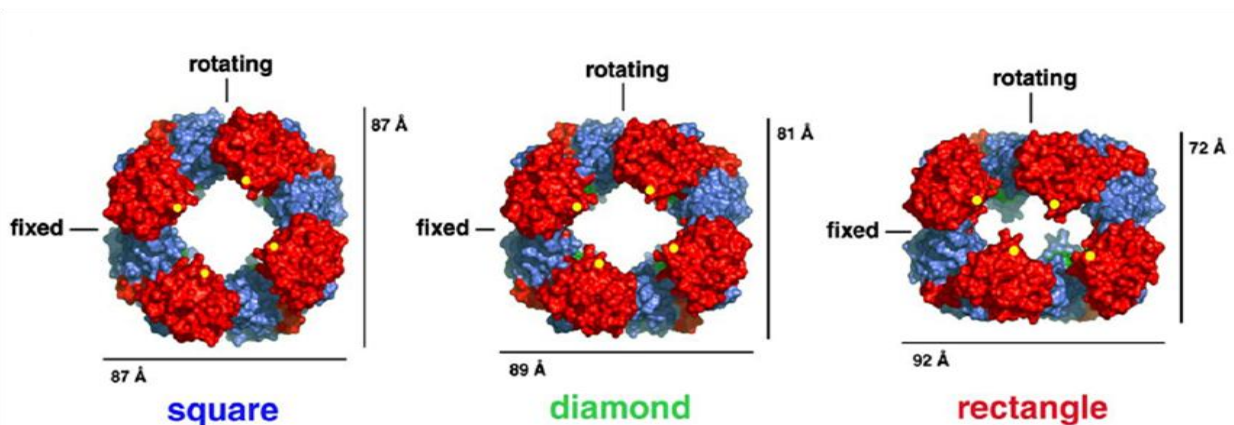


Fig.6 - Surface representations of the three conformations adopted by the truncated KtrA. Subunits are shown alternately in blue and red and ligands are shown in green. Adapted from Albright 2006.

Recently, crystal structures for the native full-length KtrA from *Bacillus subtilis* bound to both ATP and ADP were solved at 3.2 Å and 2.9 Å resolution, respectively. These new structures clearly show a different ring conformation for each ligand: a two-fold diamond shape conformation with ADP and a four-fold squared shape conformation with ATP [33] (see Figure 7). The conformation of the isolated KtrA ring with ATP is very similar to the conformation of the ATP bound KtrA ring in the KtrAB complex. This indicates that, unlike the truncated form where ligand binding is not coupled to ring conformation, in the full-length KtrA the binding of either ATP or ADP determines the conformation of the ring. These results reinforce the hypothesis of an essential role played by the C-terminus of KtrA in the conformation adopted by the KtrA octameric ring upon ligand binding.

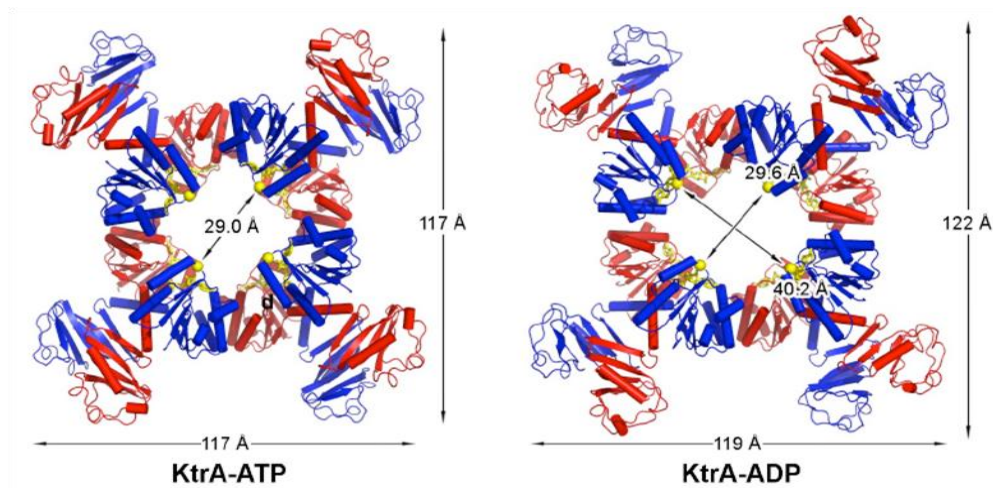


Fig.7 - Structures of the full length KtrA bound to ATP or ADP. Alternating subunits are shown in red and blue. Adapted from Vieira-Pires 2013.

KtrAB Activity

Uptake assays for KtrAB bound to ATP and ADP using rubidium labelled radioactively have shown that ATP is an activator of KtrAB [33].

Two possible activation mechanisms have been proposed, assuming the intramembrane loop of KtrB as a gate. The first model assumes that contraction or expansion of the KtrA ring occurs along the diagonal defined by the lateral contacts, since there is a direct connection between the KtrB C-terminus that interacts with KtrA and the intramembrane loop. The second model assumes that the contraction or expansion occurs along the tip contacts, where the D1-D2 loop would function as a gate controlled by KtrA and the intramembrane loop activated by another stimulus [33].

Both models have opposing evidences against them and so, to get a better understanding about the regulation of KtrAB, it is crucial to obtain structures with higher resolution. These structures would contribute to the definition of a mechanism that explains how the transporter is regulated.

Physiological role of cyclic nucleotides

Nucleotides are essential components of all living organisms, since they are needed to synthesise DNA and RNA and are used as an energy source [35, 36]. These molecules have also been shown to act as secondary messengers, by regulating signalling pathways that affect various aspects of eukaryotic and bacterial cells, as well as archaea. Among nucleotides, the first second messenger to be described was cyclic adenosine monophosphate (cAMP), which regulates several biological processes in eukaryotes, from metabolism to memory formation and immunity, functioning as a major gene expression regulator. In bacteria this molecule plays a role in carbon catabolite repression and virulence control, among other processes. It influences these processes by binding to certain proteins and changing their conformation [37].

One other nucleotide that has emerged as an important second messenger is cyclic diguanosine monophosphate (c-di-GMP), a cyclic dinucleotide obtained from the condensation of 2 molecules of GTP by the action of diguanylyl cyclases. Like cAMP, c-di-GMP also binds allosterically to receptor proteins, thus regulating several signalling pathways, namely the switch from a motile to a biofilm lifestyle in bacteria [38].

Cyclic di-AMP

Very recently, another cyclic dinucleotide is beginning to be characterized due to its relevance in bacteria. This nucleotide is cyclic diadenosine monophosphate (c-di-AMP) and, after c-di-GMP, is the second cyclic dinucleotide shown to be produced by bacteria. Although not a lot is known about this last molecule, all evidence so far indicates that these two cyclic dinucleotides regulate different processes [36].

c-di-AMP was first found in the cytosol of *Listeria monocytogenes* by Woodward and colleagues, and shown as a secreted molecule of multidrug efflux pumps (MDRs), which leads to IFN- β production [39]. Since then, it has also been found in other bacteria such as *Streptococcus pyogenes* [40], *Bacillus subtilis* [41], *Chlamydia trachomatis* [42] and *Staphylococcus aureus* [43]. This molecule is synthesised through the condensation of 2 ATP molecules by a diadenylyl cyclase (DAC) domain present in DisA proteins and degraded to 5'-Phosphoadenylyl-(3'→5')-adenosine (pApA) by GdpP, a phosphodiesterase (PDE), both widely distributed among bacteria [36].

As previously stated, the manner in which c-di-AMP regulates signalling pathways is still mostly unknown but since it is produced by DisA, a protein that scans the integrity of the DNA for double-stranded breaks, it has been suggested that it could play a role in signalling DNA damage [44]. In the last couple of years, c-di-AMP has been associated with several cellular functions: an increase in acid resistance [45, 46], as well as sporulation regulation related to DNA damage [41] in *Bacillus subtilis*, regulation of fatty acid synthesis in *Mycobacterium smegmatis* [47], the growth of *Staphylococcus aureus* in low-potassium conditions [35] and cell wall homeostasis in several species [43, 48].

Like other signalling nucleotides, c-di-AMP acts by binding to specific receptor proteins and allosterically altering their activity, thereby regulating cellular pathways. Lately, several receptors have been found: the transcription factor cyclic di-AMP receptor regulator (DarR) from de TetR (tetracycline resistant) family in *Mycobacterium smegmatis* [47], the histidine kinase KdpD, the PII-like signal transduction protein PstA in *Staphylococcus aureus* [35] and also the RCK domain proteins KtrA and cation antiporter A (CpaA) in *Staphylococcus aureus* [35].

In the case of KtrA, it was shown with a ligand assay that c-di-AMP binds specifically to the C-terminus of this RCK domain from *S.aureus* [35]. This finding is particularly interesting when coupled with the previous discoveries in the KtrA from *B. Subtilis*, which indicate that the C-terminus plays a role in the type of conformation adopted by the octameric ring; ligand binding to the C-terminal domain of KtrA would contribute to the mechanism of regulation of the transporter. Therefore it is relevant to understand if c-di-AMP also binds to the KtrA from *B.subtilis* and if so, try to determine the nature of the binding. Moreover it would also be interesting to try to discern what possibly role it could play in the regulation of the activity of the KtrAB transporter.

Goals and aims

Potassium transporters, particularly the Trk and Ktr systems, play an essential role in the regulation of vital cellular processes in prokaryotes by mediating K^+ uptake. Therefore understanding how these transporters are regulated is determinant to improve our knowledge of these organisms. In this sense, crystal structures provide a reliable source of information regarding the 3D structural organization of proteins and are, as such, important tools in the process of proposing mechanisms of function and regulation of protein systems.

In the particular case of the K^+ uptake system KtrAB, structures have already been determined for both the KtrAB complex and the RCK domain KtrA bound to its known ligands. These structures, along with biochemical assays, have been used to propose regulation mechanisms for this system. However, there still remain contradictory evidence and ideas against these proposals and higher resolution structures are needed in order to fully define these mechanisms.

One aim of this work was to develop KtrA C-terminal mutants that were able to improve the stability of the protein and the crystal lattice. Crystallization conditions for the best mutants would then be defined to possibly determine higher resolution structures for KtrA and KtrAB.

From previous studies, it has become clear that the C-terminal of KtrA plays a role in the conformation adopted by the octameric ring of the isolated protein and of the KtrAB complex. The recent discovery of a new ligand that binds specifically to the C-terminal of KtrA from *S.aureus*, cyclic di-AMP, presents a unique opportunity to determine if this nucleotide has a role in the regulation of the KtrA conformation and consequently of the whole system. Therefore, another aim of this work was to determine if c-di-AMP binds to the KtrA from *B.subtilis* and to characterize the binding properties.

Results and Discussion

Development of KtrA C-terminal mutants

The diffraction pattern of KtrAB is anisotropic [33]; it extends to 3.3 Å along the best direction and at close to 4 Å in the other two directions. Analysis of the crystal structure of KtrAB shows that the weak diffraction corresponds to the protein-protein contacts that are formed by the C-terminal domains of the KtrA rings from neighbouring channels (see Figure 8). A detailed analysis of the KtrA C-terminal domain reveals the presence of disordered regions and cavities in the core of the domain. These structural “defects” probably weaken the protein-protein contacts, impairing the formation of better diffracting crystals.

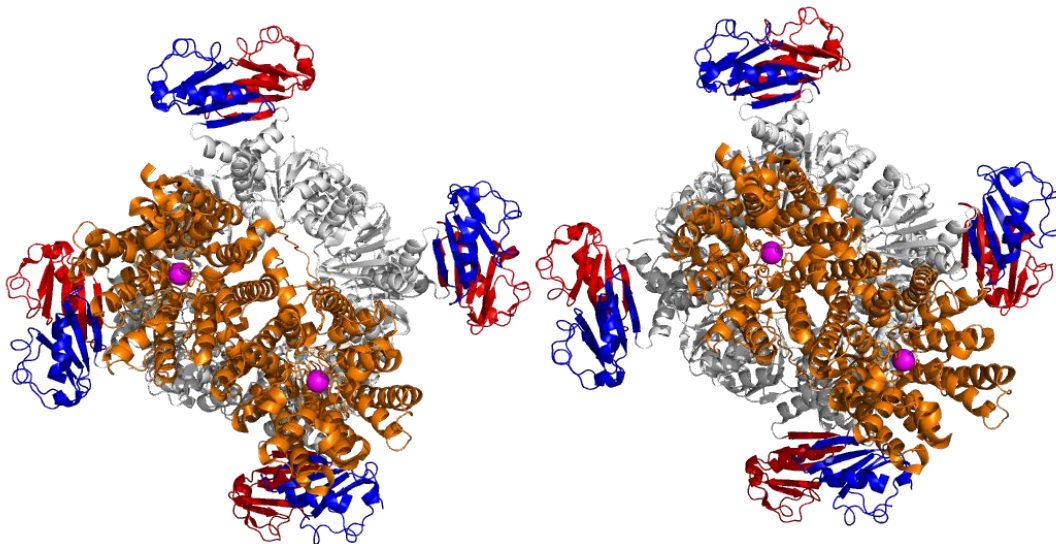


Fig.8 - Representation of the crystal lattice of KtrAB. Top view cartoon structure highlighting the contacts between two KtrAB transporters in the same crystal. The KtrB dimer is shown in orange with the potassium ion colored magenta. The KtrA octameric ring is shown at the bottom with the N-terminals colored white and the C-terminals colored alternately dark blue and red to show the dimers.

In order to improve the crystal lattice and therefore increase the quality of crystals obtained for this transporter, KtrA C-terminal mutants were developed by applying two different strategies: a cavity filling strategy and an exposed surface strategy.

Cavity Filling

The cavity filling strategy consists in mutating some key residues in order to fill cavities detected in the crystal structures previously obtained for KtrA. These cavities are present in the core of the C-terminal domain of KtrA and, by introducing a residue with similar chemical character but bulkier side-chain, it can fill the cavity, make the C-lobe more compact and therefore improve the stability of the protein. For this purpose, several different constructs were tried starting with a single (V172L) and a triple (D197Y; I198S; I199F) mutant as illustrated in Figure 9.

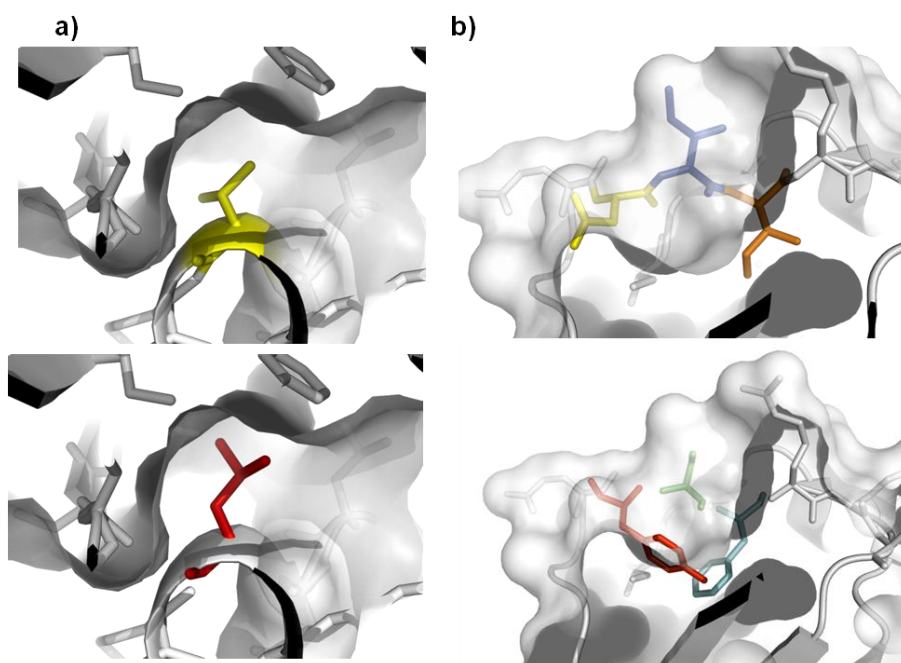


Fig.9 - KtrA C-terminal core constructs representation. Amino acids of interest are highlighted in sticks and cavities are shown in surface. **a)** Valine172 change to Leucine. Valine is shown in yellow (upper panel) and Leucine in red (lower panel). **b)** Aspartate197, Isoleucine198 and Isoleucine199 change to Tyrosine, Serine and Phenylalanine respectively. Aspartate is shown in yellow, Isoleucine198 in dark blue and Isoleucine199 in orange (upper panel). Tyrosine is shown in red, Serine in green and Phenylalanine in cyan (lower panel).

The mutants were then processed as detailed in the Methods section and the supernatant and pellet fractions for each one were analyzed by 15% SDS-PAGE, with the results being shown in Figure 10.

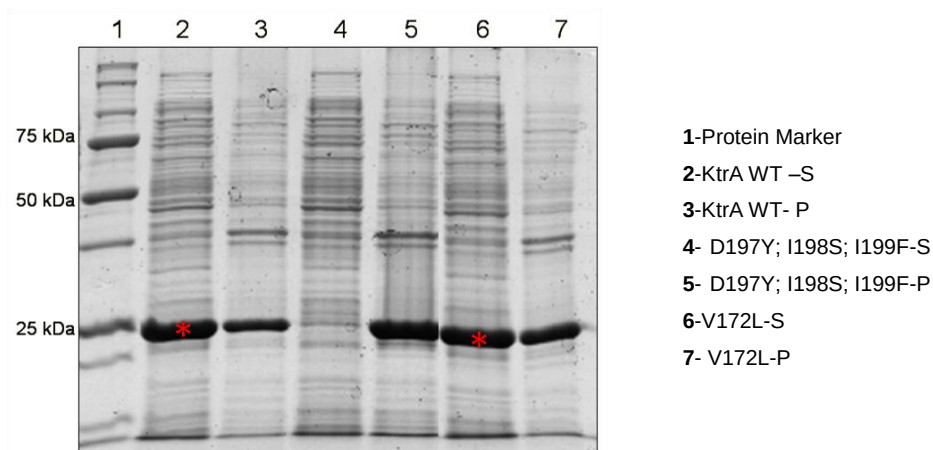


Fig.10 - SDS-PAGE gel of KtrA WT and respective C-terminal mutants. Each protein was lysed by sonication and centrifuged. Soluble fractions (Supernatant) and pellet fractions resuspended in lysis buffer were loaded on a 15% SDS gel. S refers to the supernatant fraction and P to the pellet fraction. The asterisk (*) marks the band corresponding to KtrA in the soluble fractions.

The band analysis of the gel shows that the V172L KtrA mutant is present in both the supernatant and pellet fractions, as a band with molecular weight of around 25 kDa that is similar to KtrA WT. However, the triple mutant D197Y;I198S;I199F is present only in the pellet fraction. This indicates that the mutations introduced were not tolerated by the protein, probably resulting in an abnormal conformation that caused it to become completely insoluble and remain in the pellet fraction.

Instead of changing three residues at once, an attempt was made to mutate only one of the intended residues, I199F. Nonetheless, even just a single-point mutation caused the protein to remain in the pellet fraction, as can be seen in Figure 11. This shows that KtrA is very sensitive to mutations in that area, and the D197Y;I198S;I199F and I199F mutants were not pursued further. Mutants that are able to remain partially in the supernatant (soluble) fraction like V172L were further characterized.

Within the strategy of increasing the stability of the protein, we were also concerned about the potential destabilizing effect of cysteine oxidation. There are 4 cysteines in KtrA, some of which are partially buried within the protein core. The mutant (C22V;C178V;C190S;C204S) was available in the lab and it was known to be soluble but it had not been fully characterized in terms of stability. We tried therefore to develop a combination mutant using V172L and the cysteineless KtrA. This mutant was named KtrA Cysless;V172L and, as shown in Figure 11, it doesn't render the protein insoluble, making it an interesting target for further studies.

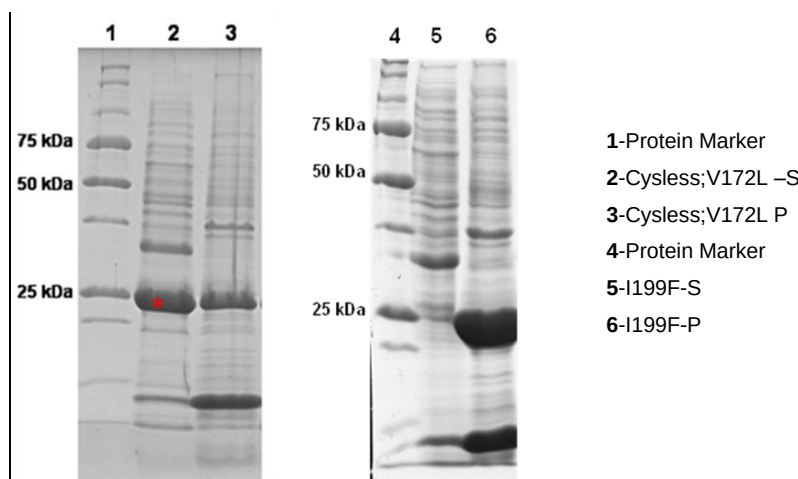


Fig.11 - SDS-PAGE gel of KtrA C-terminal Mutants. Each protein was lysed by sonication and centrifuged. Soluble fractions (Supernatant) and pellet fractions resuspended in lysis buffer were loaded on a 15% SDS gel. S refers to the supernatant fraction and P to the pellet fraction. The asterisk (*) marks the band corresponding to KtrA in the soluble fractions.

Exposed surface

This approach is based upon previous crystallography studies [49-51] that used the SER (surface entropy reduction) technique. This involves mutating residues with longer side chains at the surface of proteins and introducing residues with smaller side chains, which leads to the formation of crystals with better diffraction. The diffraction improvement occurs due to the less conformational liberty given to the side chains of residues involved in crystal contacts. By using smaller residues, the entropy necessary for crystal contacts is diminished, thereby promoting crystal formation and leading to higher resolution structures.

For this strategy, several residues with long side chains, including lysines, arginines and glutamates, exposed at the surface of the KtrA C-terminus (see Figure 12), were mutated to smaller residues. The mutations tried were: 1) R160H;K161H 2) K165H 3) E196N 4) R200H;E201D;Q202N 5) K212H, 6) K212H;R216H and 7) E201A;Q202G.

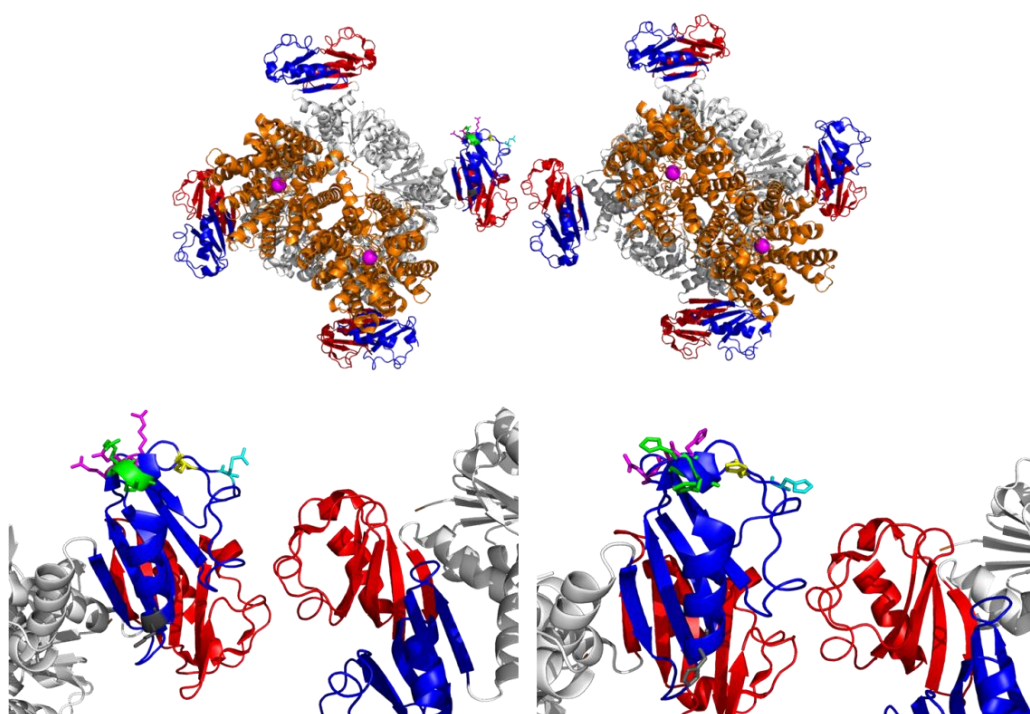


Fig.12 - Structural representation of KtrA C-terminal mutations for the exposed surface strategy. On the top panel is shown the crystal lattice representation of Figure 8 with the mutated residues highlighted in one of the C-terminals. On the lower panel is shown a close up view of the original residues on the left and of the mutated ones on the right. Each construct described in the text is represented in a different color: 1) green; 2) yellow; 3) cyan; 4) magenta and 5) gray. Constructs 6) and 7) are not shown in the figure.

The constructs were processed as for the cavity filling strategy and analyzed by SDS-PAGE as shown in Figure 13. The electrophoresis shows that, of all the mutants tried, only three are present in both the supernatant and the pellet fractions: K212H, E201A;Q202G and R200H;E201D;Q202N. All remaining mutants are only in the pellet fraction, indicating that the mutations are not tolerated, making KtrA insoluble.

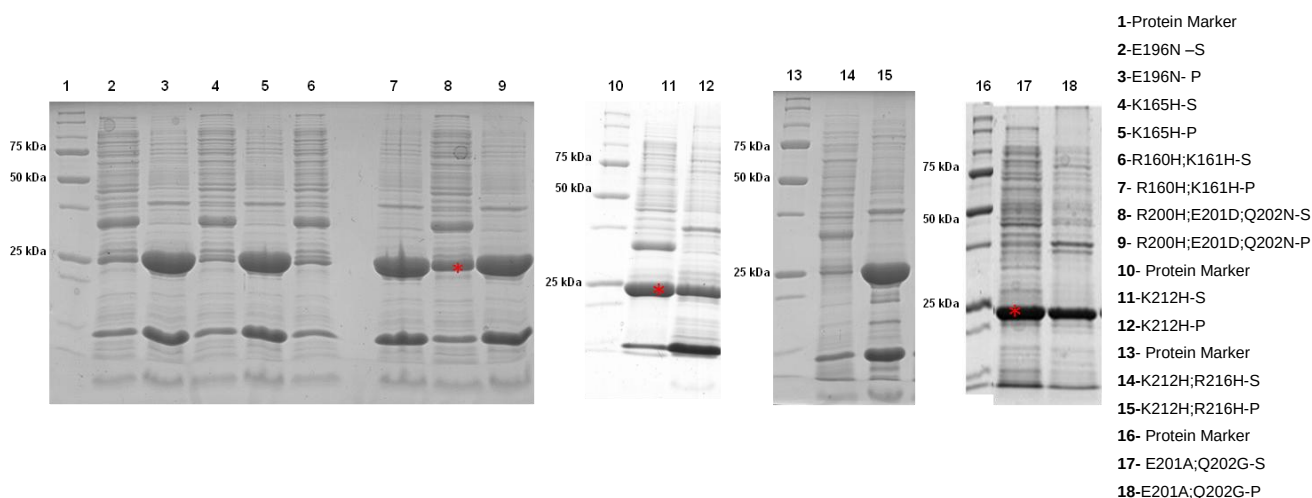


Fig.13 - SDS- PAGE gels of KtrA C-terminal exposed residues constructs. Each protein was lysed by sonication and centrifuged. Soluble fractions (Supernatant) and pellet fractions resuspended in lysis buffer were loaded on a 15% SDS gel. S refers to the supernatant fraction and P to the pellet fraction. The asterisk (*) marks the band corresponding to KtrA in the soluble fractions.

The next step consisted in creating a C-terminal mutant combining both strategies that would be the best candidate to use in crystallization studies. In order to accomplish this, the R200H;E201D;Q202N mutations that worked from the exposed surface strategy were incorporated by site directed mutagenesis in the KtrA Cysless;V172L mutant from the cavity filling strategy, generating a construct that was named KtrA CysLHDN. The new construct was processed as before and checked by SDS-PAGE. As depicted in Figure 14, CysLHDN is present in both fractions (supernatant and pellet) like KtrA WT.

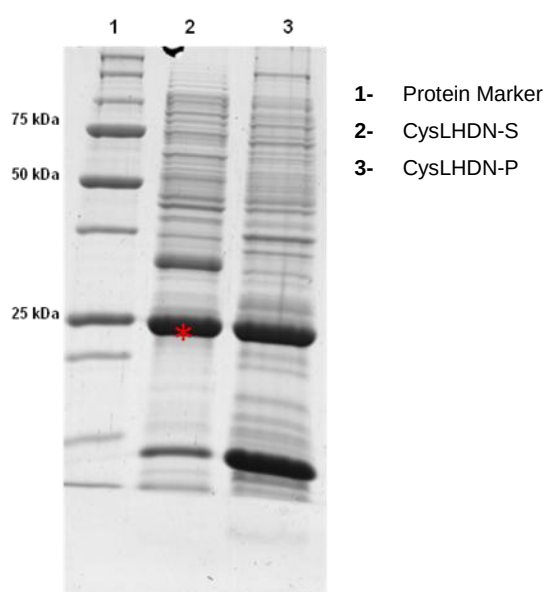


Fig.14 - SDS-PAGE gel of KtrA C-terminal mutant CysLHDN. Protein was lysed by sonication and centrifuged. Soluble fraction (Supernatant) and pellet fraction resuspended in lysis buffer were loaded on a 15% SDS gel. S refers to the supernatant fraction and P to the pellet fraction. The asterisk (*) marks the band corresponding to KtrA in the soluble fraction.

Protein Purification

For relevant constructs previously evaluated by SDS-PAGE, it was important to discern if they could be purified.

KtrA C-terminal mutants were purified as wild-type KtrA (see the Methods section). The size exclusion chromatograms obtained are shown in Figure 15.

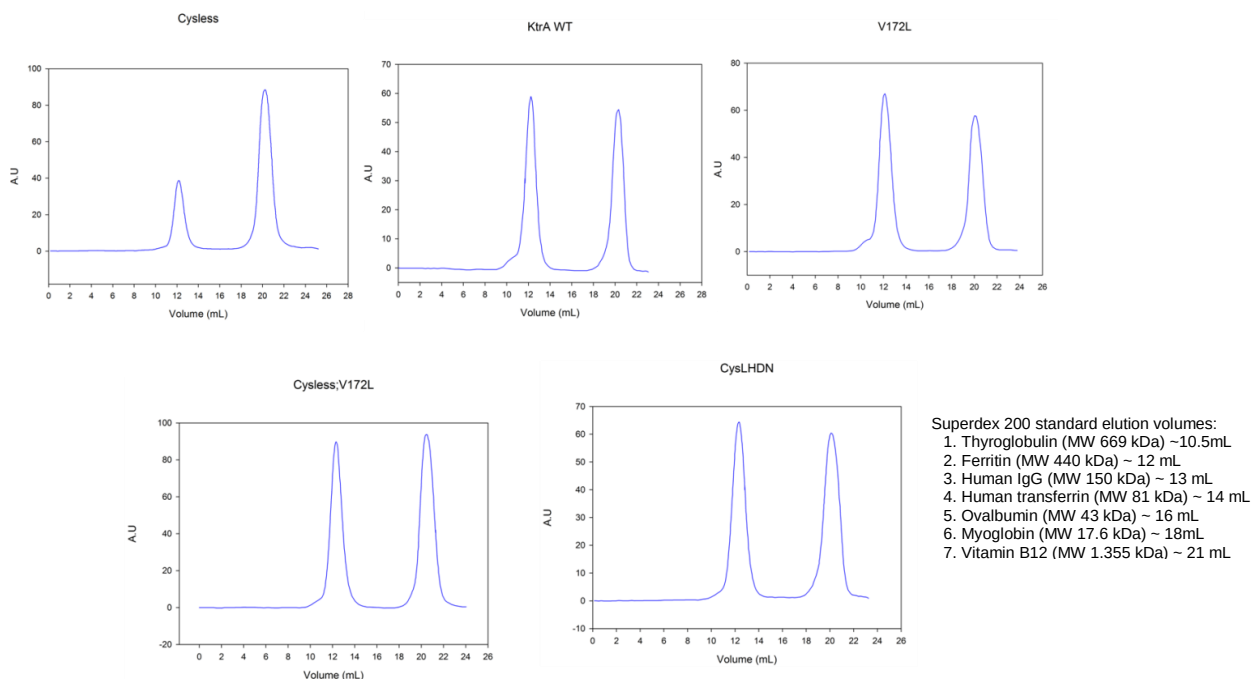


Fig.15 - KtrA C-terminal mutants size exclusion chromatography chromatograms. Protein peak elutes in the Superdex 200 column between 12 and 14 mL. Nucleotide peak elutes at around 20 mL.

As can be observed in the chromatograms, every purified mutant elutes at roughly 12.5 mL. Additionally, there is no peak in the void volume of the column (8mL), meaning that no aggregation occurs. The second peak around 20 mL corresponds to ADP or ATP.

These results clearly show that the introduced mutations don't drastically change the general behaviour of the protein, since all mutants exhibit a profile identical to the native full-length KtrA. In particular, the results indicate that the mutations do not affect greatly the assembly of the octameric ring. Therefore it is reasonable to assume that the constructs are good tools to be used in the attempt to improve the resolution of the KtrA structure.

KtrA C-terminal Mutants Stability

In order to determine if the mutations introduced were indeed improving the stabilization of the protein, KtrA WT and the most promising mutants were submitted to a thermal shift assay using the fluorescent dye Sypro Orange. When this dye is added to a properly folded protein solution, its fluorescence signal is quenched in the aqueous environment. With an increase of temperature, the protein unfolds exposing its hydrophobic regions and Sypro Orange binds to these regions, leading to an increase in the Sypro Orange fluorescence signal.

The change in fluorescence intensity is plotted as a function of the temperature, typically generating a thermal denaturation curve with sigmoid shape (melting curve). Protein stability is then evaluated by the melting temperature (T_m), which corresponds to the midpoint temperature of the transition between folded and unfolded states of the protein and is estimated from the inflexion point of the melting curve, by plotting its 1st derivative [52-54]. A protein with higher melting temperature is therefore considered more stable.

In this case, KtrA WT and its mutants were purified and incubated with Sypro Orange at 1.5 μ M concentration. Thermal stability was measured by performing a thermal denaturation curve, using a temperature range from 25 to 70°C.

The conditions for preparation of the protein for the thermal stability assay were first evaluated. In method A, the native full-length KtrA was eluted from ADP-agarose affinity beads using only ADP as a ligand. This ligand was kept throughout the whole purification process except during size exclusion chromatography, where ADP was removed from the solution. After elution, the protein was supplemented with 1mM of either ATP or ADP and used for the assay. In method B the protein was eluted from the ADP-agarose beads with either ATP or ADP. After size exclusion chromatography, the batches of protein are supplemented with 1mM of the initial ligand.

While for the protein with ADP there is no difference between the two preparation methods, the thermal stability assay reveals a 4°C difference in the melting temperatures for the protein incubated with ATP.

Table 2- Melting temperature (T_m) values obtained for KtrA WT purified with ADP and ATP with the methods A and B.

KtrA WT	T_m (°C)
ADP bound (method A)	54.97
ADP bound (method B)	54.20
ATP bound (method A)	48.63
ATP bound (method B)	44.70

This fact is an indication that some of the ADP used during the preparation of the protein still remains bound to the protein after the size exclusion chromatography. As a result, even with the addition of an excess of ATP, the bound ligand appears to not completely exchange. These results also show that ATP and ADP affect differently the thermal stability of KtrA, with ADP stabilizing the protein almost 10°C. Unfortunately, it is not possible to determine the melting temperature of the apo-protein since complete removal of ligand results in precipitation.

In light of these results, experiments with the mutants were done only with the protein prepared by method B. Figure 16 depicts the melting curves and respective first derivative for each tested protein.

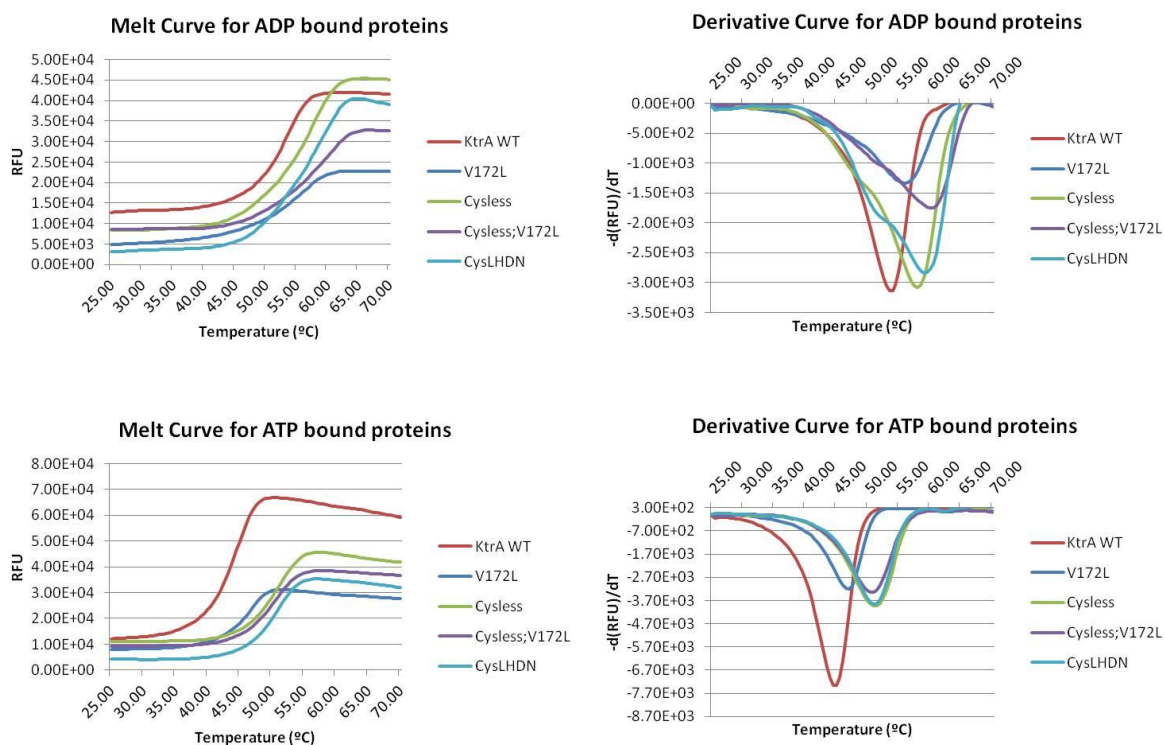


Fig.16 - Melting and derivative curves of KtrA WT and respective C-terminal mutants. On the left are represented the melting profiles obtained for each protein bound to either ADP or ATP. On the right are the respective 1st derivative curves that allow the determination of the melting temperature (T_m). A single example is shown for each protein. RFU stands for relative fluorescence units.

The melting temperatures (T_m) obtained for each protein bound to ATP or ADP are detailed in Table 2. Figure 17 shows the changes in stability by comparison with the WT protein.

Table 2 - Melting temperature (T_m) values and respective standard deviations (SD) obtained for wild-type and mutant KtrA proteins in the thermal shift assay. ADP or ATP were used at 1mM concentration for each sample and values correspond to an average of 15 repeats each.

KtrA	ADP		ATP	
	T_m (°C)	SD	T_m (°C)	SD
WT	54.20	0.32	44.70	0.65
V172L	56.10	0.39	47.20	0.56
Cysless	57.73	0.26	50.70	0.25
Cysless;V172L	60.20	0.66	50.57	0.50
CysLHDN	58.57	0.37	49.43	2.51

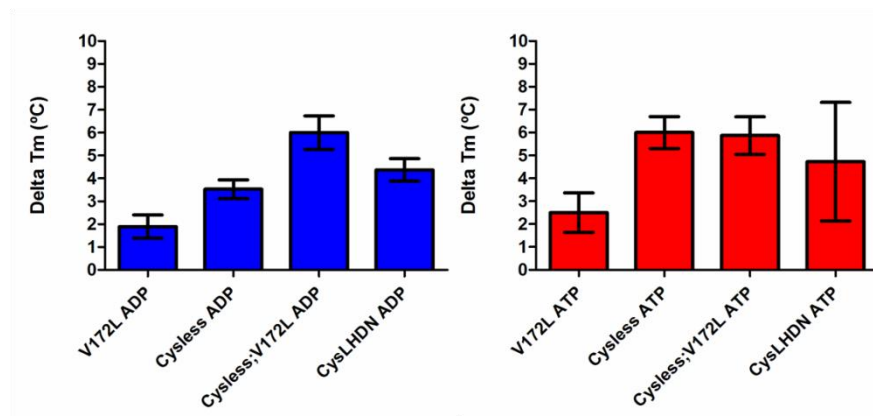


Fig.17 - Bar graph showing the difference in T_m obtained for each tested mutant by comparison with the KtrA WT either with ADP (blue) or ATP (red).

The thermal shift assay demonstrates that all the C-terminal mutants of KtrA have a higher melting temperature compared to the wild-type protein independently of the ligand bound.

In proteins bound to ADP, the minimum increase in T_m was roughly 2 degrees (for the V172L mutant), displaying a fluorescence transition from 54 to 56 °C when compared to the WT KtrA. The highest increase obtained was 6 degrees for the Cysless;V172L mutant with a change from 54 to 60 °C.

In proteins bound to ATP we also see a minimum increase in T_m of 2 degrees corresponding to the V172L mutant, and a maximum increase of 6 degrees for the Cysless;V172L mutant, transitioning from 45 to 51°C. However, with ATP there is no difference between this mutant and the Cysless mutant while with ADP we could detect a change of ~2.5°C.

These results clearly indicate that the developed mutants of the KtrA C-terminal improve the stability of the protein as expected, since the increase in T_m means that a higher temperature is needed for the protein to denature.

Interestingly, the T_m obtained for proteins bound to ADP is always higher than the one obtained for proteins bound to ATP. In the native form of KtrA a 9 degree difference in T_m is observed and this difference is more or less maintained for the mutants, showing that the protein is more stable when bound to ADP.

Complex formation of the KtrA CysLHDN mutant

Of all C-lobe KtrA mutants, CysLHDN is the one thought to be most prone to result in better diffracting crystals. This mutant was shown to greatly improve stability for both ADP and ATP bound KtrA and it includes mutations of residues exposed on the surface of the protein that diminish the entropy of crystal contacts, making the protein less prone to disturbances in the crystal lattice. This should in turn lead to the formation of a better diffracting crystal capable of generating a structure with higher resolution.

However, the ultimate goal is to increase the resolution of the KtrAB complex structure. Therefore it is necessary to ascertain if the introduced mutations impair complex formation between the KtrA octameric ring and the KtrB dimer.

In order to observe complex formation, purified KtrA CysLHDN and KtrB WT were mixed at an apparent ratio of 50:105 (w:w) respectively, since this was the ratio previously optimized for the KtrAB WT complex. Complex assembly was then evaluated by size exclusion chromatography and collected fractions run on SDS-PAGE (see Figure 18).

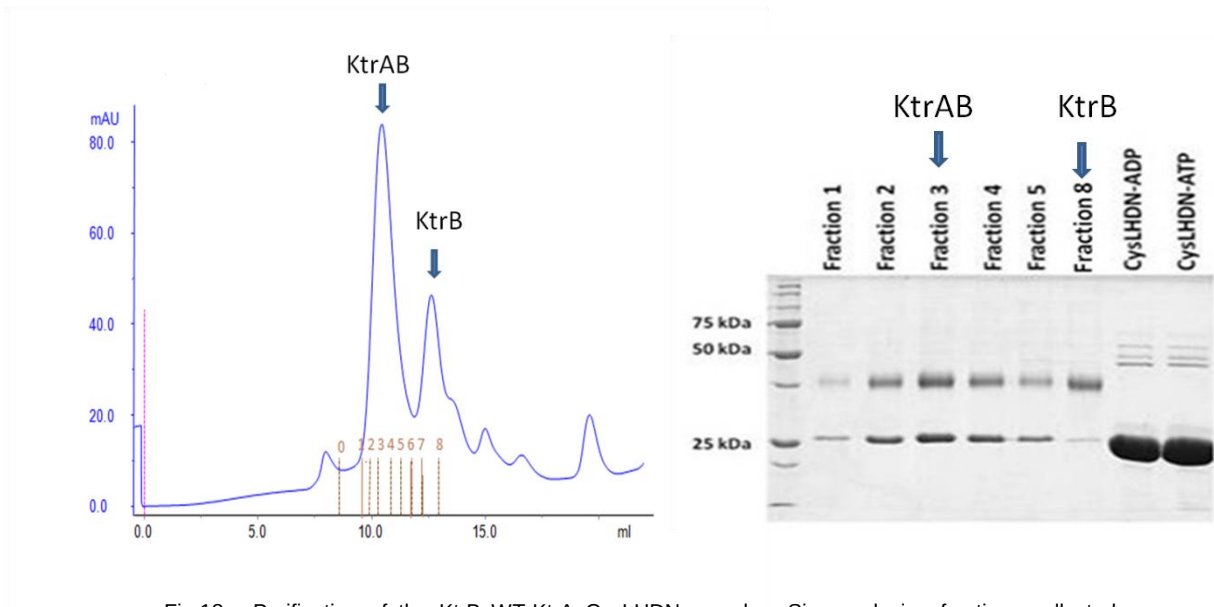


Fig.18 - Purification of the KtrB WT-KtrA CysLHDN complex. Size exclusion fractions collected are numbered in the chromatogram (left) and were analysed by 15% SDS-PAGE (right).

As depicted in the chromatogram and gel, the KtrA mutant CysLHDN assembles with KtrB to form the KtrAB complex. The complex elutes the column around 12 mL (fraction 3 in the gel) as previously seen for the KtrAB WT. However, the optimal ratio of the two proteins in this case seems to differ from the WT complex and needs to be optimized further, since in fraction 8 the peak corresponds only to KtrB WT, revealing an excess of the membrane protein.

In light of this result, this KtrA mutant was considered a good tool for crystallization studies of the full transporter.

Crystallization of KtrA CysLHDN-ATP

The structures of KtrA bound to both ATP and ADP have recently been published [33]. They were determined by X-ray diffraction with a resolution of 3.24 Å for the ATP complex and 2.93 Å for the ADP complex. In order to try to improve these resolutions, a crystallization screen was performed using the CysLHDN KtrA mutant.

For this purpose, the ATP-bound mutant was prepared, since the resolution of the original structure of the native full-length KtrA-ATP complex is lower than for the ADP complex. The sitting-drop vapour diffusion crystallization technique was applied in order to determine crystallization conditions.

Two commercially available crystallization screens, Morpheus and JCSG, were tested at room temperature in 96-well plates. Crystallization drops were prepared by mixing 1 uL of protein and 1 uL of crystallization buffer. The conditions that lead to the formation of crystals for each screen are listed in Table 3 and pictures of the crystals obtained are depicted in Figure 19.

Table 3 - Crystallization hits obtained for KtrA mutant CysLHDN bound to ATP.

Crystallization Condition		Screen
a)	0.1M Amino Acids; 0.1M Buffer System 3 pH 8.5; 10% PEG 4000; 20% Glycerol	Morpheus
b)	10% PEG 6000; 0.1M Bicine pH 9.0	JCSG
c)	40% MPD; 5% PEG 8000; 0.1M Sodium Calcodylate pH 6.5	JCSG
d)	0.1M MES pH 6.5; 1.6M MgSO ₄	JCSG

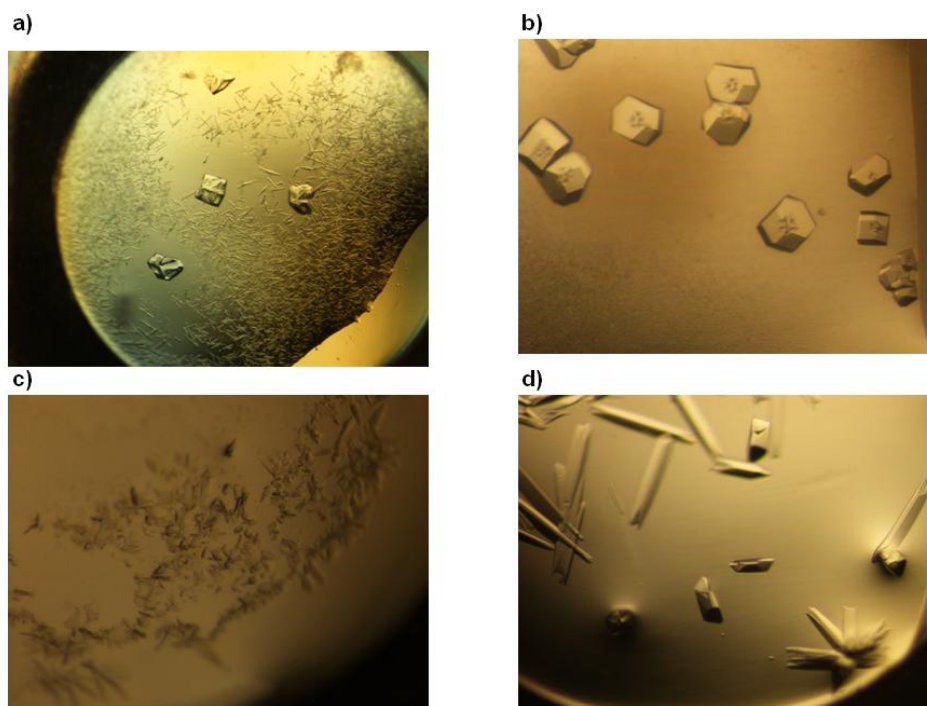


Fig.19 - Pictures of the crystals obtained for KtrA mutant CysLHDN bound to ATP. Letters a) to d) correspond to the conditions shown in Table 3.

Unfortunately, the only condition that we were able to reproduce using reagents from the laboratory was condition d).

This condition was optimized by using a range of concentrations of both MES and MgSO_4 in a 24-well plate. MES concentrations were either 0.100 M or 0.150 M at pH 6.5 whereas MgSO_4 ranged from 1.3 to 1.8 M. Crystals similar to those observed for condition d) were obtained for almost every combination in this screen but well formed single crystals were grown in the presence of 0.100 M MES; 1.5 M MgSO_4 (Figure 20).

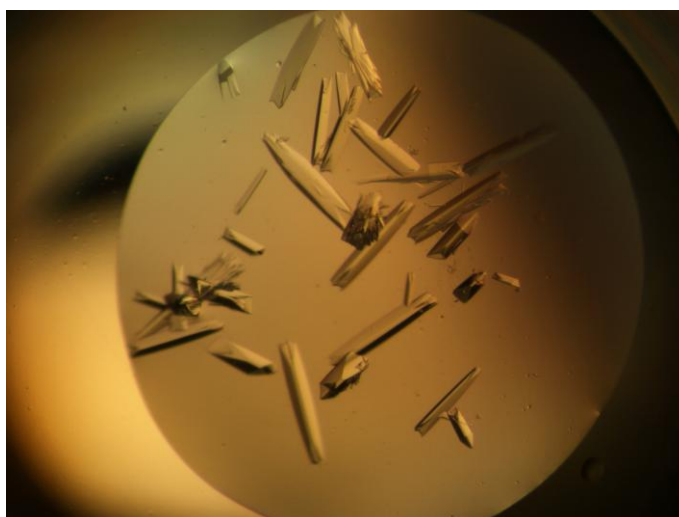


Fig.20 - KtrA CysLHDN-ATP crystals obtained in 0.100 M MES pH 6.5; 1.5 M MgSO_4 .

Several crystals from conditions b) and optimized d) were then analysed by X-ray diffraction. To do so, the crystals were taken-out of the drop and put into cryo solutions before being flash-frozen in liquid nitrogen. Cryoprotectant solutions were made in order to preserve the crystal at liquid nitrogen temperatures and minimize the effects of x-ray exposure during data collection. For crystals derived from condition d), the selected cryo solution had the following composition: 0.1M MES pH 6.5; 1.6M MgSO₄; 0.5 mM ATP; 30% Glycerol. As for the crystals from condition b), the cryo solution used was: 0.1M Bicine pH 9.0, 10% PEG 6000; 0.5 mM ATP; 30% Glycerol.

Data sets obtained, from crystals from condition b), were processed to 2.8 Å resolution, which is an improvement compared to the 3.24 Å previously obtained for KtrA WT bound to ATP.

Crystallization of KtrB WT-KtrA CysLHDN- ATP

After successfully obtaining better diffracting crystals for KtrA bound to ATP, the next step was to try to obtain a better structure of the full KtrAB transporter. In order to do so, KtrB WT and KtrA mutant CysLHDN-ATP were purified as described in the Methods section and mixed with a 50:105 (w:w) KtrA to KtrB ratio. The formed complex was then purified by size exclusion chromatography and concentrated to 7 mg/mL for crystallization.

The sitting-drop vapour diffusion method was used with the best crystallization condition previously determined for the KtrAB WT complex bound to ATP (0.1M ADA pH6.5; 20% PEG 400; 0.2 M AMS). This condition was used as a starting point for setting up a screen with the following concentration range of some of the solution components (17-22% PEG 400 and 0.150-0.220M AMS).

From this screen, two conditions resulted in the formation of crystals (see Figure 21):

- 1) 0.200M AMS; 19% PEG 400; 0.1M ADA pH6.5
- 2) 0.200M AMS; 22% PEG 400; 0.1M ADA pH6.5

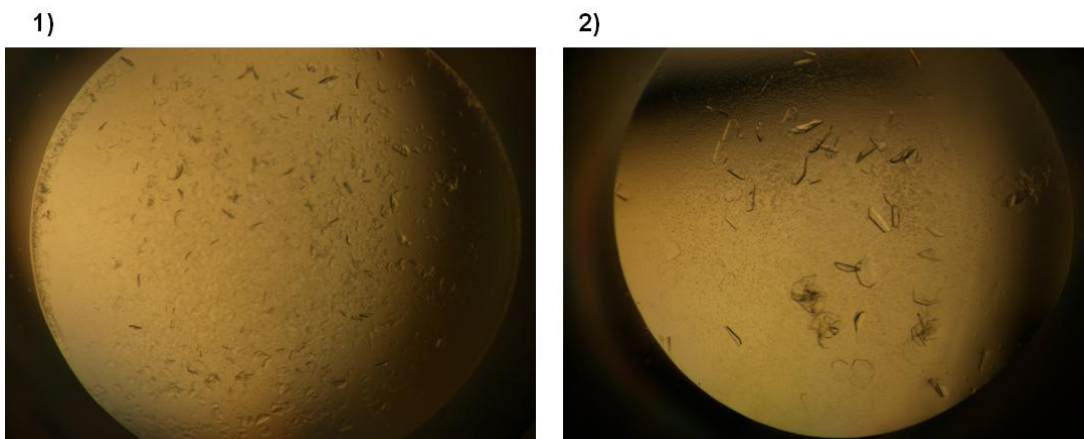


Fig.21 – Pictures of the crystals obtained for the KtrB WT-KtrA CysLHDN-ATP complex.

We also tested a different approach to improve the KtrAB crystals. This approach is called HILIDE and has been successfully used to crystallize and determine the structure of membrane proteins [55, 56]. This procedure is based on the importance of phospholipids for stability of membrane proteins and it involves the addition of different amounts of a defined phospholipid to the protein prior to the crystallization trial. The full procedure is described in the Methods section but briefly, the complex KtrB WT-KtrA CysLHDN bound to ATP was purified by size exclusion chromatography and concentrated to 9 mg/mL in the presence of the detergent Cymal-6. The transporter was then re-lipidated with the phospholipid DOPC at 0, 150 and 300 ug. Each one of these conditions was in turn incubated with 1.5; 15 or 30 mM of the detergent Cymal-6 for a total of 9 different samples. After processing, 1.2 μ L of each protein sample was mixed with 1 μ L of crystallization buffer in a 24-well plate from a screen based on the original WT crystallization condition.

The HILIDE approach resulted in very small crystals (see Figure 22) and it will be necessary to repeat the approach with other phospholipids and to test their diffraction properties at a synchrotron X-ray source.

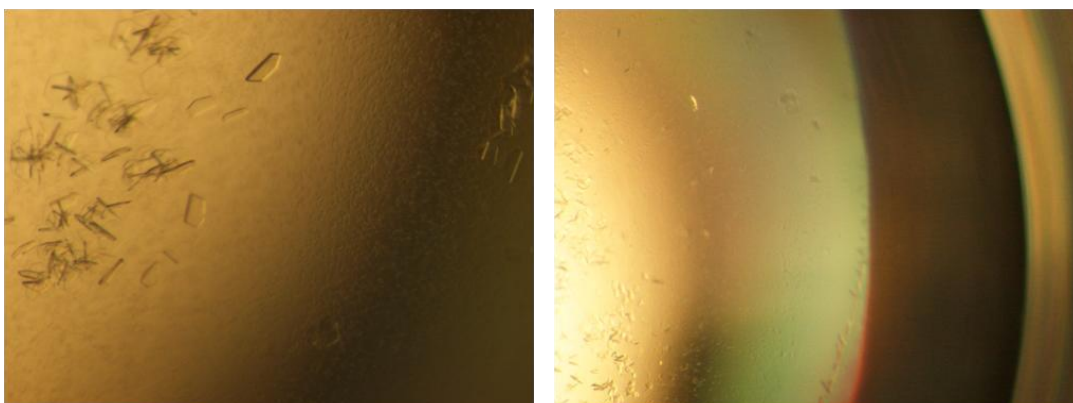


Fig.22 – Example picture of crystals obtained for the complex KtrB WT- KtrA CysLHDN-ATP in the HILIDE strategy.

Another approach attempted consisted in using an additive screen (Hampton Research HR2-428). To the crystallization condition that gave rise to the KtrAB crystals used for structure determination (1 μ L protein and 1 μ L reservoir buffer) we added 0.25 μ L of an additive (including organic solvents, divalent cations, sugars, aminoacids, lipids, among other compounds) before incubation of the drop. However no crystals resulted from this strategy.

Therefore, crystals from the two stated conditions obtained were analysed by X-ray diffraction and some data sets were obtained. However, the information didn't result in a structure of the transporter with better resolution than the one already published for the KtrAB WT complex.

Crystallization of KtrB WT-KtrA CysLHDN-ADP

The sitting-drop vapour diffusion crystallization technique was also applied in an attempt to obtain a crystallization condition for the KtrB WT-KtrA CysLHDN complex but bound to ADP instead of ATP. An initial trial was performed using an in-house crystallization screen. This screen is here referred as SB screen and mixes different salts, with six different pH and two different PEGs in each condition.

This screen revealed three crystal forming conditions shown in Figure 23:

- 1) 0.1M KNa; 10% PEG 4000; 0.1M Na Acetate pH 4.6
- 2) 1M NaCl; 10% PEG 4000; 0.1M HEPES pH 7.5
- 3) 1M NaCl; 10% PEG 4000; 0.1M ADA pH 6.5

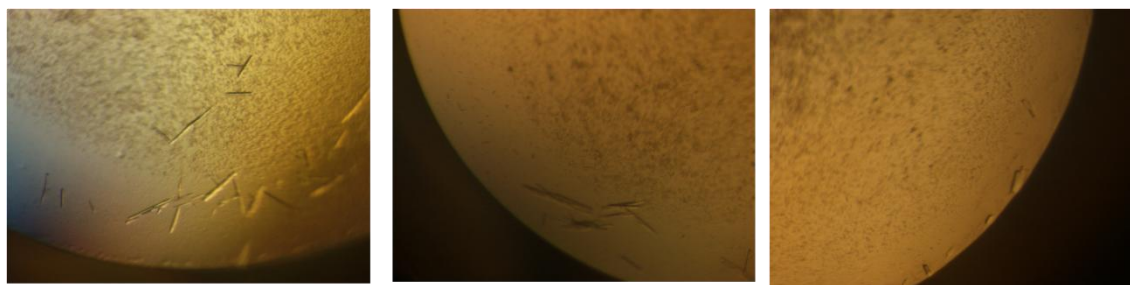


Fig.23 - Crystals obtained with SB screen for the KtrB WT-KtrA CysLHDN-ADP complex. From left to right are represented conditions 1, 2 and 3, respectively.

This work is still in progress, since the crystals obtained still need to be optimized for X-ray analysis.

Cyclic di-AMP stability effect on KtrA

Recently, Corrigan and colleagues have shown that c-di-AMP does not bind to the N-terminal of KtrA from *S.aureus* [35]. Instead, they showed that this nucleotide binds to a truncated form of KtrA containing only the C-terminal region [35]. These findings made us wonder if this binding occurs in the KtrA from *B.subtilis* and, if so, what might be the nature of the binding site and how does the nucleotide affect the regulation of the transporter.

In order to answer some of these questions, we started by evaluating the effect of cyclic dinucleotides in the thermal stability of native full-length KtrA using the thermal shift assay described above. This technique has been used before to determine if small molecules, including c-di-GMP, bind to proteins [57-60]. KtrA was purified with either ATP or ADP at 1 mM concentration and supplemented with 250 μ M of different cyclic nucleotides. The nucleotides tested were: c-di-AMP, c-di-GMP, c-di-IMP and cAMP. Although this last one is not a cyclic dinucleotide, it is a known relevant biological regulator and it would be interesting to see if the protein shows specificity for the dinucleotide form or not.

The fluorescence transitions observed in the form of melting curves are shown in Figure 24 and the melting temperatures obtained from this study are shown in Table 4. Figure 25 illustrates the difference in T_m obtained for each combination of KtrA with either ATP or ADP and cyclic nucleotide in comparison with the non-treated protein.

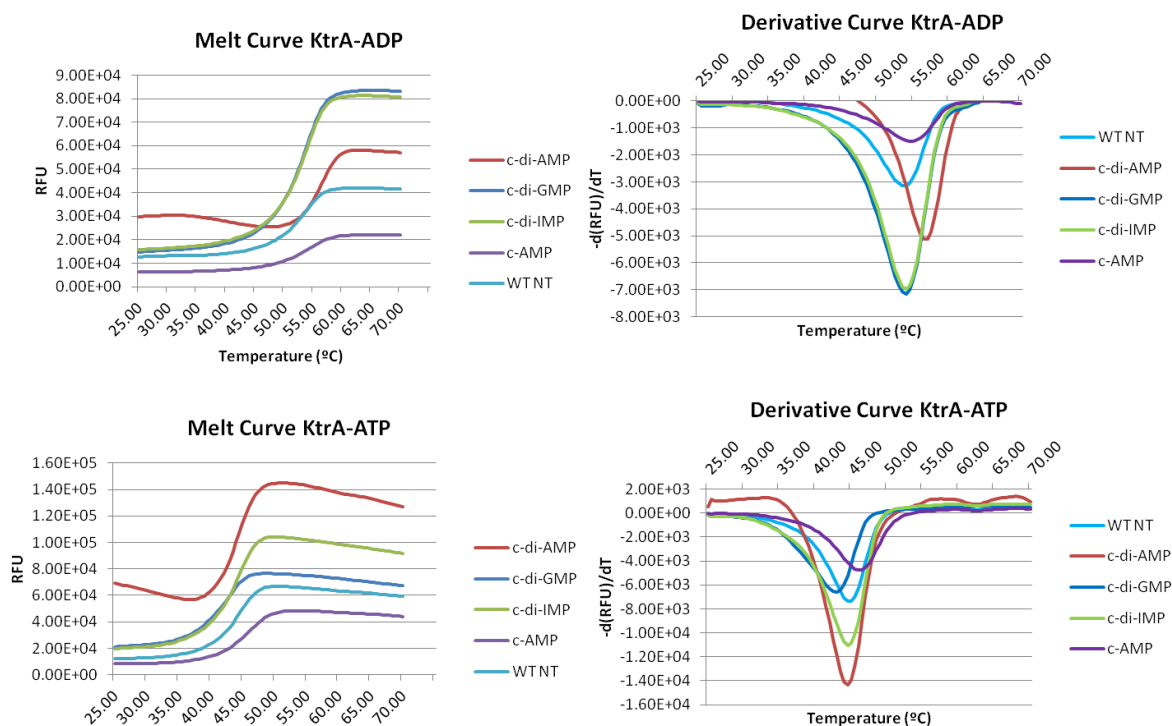


Fig.24 - Melting and corresponding 1st derivative curves for KtrA WT with and without cyclic nucleotides. On the left are the melting profiles obtained for treated and non-treated KtrA bound to either ADP or ATP. On the right are the respective 1st derivative curves that allow the determination of the melting temperature(T_m). A single example is shown for each protein. RFU stands for relative fluorescence units.

Table 4- Melting temperature (T_m) values and respective standard deviations (SD) obtained in the thermal shift assay. ADP or ATP were at a concentration of 1mM in each sample and values correspond to an average of 15 repeats for non treated (NT) native KtrA and of 6 repeats for KtrA treated with nucleotides.

KtrA-WT	ADP		ATP	
	T_m (°C)	SD	T_m (°C)	SD
NT	54.20	0.32	44.70	0.65
c-di-AMP	56.92	0.20	44.17	0.61
c-di-GMP	54.00	0.00	43.58	0.58
c-di-IMP	53.92	0.20	44.00	2.21
c-AMP	55.00	0.00	46.33	0.86

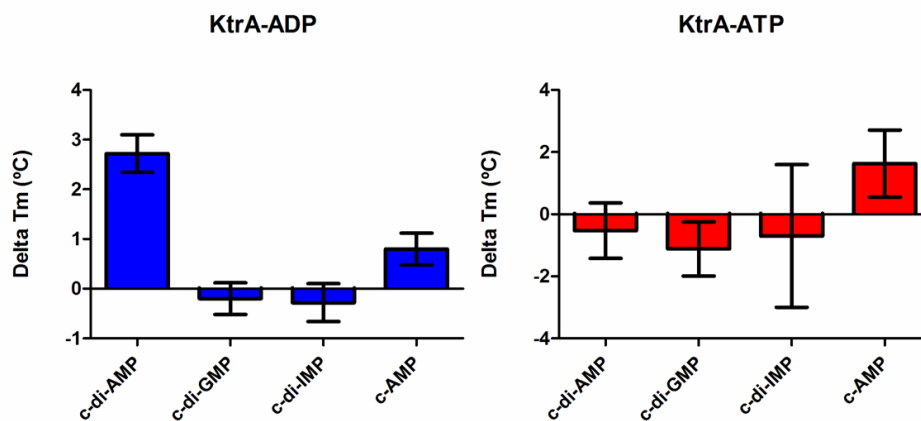


Fig.25 - Bar graphs illustrating the difference in melting temperatures (T_m) obtained for KtrA WT treated with various cyclic dinucleotides and cAMP, in comparison with non-treated protein. The left graph refers to KtrA with ADP and the right graph to KtrA with ATP.

These results clearly show that, when KtrA is bound to ADP, c-di-AMP contributes to the stabilization of the protein, leading to a change in T_m from ~54 to ~57 °C. Moreover, this effect is specific to this cyclic dinucleotide since all other tested display roughly the same melting temperature as the non-treated (NT) KtrA. In the case of cyclic AMP, a slight increase is observed when compared to the non treated KtrA, however this increase is clearly lower since it amounts to less than 1°C, probably meaning that the KtrA binding affinity is lower for cAMP than c-di-AMP.

In contrast, with KtrA bound to ATP all cyclic dinucleotides caused a decrease in the melting temperature. cAMP was the only molecule tested that increased (less than 2°C) the T_m when compared with the non-treated protein.

In light of these results, we conclude that the only cyclic dinucleotide tested that significantly increases the stability is c-di-AMP. However this only occurs when the protein is bound to ADP since no effect is observed in the ATP bound protein.

These results suggest that KtrA binds c-di-AMP and that the conformational change in the octameric ring induced by ATP alters the binding site of c-di-AMP reducing the ligand's affinity.

Binding of c-di-AMP to KtrA

In order to better understand the binding of c-di-AMP to KtrA, an isothermal titration calorimetry (ITC) assay was used. This technique measures the heat change associated with the mixing of two components, usually a protein and a ligand. The ligand is titrated into the protein solution through several injections and the heat, either released or taken-up, is measured by determining the power needed to maintain a constant temperature in relation to a reference cell containing water. This heat is proportional to the amount of ligand that binds to the protein in each injection. With the progress of the titration, the binding sites eventually get saturated and the reaction heats tend to zero. The obtained binding isotherm can then be used to retrieve several parameters of the interaction: the binding enthalpy, the equilibrium binding affinity, K_a ($K_a=1/K_d$), and the stoichiometry of interaction or number of binding sites (n) [61].

Native full-length KtrA was purified with either ATP or ADP as described in the Methods section. The protein concentration was at 30 μ M and ATP or ADP were at 1mM concentration. This protein solution was titrated at 25°C with c-di-AMP at a concentration of 300 μ M.

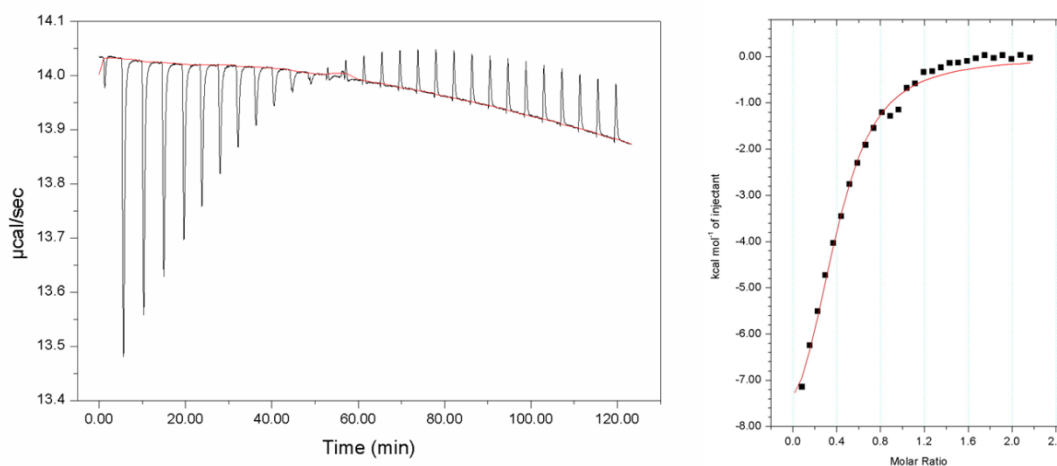


Fig.26 - Interaction of KtrA bound to ADP with c-di-AMP by isothermal titration calorimetry (ITC). A representative assay is shown. On the left, the thermogram (thermal power vs. time) is shown after baseline correction. The right panel shows the integrated heat release as a function of the molar ratio of c-di-AMP to KtrA-ADP.

Regarding KtrA WT bound to ADP, the experiment shows an exothermic profile with c-di-AMP binding to the protein (see Figure 26). The integrated heat release was normalized according to the heat of dilution of c-di-AMP determined by titrating buffer alone with the compound (see Figure 27) and fitting was achieved with a one binding site model in Origin.

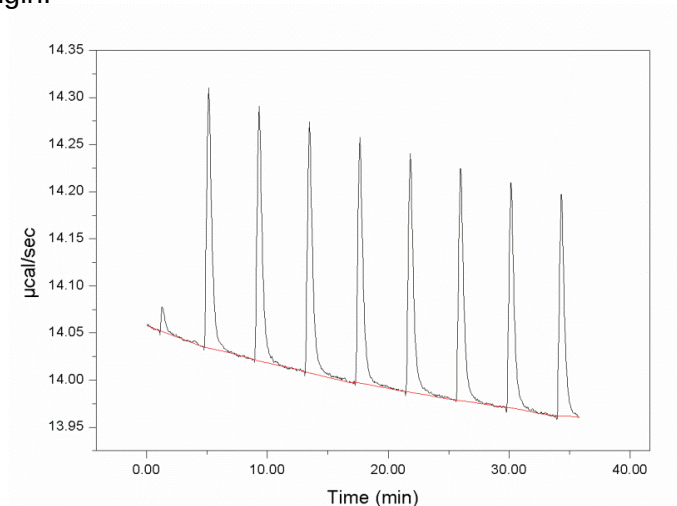


Fig.27 - Thermogram depicting the heat of dilution of c-di-AMP titrated into buffer. A representative assay is shown.

The estimated enthalpy for the reaction is of -12 kcal/mol, with a K_d of 12 μ M and a stoichiometry of interaction (n) of 0.5.

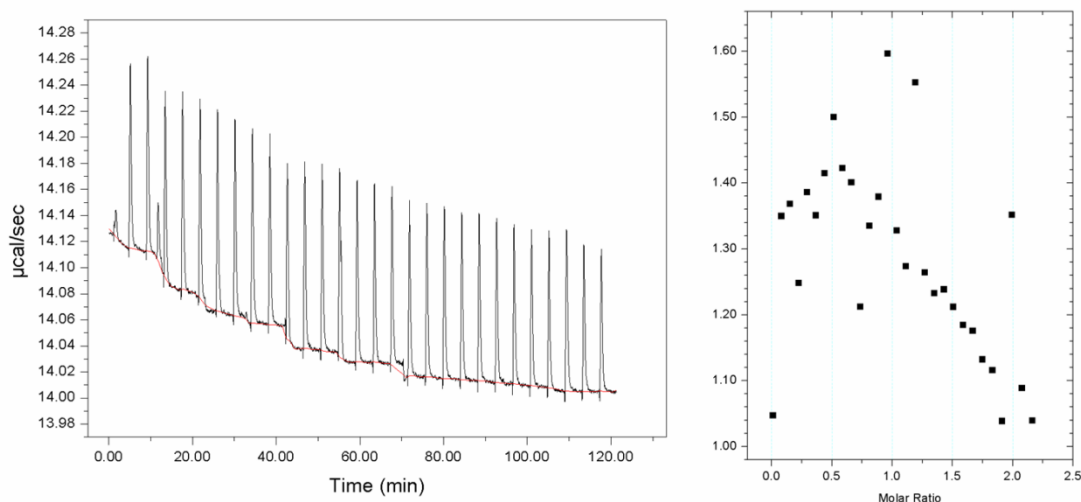


Fig.28 - Interaction of KtrA bound to ATP with c-di-AMP by isothermal titration calorimetry (ITC). A representative assay is shown. On the left, the thermogram (thermal power vs. time) is shown after baseline correction. The right panel shows the heat release as a function of the molar ratio of c-di-AMP to KtrA-ATP.

In the assay performed with KtrA bound to ATP, using similar conditions, the amount of heat exchanged with the c-di-AMP titration is much smaller than for KtrA-ADP (see left panel in Figure 29). This is consistent with the thermal shift assay results where c-di-AMP does not improve the stability of the protein, when KtrA is bound to ATP and it strongly indicates that c-di-AMP does not bind to KtrA-ATP.

Overall, these results confirm the binding of c-di-AMP to KtrA-ADP but not to KtrA-ATP. However, the affinity determined by us is much lower than the one previously seen in *S.aureus*, where the K_d obtained was roughly 64 nM [35]. The basis for this difference is not clear at the moment but it may just reflect differences in amino acid sequence between the two proteins.

A simple analysis of the KtrA structure led us to propose a putative binding site for c-di-AMP that explains some of the properties that we have determined. There is a cavity formed by the C-terminal regions of the KtrA dimers in the octameric ring (see Figure 29) which appears to have the right volume and shape to accommodate a cyclic dinucleotide molecule.

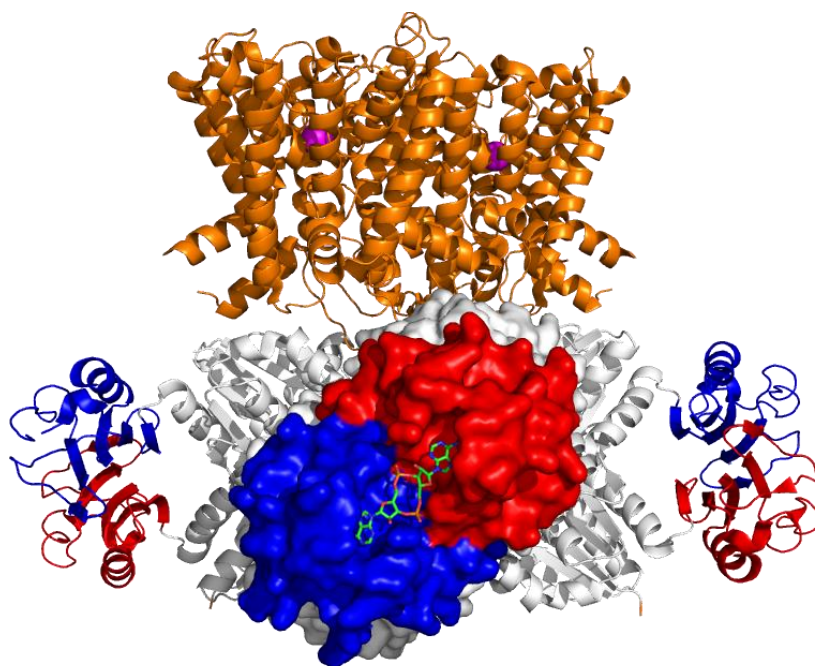


Fig.29 - C-di-AMP putative binding pocket in the C-terminal of KtrA. The KtrAB transporter is represented in a side view, with KtrB dimer colored orange and potassium ions in magenta. KtrA octameric ring is shown with the N-terminals colored white and the C-terminals colored alternately blue and red to evidence the KtrA dimers. The putative binding pocket is highlighted in surface and c-di-AMP is shown in sticks.

This site is shared between the two C-terminal regions of a KtrA dimer within the octameric ring, providing therefore an explanation for the stoichiometry of 0.5 determined by ITC. In addition the position of the binding site also can explain the differences between cyclic di-AMP binding to KtrA-ADP or KtrA-ATP since the structural relationship between the two C-terminal regions of a KtrA dimer changes when the protein binds one or the other ligand and this is reflected in the structural features of this putative binding site. This putative ligand binding site could be explored through mutagenesis in the future.

Crystallization of KtrA WT-ADP with c-di-AMP

The results from both the thermal shift assay and the ITC show binding of c-di-AMP to KtrA when the protein is bound to ADP and not ATP. Therefore we tried to obtain a crystallization condition for this form of the protein in order to discern the exact binding site of the cyclic dinucleotide.

Several crystallization screens were tested using the sitting-drop vapour diffusion method (Table 5) and the crystallization conditions found are shown in Table 6 and Figure 30:

Table 5 - Crystallization screens tested with KtrA-ADP-c-di-AMP.

Screens tested	c-di-AMP Concentration (uM)	ADP Concentration (mM)	Protein Concentration (mg/mL)	Temperature (°C)
Morpheus	300	1	10	20
Wizard	300	1	10	20
JCSG	300	1	10	20
Handmade Screen	300	1	10	20

Table 6 - Crystallization hits obtained for KtrA-ADP-c-di-AMP.

Crystallization Condition	Screen
0.12M Ethylene Glycols; 0.1M Buffer System 1 pH 6.5; 10% PEG 4000; 20% Glycerol	Morpheus
0.12M Monosaccharides; 0.1M Buffer System 1 pH 6.5; 10% PEG 4000; 20% Glycerol	Morpheus
0.12M Amino Acids; 0.1M Buffer System 1 pH 6.5; 10% PEG 4000; 20% Glycerol	Morpheus
0.2 M KCl; 8% PEG 4000; 0.1M ADA pH 6.5	Handmade Screen
0.2 M KCl; 11% PEG 4000; 0.1M NaCitrate pH 5.6	Handmade Screen

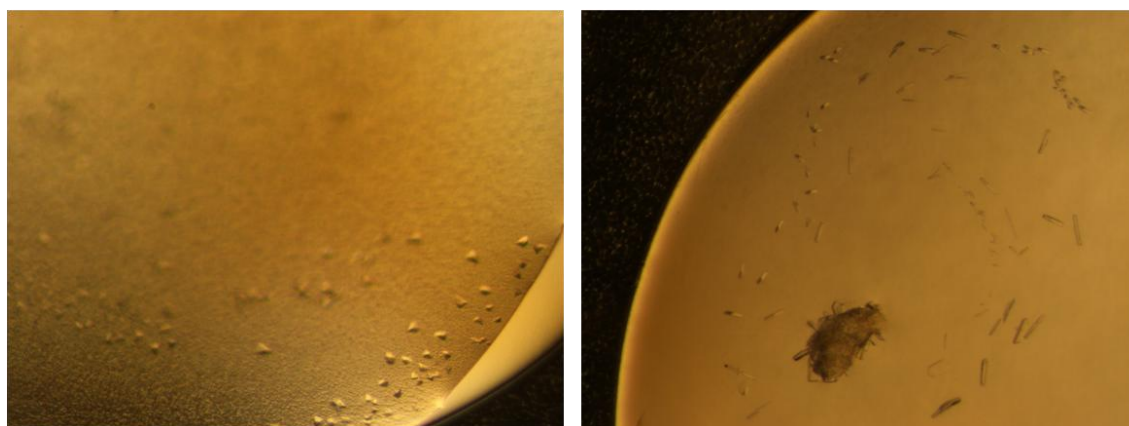


Fig.30 - Example of the crystals obtained for KtrA-ADP-c-diAMP. On the left is a picture of crystals formed with conditions from the Morpheus screen. On the right is a picture of crystals formed with the handmade screen.

Hits were further refined using three different strategies carried out in 24-well plates, see full description in the Methods section.

The first strategy was based in the crystallization condition 0.2 M KCl; 11% PEG 4000; 0.1M NaCitrate pH 5.6. Conditions screened were 0.1M NaCitrate pH 5.6, 7-10% PEG 4000, 50-225 mM KCl and either 0, 10 or 20% Glycerol. The second strategy was based on the crystallization condition 0.2 M KCl; 8% PEG 4000; 0.1M ADA pH 6.5. Screened conditions were similar to the previous screen except that 0.1M ADA pH 6.5 was used instead of 0.1M NaCitrate pH 5.6.

The final strategy was based on the conditions that resulted from the Morpheus screen, which are all very similar. In this case, screened conditions used included 6-11% PEG 4000 and 5-20% Glycerol.

Unfortunately, this refinement did not improve the crystals formed, since they were very similar to the initial ones.

These crystals were then taken out of the drop, immersed in a cryoprotectant solution (10% PEG 4000, 0.1M ADA pH 6.5, 0.5 mM ADP, 150 uM c-di-AMP and 30% Glycerol) and flash-frozen in liquid nitrogen.

Crystals were then analysed by X-ray diffraction, but the data sets collected have a low resolution that doesn't allow the detection of c-di-AMP in the structure. Therefore it is necessary to improve these crystallization conditions further or find better ones in order to be able to determine the KtrA binding site for this nucleotide.

Future Perspectives

Future work on the KtrAB project entails the optimization of crystallization conditions for both the KtrB WT-KtrA CysLHDN complex and KtrA-ADP-c-di-AMP, in order to get a structure of the transporter with better resolution than the existing one in the first case, and to discern the binding site of c-di-AMP in KtrA in the second case. This can be achieved by testing new crystallization screens and applying different crystallization techniques such as hanging drop, at different temperatures.

Moreover, it is still imperative to understand better the binding of c-di-AMP to KtrA. In particular it would be interesting to determine if binding affinity of ATP and ADP vary with the presence of c-di-AMP. This would also help to clarify if the binding is dependent on the KtrA octameric ring conformation, as suggest by the results shown here for KtrA-ADP and KtrA-ATP.

It would also be interesting to see if c-di-AMP plays a role in the function of the transporter KtrAB and that can be discerned, for example, through radioactive flux assays.

Methods

KtrA constructs mutagenesis

KtrA C-terminal mutants were made by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit from Agilent. KtrA from *B.subtilis* is cloned in a pET-24d vector. The reaction was the following: 5 uL 10X *Pfu* Buffer; 3 uL (30 ng) dsDNA template; 1uL (125ng) of oligonucleotide primers forward and reverse; 2.5 uL DMSO; 1 uL dNTPmix; ddH₂O to a final volume of 50 uL and 1 uL *PfuTurbo* DNA polymerase (2.5 U/uL). The prepared reactions were then subjected to a temperature cycling using a T100 Thermal Cycler (Bio-Rad) with the following conditions:

Table 5- PCR conditions used to prepare the KtrA C-terminal mutants.

Segment	Cycles	Temperature	Time
1	1	95 °C	4 minutes
2	17	95 °C	1 minute
		55°C	1 minute
		68°C	15 minutes
3	1	68 °C	10 minutes

Afterwards, each reaction was incubated with 1 uL *Dpn* I restriction enzyme (10 U/uL) for 2h at 37 °C to digest the parental (nonmutated) dsDNA. Each PCR product was then transformed in *E.coli* DH5- α strain and a resulting colony was grown in LB with 50 ug/mL Kanamycin at 37 °C for 16h at 225 rpm. DNA was isolated using a ZR Plasmid Miniprep Classic kit (Zymo Research) following the manufacturer's instructions.

The nucleotide sequence of each mutant was verified by DNA sequencing to confirm that the desired mutation was made and no other mutation was incorporated during the polymerase chain reaction (PCR).

The primers used to make each mutant were designed with the QuikChange Primer Design online tool from Agilent and are shown in the following table. Bases changed are highlighted in red:

Table 6- Primers and Templates used to prepare the KtrA C-terminal mutants.

KtrA Mutant	Template	Primers
V172L	KtrA WT	Forward: 5'-TTGATTCAAATCGATTATTGACCTGAATTAAAGAGCAAAGTACGG-3' Reverse: 5'-CCGTACTTTGCTCTTAAATTCAGGTCAATAATCGATTTTGAATCAA-3'
I199F	KtrA WT	Forward: 5'- CGCGCCTGAAGACATCTTCGCGAGCAAGATTG -3' Reverse: 5'- CAATCTTGCTCGCGAAAGATGTCTTCAGGCGCG -3'
E201A; Q202G	KtrA WT	Forward: 5'-CCTGAAGACATCATTCGCGCGGAGATTGCCTGGTGATCATG-3' Reverse: 5'-CATGATCACCAGGCAATCTCCGCGCGAATGATGTCTTCAGG-3'
D197Y; I198S; I199F	KtrA WT	Forward: 5'-TATCTGTCTTTCCCCGCGCCTGAATATAGCTTTCGCGAGCAAGATTG-3 Reverse: 5'-CAATCTTGCTCGCGAAAGCTATATTCAGGCGCGGGGAAAGACAGATA-3'
V172L;E201A;Q202G	E201A;Q202G	Forward: 5'-TTGATTCAAATCGATTATTGACCTGAATTAAAGAGCAAAGTACGG-3' Reverse: 5'-CCGTACTTTGCTCTTAAATTCAGGTCAATAATCGATTTTGAATCAA-3'
Cysless;V172L	Cysless	Forward: 5'-TTGATTCAAATCGATTATTGACCTGAATTAAAGAGCAAAGTACGG-3' Reverse: 5'-CCGTACTTTGCTCTTAAATTCAGGTCAATAATCGATTTTGAATCAA-3'
K212H	KtrA WT	Forward: 5'- CCTGGTGATCATGGGCCACAAACATGATATTAACGTTTTGAAAACGA -3' Reverse: 5'- TCGTTTTCAAACGTTTAATATCATGTTTGTGGCCCATGATCACCAGG -3'
K165H	KtrA WT	Forward: 5'- TGGCTACGCGCAAGCTTGATTCAATTCGATTATTGACCTG -3' Reverse: 5'- CAGGTCAATAATCGAATGTGAATCAAGCTTGCGCGTAGCCA -3'
E196N	KtrA WT	Forward: 5'- CTTTCCCCGCGCCTAATGACATCATTCGCGAG -3' Reverse: 5'- CTCGCGAATGATGTCATTAGGCGCGGGGAAAG -3'
K212H;R216H	K212H	Forward: 5'- GGGCCACAAACATGATATTAACATTTGAAAACGAAGGGATGTAG -3' Reverse: 5'- CTACATCCCTTCGTTTTCAAATGTTTAATATCATGTTTGTGGCCC -3'
R160H;K161H	KtrA WT	Forward: 5'- TTGTCGAGCTGCTGGCTACGCAACCATCTTGATTCAAATCGATTATTG -3 Reverse: 5'- CAATAATCGATTTTGAATCAAGATGGTGCGTAGCCAGCAGCTCGACAA -3'
R200H,E201D;Q202N	KtrA WT	Forward: 5'- CGCGCCTGAAGACATCATTCACGATAATGATTGCCTGGTGATCATGGG -3' Reverse: 5'- CCCATGATCACCAGGCAATCATTATCGTGAATGATGTCTTCAGGCGCG -3'
CysLHDN	Cysless;V172L	Forward: 5'- CGCGCCTGAAGACATCATTCACGATAATGATAGCCTGGTGATCATGGG -3' Reverse: 5'- CCCATGATCACCAGGCTATCATTATCGTGAATGATGTCTTCAGGCGCG -3'

Protein expression and purification

Tag-less KtrA and respective mutants were over-expressed in *E. coli* BL21 (DE3) strain after growth to an OD₆₀₀ (optical density at 600 nm) of 0.6 at 37 °C in LB media and approximately 16 h induction by 0.5 mM IPTG and 2% ethanol at 20 °C. Cell lysis was done in Buffer A (50 mM Tris-HCl pH 7.5, 50 mM KCl, 5mM DTT) supplemented with protease inhibitors (1ug/mL Leupeptine and Pepstatine A and 1mM PMSF) using a prechilled cell cracker Emulsiflex-C5 (Avestin). For solubility tests, lysate was analyzed immediately in SDS-PAGE. Otherwise, spin-cleared lysate was loaded into an anion exchange column Hi-Trap Sepharose Q HP (5mL) attached to an ÄKTA purifier UP-10. KtrA was eluted using a KCl gradient (50mM to 1 M). Eluted fractions containing KtrA were pooled and incubated with an ADP-agarose resin (Innova Biosciences) overnight at 4 °C with gentle agitation. Beads were then washed thoroughly with Buffer B (50 mM Tris-HCl pH 8.0, 150 mM KCl, 1mM TCEP) and protein was eluted in buffer B supplemented with either 5 mM ADP or ATP. Protein was then concentrated to ~5 mg/ml in a Sartorius Vivaspinn column (15mL with Mw-cutoff of 30 kDa) and further purified by size exclusion chromatography with a Superdex-S200 (GE Healthcare) column using buffer C (50 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM DTT). Eluted KtrA was supplemented with 1 mM ATP or ADP from a 100 mM stock in 1 M Tris-HCl pH 7.5. Protein concentration was determined with a colorimetric assay (Bio-rad) and measuring A_{595nm} or by the Direct Detect Spectrometer (EMD Millipore).

'N-His₆-KtrB cloned in a modified pET-24d vector (Novagen) was over-expressed in *E. coli* BL21 (DE3) strain. Cells were grown in LB media at 37 °C to an OD₆₀₀ of 1.1 and induced by 0.5 mM IPTG and 1mM BaCl₂ for 2.5 h. Cells were then lysed as described for KtrA in buffer D (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 30 mM KCl) supplemented with protease inhibitors. KtrB was extracted from the cell lysate with 40 mM DDM (*n*-dodecyl-β-D-maltoside, solgrade from Anatrace) overnight at 4 °C with gentle agitation. Spin-cleared lysate was loaded into a Talon metal affinity resin (Clontech) and washed with Buffer E (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 30 mM KCl, 1 mM DDM). The column was then washed with buffer D supplemented with 20 mM imidazole. KtrB was eluted with buffer D supplemented with 150 mM imidazole and immediately diluted in buffer D supplemented with 15 mM DTT and 1.5 mM TCEP (both wash and elution were monitored with a UV-detector-attached chart-recorder). Eluted fractions were concentrated to ~2.5 mg/ml and incubated overnight at 4 °C with

thrombin for his-tag cleavage. At this point the protein was directly used for KtrAB complex assembly.

KtrAB complex was assembled by mixing individually purified KtrA and KtrB batches at a pre-defined ratio. The complex was concentrated and further purified by size exclusion chromatography in a Superdex-S200 column (GE Healthcare) in Buffer F (20 mM Tris-HCl pH 8.0; 120 mM NaCl; 30 mM KCl; 5 mM DTT; 1 mM DDM). For crystallization, 1mM DDM in Buffer F was replaced by 1.5 mM 6-cyclohexyl-1-hexyl- β -D-maltoside (cymal-6, anagrade from Anatrace).

Solubility Assay

For solubility assays, cells were lysed in Buffer G (50 mM Tris-HCl pH 7.5, 50 mM KCl, 0.5 mg/mL Lysozyme) supplemented with protease inhibitors, using a Branson sonifier. Sonication conditions used were: output power of 5, 50% duty cycle. Cells were sonicated for a total of 6 mins (15 seconds ON/OFF) for each sample. 1mL of sonicated sample was then incubated with 0.2 mg/mL DNase in gentle rotation for 30 minutes, at room temperature, and then centrifuged for 10 minutes at 11500 rpm. Supernatants and pellets (ressuspended in 1mL Buffer G) were run on a 15% SDS-PAGE gel.

Thermal Shift Assay

KtrA WT and respective mutants were purified as described in the Protein expression and purification section. Samples were prepared in 40 μ L reactions containing 1.5 μ M final concentration of protein, with 1mM of either ATP or ADP, and 6-fold dilution final concentration of SYPRO Orange Protein Gel Stain (Sigma-Aldrich) in Buffer C. In the assays performed with c-di-NMPs (Biolog) or cAMP (Sigma-Aldrich), the compounds were used at 250 μ M final concentration. The reactions were assembled in a 96-well PCR white plate (Bio-Rad) which was then sealed and covered in foil paper. The plate was centrifuged for 1min at 1500 rpm to ensure good mixing and the assay was performed using the iQ5 real-time PCR detection system (Bio-Rad) with the Cy3 optical filter for Sypro Orange detection (Ex: 490/Em: 575 nm). Temperature was varied in the 25 to 70 $^{\circ}$ C range, with an increment of 0.5 $^{\circ}$ C and a dwell time at each step of 30 seconds. Recorded data was analysed with the CFX Manager (Bio-Rad) and GraphPad Prism 5 softwares.

Isothermal Titration Calorimetry (ITC)

For ITC experiments, KtrA WT was purified as described in the Protein expression and purification section and then dialyzed overnight against Buffer H (50 mM HEPES pH 7.5; 150 mM KCl; 1mM TCEP; 1mM ATP or ADP).

Titration were performed using a VP-ITC calorimeter (MicroCal, LLC, Northampton, MA, USA) at 25 $^{\circ}$ C. In the sample cell KtrA WT was present at 30 μ M with 1mM of ADP or ATP. Cyclic di-AMP (c-di-AMP) was loaded into the injection syringe at 300 μ M. All samples were centrifuged to remove possible precipitates and thoroughly degassed.

In all titration runs, the first injection had a volume of 2 μ L and was followed by injections of 10 μ L volume. Consecutive injections were separated by 250 seconds to allow the peak to return to baseline levels. Heats of dilution were measured in a titration of c-di-AMP into buffer alone and subtracted from the titration heats to yield the heats of interaction. Data was analyzed with a single binding site model using Origin 7.0 (Origin Lab Corp., Northampton, MA, USA) software.

Crystallization

Crystallization screens were carried out at room temperature using the sitting-drop vapour diffusion method. Experiments were performed in 24 (Cryschem, Hampton Research) or 96-well (MRC 2, Swisisci, Hampton Research) plates, sealed with high performance crystal clear tape (Duck) and stored at 22 °C. Sitting drops were assembled by mixing protein solution and reservoir buffer in a 1:1 ratio for a final 2 µL volume drop. For 96-well plates, 75 µL of crystallization solution was placed in the reservoir and for 24-well plates, 500 µL were used.

KtrA CysLHDN-ATP

Protein was prepared as described in the Protein expression and purification section and concentrated to 10 mg/mL. Initial screening was performed in 96-well plates using commercial crystallization screens: Morpheus (Molecular Dimensions) and JCSG (Molecular Dimensions) with a total of 96 different buffer conditions each.

The crystal forming condition with the better looking crystals (0.1M MES pH 6.5; 1.6M MgSO₄) was then refined by using a 24-well plate with the reservoir composition shown below, in order to obtain a good amount of crystals for X-ray analysis:

Table 7 - Reservoir composition of the 24-well plate used to refine the best crystals of KtrA CysLHDN-ATP.

MES pH6.5/MgSO ₄	MgSO ₄ 1.3 - 1.8 (M)					
MES pH 6.5 0.100 - 0.150 (M)	0.100/ 1.3	0.100/ 1.4	0.100/ 1.5	0.100/ 1.6	0.100/ 1.7	0.100/ 1.8
	0.150/ 1.3	0.150/ 1.4	0.150/ 1.5	0.150/ 1.6	0.150/ 1.7	0.150/ 1.8
	0.100/ 1.3	0.100/ 1.4	0.100/ 1.5	0.100/ 1.6	0.100/ 1.7	0.100/ 1.8
	0.150/ 1.3	0.150/ 1.4	0.150/ 1.5	0.150/ 1.6	0.150/ 1.7	0.150/ 1.8

Crystals were collected from the drop and immersed in a cryoprotectant solution with the following composition: 0.1M MES pH 6.5; 1.6M MgSO₄; 0.5 mM ATP; 30% Glycerol. The crystals were then flash-frozen in liquid nitrogen and analysed by X-ray diffraction.

KtrB WT+ KtrA CysLHDN-ATP complex

Initial Screen

KtrB WT and KtrA mutant CysLHDN-ATP were purified as described in the Protein expression and purification section and mixed at a 50:105 (w:w) KtrA to KtrB ratio. The complex was then purified by size exclusion chromatography and concentrated to 7 mg/mL. Initial screening was based in the best crystallization condition already obtained for the KtrAB WT complex bound to ATP: 0.1M ADA pH6.5; 20% PEG 400; 0.2M AMS and performed in two 24-well plates (tables 8 and 9) with the following reservoir composition (all wells contain 0.1M ADA pH 6.5):

Table 8- Reservoir composition of the first 24-well plate used to obtain crystallization conditions of the KtrB WT+KtrA CysLHDN-ATP complex.

AMS/PEG 400	PEG 400 17 – 22 % (v/v)					
AMS 0.150 - 0.220 (M)	0.150/ 17	0.150/ 18	0.150/ 19	0.150/ 20	0.150/ 21	0.150/ 22
	0.180/ 17	0.180/ 18	0.180/ 19	0.180/ 20	0.180/ 21	0.180/ 22
	0.200/ 17	0.200/ 18	0.200/ 19	0.200/ 20	0.200/ 21	0.200/ 22
	0.220/ 17	0.220/ 18	0.220/ 19	0.220/ 20	0.220/ 21	0.220/ 22

Table 9 - Reservoir composition of the second 24-well plate used to obtain crystallization conditions of the KtrB WT+KtrA CysLHDN-ATP complex.

AMS/PEG 400	PEG 400 17 – 22 % (v/v)					
AMS 0.180 – 0.210 (M)	0.180/ 17	0.180/ 18	0.180/ 19	0.180/ 20	0.180/ 21	0.180/ 22
	0.190/ 17	0.190/ 18	0.190/ 19	0.190/ 20	0.190/ 21	0.190/ 22
	0.200/ 17	0.200/ 18	0.200/ 19	0.200/ 20	0.200/ 21	0.200/ 22
	0.210/ 17	0.210/ 18	0.210/ 19	0.210/ 20	0.210/ 21	0.210/ 22

Crystals were collected from the drop and immersed in a cryoprotectant solution with the following composition: 0.1M ADA pH 6.5; 45% PEG 400; 0.2M AMS; 1.5 mM cymal-6 and 0.5 mM ATP. The crystals were then flash-frozen in liquid nitrogen and analysed by X-ray diffraction.

HILIDE Screen

KtrB WT and KtrA mutant CysLHDN-ATP were purified as described in the Protein expression and purification section and mixed at a 50:105 (w:w) KtrA to KtrB ratio. The complex was then purified by size exclusion chromatography and concentrated to 9 mg/mL.

Nine samples with different lipid/detergent concentration ratios were prepared for the screen according to table 10:

Table 10 –Lipid/detergent ratios used for the HILIDE Screen.

Cymal-6/ DOPC	0 ug	150 ug	300 ug
1.5 mM	0 uL (Cymal-6) 0 uL (DOPC)	0 uL (Cymal-6) 150 uL(DOPC)	0 uL (Cymal-6) 300uL (DOPC)
15 mM	6.75 uL(Cymal-6) 0 uL (DOPC)	6.75 uL (Cymal-6) 150 uL(DOPC)	6.75 uL (Cymal-6) 300 uL(DOPC)
30 mM	14.25 uL(Cymal-6) 0 uL (DOPC)	14.25 uL(Cymal-6) 150 uL(DOPC)	14.25 uL (Cymal-6) 300 uL(DOPC)

Re-lipidation:

57 μ L of concentrated protein was added to nine glass tubes pre-treated with a thin film of the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) from Avanti. The film was prepared by placing DOPC, dissolved at 1 mg/mL in chloroform, corresponding to 0, 150 and 300 μ g, into three glass tubes for each lipid concentration. The chloroform was then evaporated using an argon gas stream at room temperature.

Lipidated protein samples were supplemented with 1.5, 15 or 30 mM of the detergent 6-cyclohexyl-1-hexyl- β -D-maltoside (cymal-6, anagrade from Anatrache) from a 100mM stock.

The nine resulting sample tubes were then covered with parafilm and incubated for 16 h at 4°C with agitation at 300 rpm. Afterwards, samples were centrifuged for 15 minutes at 25 psi in an airfuge; resulting supernatants were used for crystallization.

Each different sample was incubated in a 24-well plate with the reservoir composition shown in Table 8. In this case, 1.2 μ L of protein solution was mixed with 1 μ L of reservoir buffer to assemble the drop.

Additive Screen

KtrB WT and KtrA mutant CysLHDN-ATP were purified as described in the Protein expression and purification section and mixed at a 50:105 (w:w) KtrA to KtrB ratio. The formed complex was then purified by size exclusion chromatography and concentrated to 7 mg/mL.

The screen was carried out using 24-well plates with the reservoir composition shown in Table 8. The drop (1 μ L protein solution + 1 μ L reservoir buffer) was supplemented with 0.25 μ L of additive solution, from the additive screen Hampton Research HR2-428 for a total of 96 different additive conditions.

KtrB WT+ KtrA CysLHDN-ADP complex

KtrB WT and KtrA mutant CysLHDN-ADP were purified as described in the Protein expression and purification section and mixed at a 50:105 (w:w) KtrA to KtrB ratio. The complex was then purified by size exclusion chromatography and concentrated to 7 mg/mL.

The crystallization screening was performed in 24-well plates using a handmade screen previously developed in the laboratory, named SB screen. This screen contains 480 different conditions. The screen composition is listed in the Appendix.

KtrA WT-ADP-c-di-AMP

Protein was prepared as described in the Protein expression and purification section, concentrated to 10 mg/mL and supplemented with c-di-AMP at 300 μ M. Initial screening was performed in 96-well plates using commercial crystallization screens: Morpheus (Molecular Dimensions), JCSG (Molecular Dimensions) and Wizard (Emerald BioSystems) with a total of 96 different buffer conditions each. Additionally, a handmade screen developed by us in the laboratory with 672 conditions was also tested. The screen composition is detailed in the Appendix.

Conditions with crystals were refined by using 24-well plates with the reservoir composition shown in the tables below:

Table 11 - Reservoir composition of the first 24-well plate used to refine the crystallization conditions of KtrA WT-ADP with c-di-AMP. Three plates were made with wells containing 0.1M Sodium citrate pH 5.6 and either 0, 10 or 20% Glycerol.

PEG 4000 / KCl	KCl 50 – 225 (mM)					
PEG 4000 7 – 10 % (w/v)	7%/ 50	7%/ 100	7%/ 150	7%/ 175	7%/ 200	7%/ 225
	8%/ 50	8%/ 100	8%/ 150	8%/ 175	8%/ 200	8%/ 225
	9%/ 50	9%/ 100	9%/ 150	9%/ 175	9%/ 200	9%/ 225
	10%/ 50	10%/ 100	10%/ 150	10%/ 175	10%/ 200	10%/ 225

Table 12 - Reservoir composition of the second 24-well plate used to refine the crystallization conditions of KtrA WT-ADP with c-di-AMP. Three plates were made with wells containing 0.1M ADA pH 6.5 and either 0, 10 or 20% Glycerol.

PEG 4000 / KCl	KCl 50 – 225 (mM)					
PEG 4000 7 – 10 % (w/v)	7%/ 50	7%/ 100	7%/ 150	7%/ 175	7%/ 200	7%/ 225
	8%/ 50	8%/ 100	8%/ 150	8%/ 175	8%/ 200	8%/ 225
	9%/ 50	9%/ 100	9%/ 150	9%/ 175	9%/ 200	9%/ 225
	10%/ 50	10%/ 100	10%/ 150	10%/ 175	10%/ 200	10%/ 225

Table 13 - Reservoir composition of the third 24-well plate used to refine the crystallization conditions of KtrA WT-ADP with c-di-AMP. Three plates were made with wells containing 0.1M ADA pH 6.5.

Glycerol / PEG 4000	PEG 4000 6 – 11 % (w/v)					
Glycerol 5 - 20 % (v/v)	5%/ 6%	5%/ 7%	5%/ 8%	5%/ 9%	5%/ 10%	5%/ 11%
	10%/ 6%	10%/ 7%	10%/ 8%	10%/ 9%	10%/ 10%	10%/ 11%
	15%/ 6%	15%/ 7%	15%/ 8%	15%/ 9%	15%/ 10%	15%/ 11%
	20%/ 6%	20%/ 7%	20%/ 8%	20%/ 9%	20%/ 10%	20%/ 11%

Crystals were collected from the drop and immersed in a cryoprotectant solution with the following composition: 10% PEG 4000, 0.1M ADA pH 6.5, 0.5 mM ADP, 150 uM c-di-AMP and 30% Glycerol.

The crystals were then flash-frozen in liquid nitrogen and analysed by X-ray diffraction.

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Appendix

Table 14 – Handmade Crystallization Screen Composition.

Conditions: 1- 48		Precipitant: PEG 2000		Salt: Lithium sulphate 0.2M	
PEG / pH		pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5	
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5	
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5	
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5	
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5	
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5	
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5	
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5	
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5	
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5	
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5	
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5	
Conditions: 49- 96		Precipitant: PEG 4000		Salt: Lithium sulphate 0.2M	
PEG / pH		pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5	
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5	
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5	
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5	
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5	
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5	
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5	
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5	
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5	
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5	
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5	
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5	

Conditions: 97- 144 Precipitant: PEG 2000 Salt: Ammonium sulphate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 145- 192 Precipitant: PEG 4000 Salt: Ammonium sulphate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 192-240 Precipitant: PEG 2000 Salt: Ammonium formate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5

Conditions: 241- 288 Precipitant: PEG 4000 Salt: Ammonium formate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 289- 336 Precipitant: PEG 2000 Salt: Potassium-sodium tartrate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 337- 384 Precipitant: PEG 4000 Salt: Potassium-sodium tartrate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5

Conditions: 385- 432 Precipitant: PEG 2000 Salt: Calcium chloride 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 433- 480 Precipitant: PEG 4000 Salt: Calcium chloride 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 481- 528 Precipitant: PEG 2000 Salt: Magnesium acetate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5

Conditions: 529- 576 Precipitant: PEG 4000 Salt: Magnesium acetate 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 577- 624 Precipitant: PEG 2000 Salt: Potassium chloride 0.2M				
PEG / pH	pH 5.6 – 8.5			
PEG 2000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5
Conditions: 625- 672 Precipitant: PEG 4000 Salt: Potassium chloride 0.2M				
PEG /pH	pH 5.6 – 8.5			
PEG 4000 6 – 17 % (w/v)	6%/ 5.6	6%/ 6.5	6%/ 7.5	6%/ 8.5
	7%/ 5.6	7%/ 6.5	7%/ 7.5	7%/ 8.5
	8%/ 5.6	8%/ 6.5	8%/ 7.5	8%/ 8.5
	9%/ 5.6	9%/ 6.5	9%/ 7.5	9%/ 8.5
	10%/ 5.6	10%/ 6.5	10%/ 7.5	10%/ 8.5
	11%/ 5.6	11%/ 6.5	11%/ 7.5	11%/ 8.5
	12%/ 5.6	12%/ 6.5	12%/ 7.5	12%/ 8.5
	13%/ 5.6	13%/ 6.5	13%/ 7.5	13%/ 8.5
	14%/ 5.6	14%/ 6.5	14%/ 7.5	14%/ 8.5
	15%/ 5.6	15%/ 6.5	15%/ 7.5	15%/ 8.5
	16%/ 5.6	16%/ 6.5	16%/ 7.5	16%/ 8.5
	17%/ 5.6	17%/ 6.5	17%/ 7.5	17%/ 8.5

SB screen composition:

Table 15 - SB Crystallization Screen Composition.

Conditions: 1-24		Precipitant: PEG400		Salt: Ammonium sulphate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 25-48		Precipitant: PEG4000		Salt: Ammonium sulphate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 49-72		Precipitant: PEG400		Salt: Calcium chloride 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 73-96		Precipitant: PEG4000		Salt: Calcium chloride 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5

Conditions: 97-120		Precipitant: PEG400		Salt: Lithium-sodium sulphate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 121-144		Precipitant: PEG4000		Salt: Lithium-sodium sulphate 0.2M		
PEG /pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 145-168		Precipitant: PEG400		Salt: Ammonium formate 1M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 169-192		Precipitant: PEG4000		Salt: Ammonium formate 1M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5

Conditions: 193-216		Precipitant: PEG400		Salt: Magnesium acetate 0.05M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 217-240		Precipitant: PEG4000		Salt: Magnesium acetate 0.05M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 241-264		Precipitant: PEG400		Salt: Sodium chloride 1M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 265-288		Precipitant: PEG4000		Salt: Sodium chloride 1M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 289-312		Precipitant: PEG400		Salt: Potassium phosphate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 313-336		Precipitant: PEG4000		Salt: Potassium phosphate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5

Conditions: 337-360		Precipitant: PEG400		Salt: Potassium chloride 0.5M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 361-384		Precipitant: PEG4000		Salt: Potassium chloride 0.5M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 385-408		Precipitant: PEG400		Salt: No salt		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 409-432		Precipitant: PEG4000		Salt: No salt		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
Conditions: 433-456		Precipitant: PEG400		Salt: Potassium-sodium tartrate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 400 15 – 30 % (v/v)	15%/ 4.6	15%/ 5.5	15%/ 6.5	15%/ 7.5	15%/ 8.5	15%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5
	25%/ 4.6	25%/ 5.5	25%/ 6.5	25%/ 7.5	25%/ 8.5	25%/ 9.5
	30%/ 4.6	30%/ 5.5	30%/ 6.5	30%/ 7.5	30%/ 8.5	30%/ 9.5
Conditions: 453-480		Precipitant: PEG4000		Salt: Potassium-sodium tartrate 0.2M		
PEG / pH	pH 4.6 – 9.5					
PEG 4000 2 – 20 % (w/v)	2%/ 4.6	2%/ 5.5	2%/ 6.5	2%/ 7.5	2%/ 8.5	2%/ 9.5
	5%/ 4.6	5%/ 5.5	5%/ 6.5	5%/ 7.5	5%/ 8.5	5%/ 9.5
	10%/ 4.6	10%/ 5.5	10%/ 6.5	10%/ 7.5	10%/ 8.5	10%/ 9.5
	20%/ 4.6	20%/ 5.5	20%/ 6.5	20%/ 7.5	20%/ 8.5	20%/ 9.5