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# DISSERTATION

Titel der Dissertation

„The development of strategies to facilitate the  
crystallization of the prokaryotic peptide transporter  
YdgR“

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt: A 091 490

Dissertationsgebiet lt. Studienblatt: Molekulare Biologie

Betreuerin / Betreuer: PhD Peggy Stolt-Bergner



# Acknowledgements

I had an amazing time during the last years and that was due to a lot of great people.

I want to thank my supervisor Peggy for the chance to do my PhD in her lab and her support throughout the last years. She was open for all my ideas and fruitful scientific discussions have deepened my general understanding. I want to thank her sincerely for her guidance during the last years.

I am grateful to Kristina Djinovic-Carugo and Klaas Martinus Pos for the time they took to read and evaluate my thesis. Additionally I want to thank Tim Clausen and Thomas Marlovits for their helpful discussions as members of my PhD committee.

I want to thank the members of the Stolt-Bergner Lab and the Clausen Lab for their help and support. Especially Eli and Hongwen have contributed to my work.

I am grateful to the members of the Djinovic-Carugo lab, the Konrat lab and the Marlovits lab that use different methods for structure determination and helped me to see my project from different perspectives. Additionally I want to thank the MFPL antibody facility for doing a great job.

I want to thank my parents, my sisters Karin and Monika and my brother Karl who have supported me throughout my life. I am convinced that strong roots are necessary for all challenges in life especially when the outcome is not that clear. I also want to thank my small niece Juliane for bringing a lot of sunshine in my life.

I also found a lot of good friends and hopefully they will also be part of the next phase of my life. A big hug to Bruno, Romana, Eli, Juliane K., Anita, Juliane T., Julia and Lisa.



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# 1 Zusammenfassung

Um den Mechanismus der Substrattranslokation von Transportern durch die Membran zu verstehen, sind Studien betreffend deren Struktur und die Funktion unerlässlich. Unser Labor beschäftigt sich seit längerem mit einem prokaryotischen Mitglied der Peptid Transporter (PTR) Familie von Symportern. Diese Familie ist für den Transport von Di- und Tripeptiden in die Zelle verantwortlich, transportiert aber auch Medikamente und peptid-ähnliche Stoffe. Die Strukturbestimmung von Membranproteinen ist ein schwieriges Unterfangen, deshalb wurden zwei Strategien verfolgt, um die Struktur von YdgR zu lösen.

Es wurde bereits gezeigt, dass Co-Kristallisation von Membranproteinen mit monoklonalen Antikörperfragmenten (mFab) in gut streuenden Proteinkristallen resultieren können [42]. Die Detergentmizelle schirmt den Grossteil des solubilisierten Membranproteins ab, was zur Beschränkung von zugänglichen Bereichen führt, die Kristallkontakte formen könnten. Im Komplex mit mFab Fragmenten werden diese Bereiche vergrößert und erhöhen die Wahrscheinlichkeit von gut geordneten Proteinkristallen. Deshalb wurden zwei konformationspezifische Antikörper gegen YdgR generiert, um sie für Co-Kristallisation zu nutzen. Die resultierenden Kristalle streuten bis 8-10 Å.

Ausserdem wurde gezeigt, dass die erhöhte Thermostabilität von Proteinen die Wahrscheinlichkeit für gut streuende Kristalle verbessert [52]. Nach der Mutagenese von YdgR [35] wurden die Mutanten mit einer „high-throughput“ Methode auf ihre Thermostabilität getestet. Zwei Mutanten, deren Thermostabilität um wenigstens 3°C erhöht war, konnten identifiziert werden. Ein weiter Assay, der auf der Fraktion von gefalteten Protein nach einer Inkubation auf 50°C basiert, konnte zeigen, dass sowohl die Einzelmутanten als auch die Doppelmutante erhöhte Thermostabilität zeigen und ihre Toleranz für raue Detergentien wie LDAO erhöht ist. Unglücklicherweise formten die Mutanten keine Kristalle. Der Vergleich der Mutanten mit dem PTR-Transporter  $\text{PepT}_{\text{so}}$ , dessen Struktur bereits gelöst ist [39], zeigte, dass selbst nach der Selektion von thermostabilen YdgR Mutanten aus *E. coli*, diese weniger thermostabil sind als das Homolog  $\text{PepT}_{\text{so}}$  aus *Shewanella oneidensis*.

Interessanterweise kann einer der Antikörper nur mit dem N-terminal His-tag nativen YdgR interagieren, während der zweite mAb auch mit allen Mutanten unabhängig von der Orientierung des His- tags interagieren kann. Wahrscheinlich können die thermostabilen Mutanten nur eine Konformation annehmen, in der das Antigen des ersten mAb nicht zugänglich ist. Um dies Annahme zu überprüfen, wurde die zugänglichen Lysine im nativen Protein und in den Mutanten acetyliert und das Acetylierungsmuster mit Massenspektrometrie analysiert. Die thermostabilen Mutanten scheinen hauptsächlich die „in-ward facing“ Konformation anzunehmen.

## 2 Abstract

Structural and functional studies on transmembrane transporters are crucial to understanding mechanisms of substrate translocation. Our lab has begun structural studies on YdgR, a prokaryotic member of the peptide transporter (PTR) family of symporters. This transporter family takes up di-/tri-peptides, as well as many drugs and peptidomimetics. As membrane protein crystallization is inherently difficult, two strategies were used to approach this problem.

First, it has been shown that co-crystallization of monoclonal antibody (mFab) fragments with membrane proteins can give rise to well diffracting crystals [42]. Due to the detergent micelle surrounding the protein, membrane proteins have restricted accessible areas that are able to form crystal contacts. When in complex with mFab fragments, the accessible surface area is increased and supports the formation of crystal contacts. Therefore two conformationally specific monoclonal antibodies against YdgR were produced to use as tools to improve crystallization. Co-crystallization of the target protein YdgR with the proteolytically generated mFab fragments gave rise to crystals that diffracted to 8-10 Å.

Second, improvement of protein thermostability has been shown to increase the probability of yielding crystals of membrane proteins [52]. Random mutagenesis of YdgR was carried out [35] and testing for thermostability of the target protein was performed in a high-throughput manner to identify mutants that are more thermostable and therefore may have a higher propensity to crystallize. Two mutants with an increase in thermostability of at least 3° C were identified. An additional thermostability screen, based on gel filtration analysis of the fraction of folded protein remaining after incubation at 50°C, revealed that the single mutants as well as a double mutant show increased thermostability and increased tolerance to the harsh detergent LDAO. Unfortunately the mutants did not give rise to crystals. Comparison with the PTR transporter PepT<sub>so</sub>, whose structure has been solved [39], shows that even after selection for thermostability the mutants of YdgR from *E.coli* are less thermostable than the homologue PepT<sub>so</sub> from *Shewanella oneidensis*.

Most interesting, one of the generated mAb is only able to interact with N-terminally His-tagged native YdgR while the second mAb interacts with the native protein as well as with the mutants, independent of the location of the His-tag. This suggests that the thermostable mutants are locked in one conformation, in which the antigen recognized by the first mAb is not exposed. Acetylation of accessible lysine residues coupled with analysis via mass spectrometry indicates that the thermostable mutants S166G and N196K may be locked in the inward facing conformation. Additional experiments to support this conclusion are underway.

## 3 Introduction

### 3.1 Transport across membranes

The barrier function of membranes is crucial for life since the membrane-enclosed compartments allow for the build-up of differences in solute concentrations that are necessary for driving metabolic processes. These differences form the basis for the intra- or extra-cellular gradient or gradients. Small hydrophobic molecules ( $O_2$ ,  $CO_2$ ,  $N_2$ ...) can freely traverse the membrane while larger molecules and especially charged molecules and ions have to be transported across this barrier in a passive or active manner depending on the physiological needs of the cell. Controlled signal transfer and exchange of solutes with the environment and between cell compartments can be achieved by a wide variety of membrane proteins that have evolved to serve different tasks, such as exchange of nutrients and metabolic waste products or ions necessary for the cell's membrane potential (Figure 3.1).

Channels and carrier proteins (also known as carriers or permeases) allow the transport of solutes that are normally not able to cross the membrane due to their size or charge. Channels and many carrier proteins facilitate transport “downhill”, or in the direction of the solute gradient, and therefore no energy input has to be used to feed this passive transport. Channels can be open or closed and serve as selective filters across the membrane.

On the other hand there is also a variety of active transporters that use energy either in the form of ATP, from coupling with gradients, from redox processes in mitochondria, or from light for transport [2].

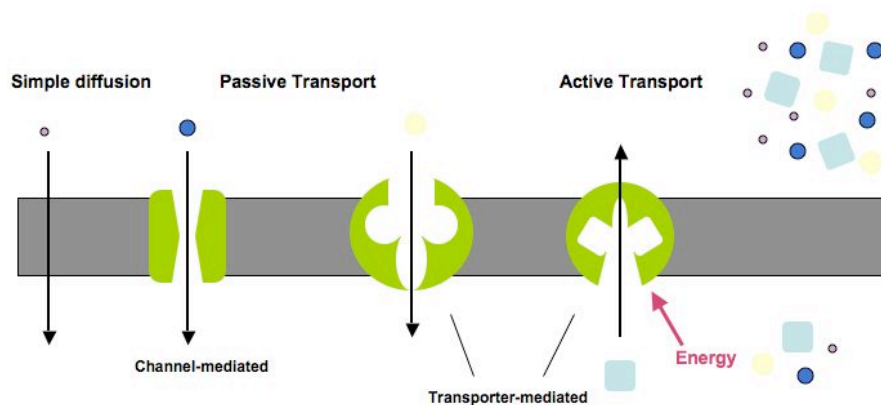


Figure 3.1: Schematic figure of passive and active transport

### 3.2 Primary active transporters

For active transport energy is needed to transport substrate across the membrane against the substrate gradient in an “uphill” manner. These transporters are also known as primary active transporters. [37] Depending on the source of energy used different forms of transporters can be distinguished.

Some transporters, such as bacteriorhodopsin in archae use energy in the form of light for proton transport, but ATP-driven transport is widely used in all organisms.

Two major groups of transporters use ATP-driven transport, namely ATP-binding cassette (ABC) transporters and P-type ATPases. Vacuolar H<sup>+</sup> ATPases (V-type ATPases) also use ATP to pump protons across membranes.

ABC-transporters consist of two transmembrane domains (TMD) and two cytoplasmic nucleotide-binding domains (NBD) (Figure 3.2b). While the TMDs form a passage-way through the membrane, the NBDs can bind and hydrolyze ATP. The NBDs come close together and form two ATP binding sites when ATP is available, but undergo a conformational change when ATP is hydrolyzed to ADP. This conformational change due to ATP hydrolysis is transferred to the TMDs via connecting helices that seem to underlie a conserved architecture. [21] Since ABC transporters are linked to hereditary diseases, such as cystic fibrosis, and also play a role in extrusion of chemotherapeutic drugs, they are of high interest what is reflected by the high number of solved crystal structures [21].

Prominent members of the group of the P-type ATPases are the Sodium-Potassium pump that is crucial for establishing the gradient of sodium and potassium ions across the cell membrane and the Ca<sup>2+</sup> pump, which together consume one third of the energy used in humans [41]. These pumps consist of three cytosolic domains: N (nucleotide binding), P (phosphorylation) and A (actuator) together with a transmembrane domain (TMD) varying in the number of transmembrane helices depending on the subtype (Figure 3.2a). The phosphorylation and dephosphorylation of the P domain leads to a major rotation of the A domain, which is transferred to the TMD that provides the opening and closing of a transport cavity [37].

Many primary active transporters use ATP to set up a gradient of ion and/or solute gradients whose free energy can be used by secondary active transporters.

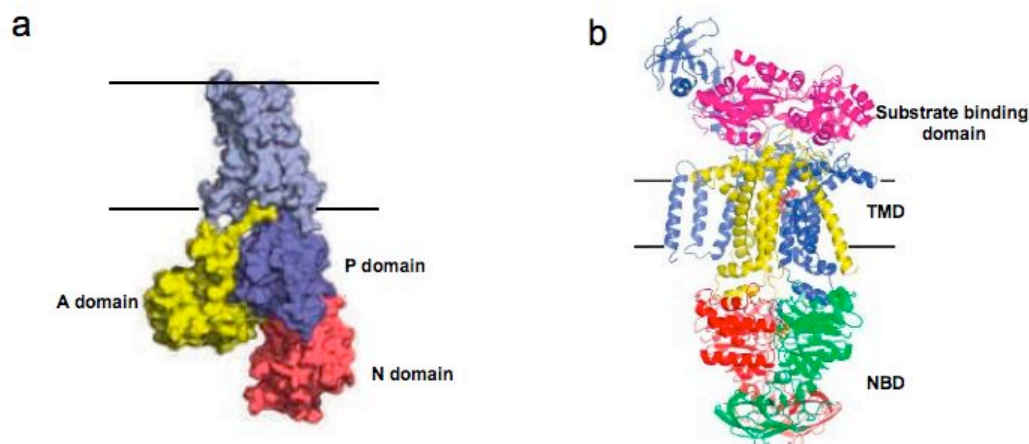


Figure 3.2: Schematic drawing of a. the Na/K pump and b. the maltose ABC transporter; modified from Oldham et al. 2007 and Morth, Pedersen et al. 2011

### 3.3 Secondary active transporters

Secondary active transporters are widely spread through all kingdoms of life. They transport a wide variety of compounds. Accumulation of the transported substrate is achieved by coupling the transport with co-transport of another ion or solute down its concentration gradient. In that way the free energy of another gradient is used to transport the target substrate.

Secondary active transporters can act as symporters or antiporters. Symporters translocate two or more substrates in the same direction, making use of the gradient of one substrate, while antiporters transport two or more substrates in opposite directions [30]. The general model of secondary active transport is based on the alternating access hypothesis. The transporter undergoes conformational changes that provide alternating pathways on either side of the molecule. These conformations include at least an inward-facing and an outward-facing, and a transition state (Figure 3.3), which provides no pathway to either side [21].

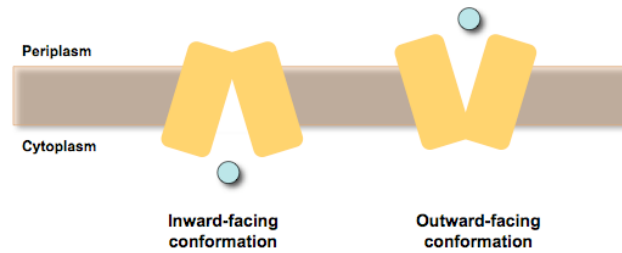


Figure 3.3: Alternating access model (transporter in yellow and substrate in blue)

The secondary active transporters seem to be based on a common transport mechanism since similar features in transmembrane helix packing can be observed (Figure 3.4). The structures of a growing number of secondary active transporters have been solved in the last years. Nearly all of them have inverted repeats that are parallel or anti-parallel, which seems to be a common feature [14; 47; 62; 63]. In all known structures so far, helix bundles are related by a pseudo-two-fold axis located in the plane of the membrane despite the lack of detectable sequence conservation [63].

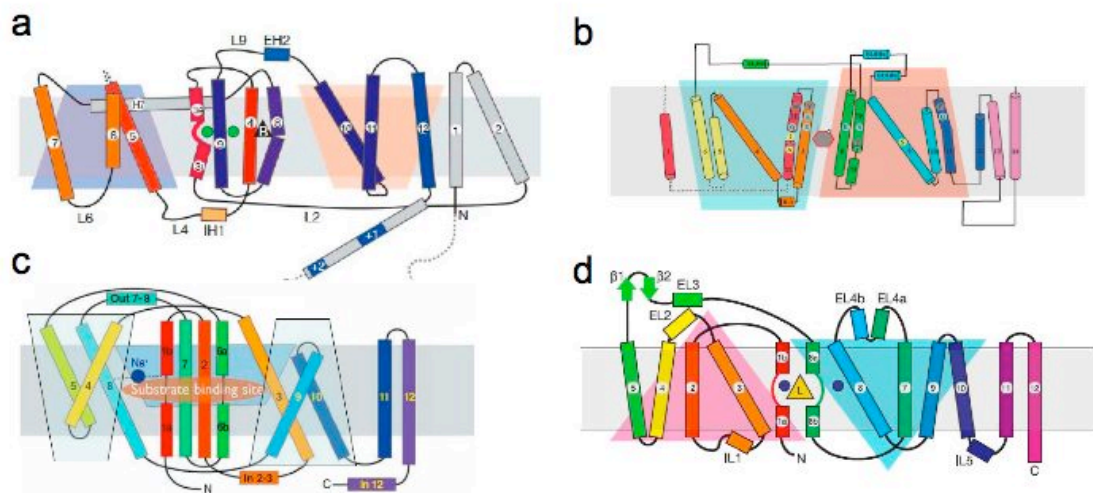


Figure 3.4: Inverted repeats with pseudo-2-fold axis indicated in coloured triangles or trapezoids; Topology of a. BetP b. vSGLT c. Mhp1 d. LeuT; Figure modified from Ressler et al. 2009; Faham et al 2008; Weyand et al. 2008 and Yamashita et al. 2005

### 3.4 Comparison of Sodium-coupled secondary active transporter structures

Unfortunately most structures of individual secondary active transporters are only available in the inward-facing, outward-facing or the substrate occluded-state. To

learn more about the transport mechanism structures of different proteins must be compared. The structures of several sodium-coupled secondary transporters are available. The comparison of the structure of outward-facing LeuT<sub>Aa</sub> [63], outward-facing Mhp1 [62], inward-facing vSGLT [14] and BetP in an inward-facing occluded state [46] proposes a mechanism of transport. The rotational movement of a central four-helix bundle occludes the substrate-binding site to change from an outward-facing conformation to an occluded state. The helices in the scaffold surrounding the four helix-bundle straighten and open a passageway for the substrate resulting in an inward-facing conformation. Therefore the conformational changes necessary for transport can be described as concerted movements of the four-helix bundle and the surrounding scaffold [46]. Most interesting, conserved glycine residues are located close to the pivot points of movement and seem to provide the necessary flexibility [46].

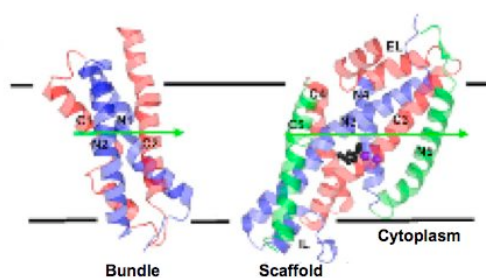


Figure 3.5: Bundle and scaffold (Pseudo-2-fold axis as green arrows); modified from Forrest and Kramer et al. 2010

The only secondary active transporter whose structure in both the inward and the outward-facing conformation is available is the sodium/aspartate symporter Glt<sub>Ph</sub> from *Pyrococcus horikoshii* [47]. The outward-facing structure of Glt<sub>Ph</sub> was already solved [64], while cysteine crosslinking was applied to solve the structure of Glt<sub>Ph</sub> locked in an inward-facing conformation.

Comparison of the inward and the outward-facing conformation proposes a mechanism of transport. Glt<sub>Ph</sub> forms a trimer and therefore some parts of the protein make intermolecular contact while another part forms a relatively rigid transport domain. Conversion of the outward- to the inward-facing conformation involves movement and rotation of the transport domain towards the peripheral trimerization scaffolding helices. This movement is permitted by the partial unwinding and

refolding of helices connecting the transport domain with the scaffold domain. This is facilitated by conserved glycine residues, which allow the unfolding/refolding events and may serve as hinges. The gating mechanism is based on the movement of two hairpin helices (HP1 and HP2) that close the intra- or extracellular gate [47].

Therefore the rotational movement of a flexible domain against a stable scaffold seems to be a general feature of the transport mechanism of secondary active sodium driven transporters.

In general, discontinuous helices and hairpin helices can be found in many secondary active transporters as well as in ion pumps [49]. These motifs seem to be connected with the ion translocation function, and ensure that the ion binding site is deeply buried in the non-polar membrane core. Ion desolvation is therefore necessary and ensures high selectivity of binding [49].

### **3.5 Proton- driven secondary active transporters**

Most secondary active transporters are coupled to a proton or sodium gradient [56]. In comparison with sodium-coupled secondary active transporters only a few structures of proton-driven transporters have been solved. One of the solved structures is the structure of the well-characterized lactose permease LacY.

#### **3.5.1 LacY**

LacY belongs to the major facilitator superfamily (MFS). It carries out the coupled transport of a galactoside with a proton. LacY is selective for disaccharides containing a D-galactopyranoyl ring as well as D-galactose, but has no affinity for D-glucopyranoside or D-glucose [19]. The transport mechanism was extensively studied with different methods, but unique insight was gained when the structure of the LacY mutant C154G was solved in the inward-facing conformation [1]. The structure of native LacY has also been solved and shows the same inward-facing conformation [20]. LacY consists of 12 transmembrane helices that form an N- and a C-terminal six-helix bundle positioned by a pseudo-2-fold symmetry axis running perpendicular to the membrane, connected with a long loop between helix VI and VII [1] (Figure

3.6). In the structure LacY C154G, a lactose analogue,  $\beta$ -D-galactopyranosyl-1-thio- $\beta$ -D-galactopyranoside (TDG), is bound to the transporter [1]. This revealed the residues involved in substrate binding (Figure 3.6).

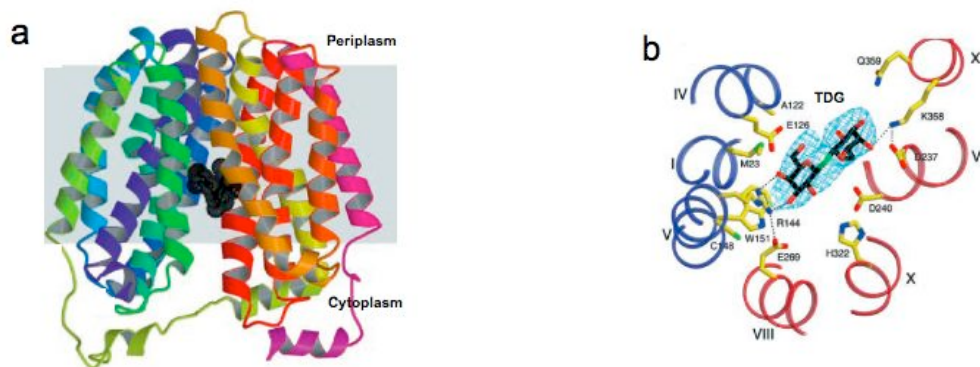


Figure 3.6: a. LacY structure with N-terminal six-helix bundle (blue and green) and C-terminal six-helix bundle (in yellow and red); b Substrate binding site in LacY; both modified from Abramson et al. 2003

Arg-144 interacts with the O<sub>3</sub> and O<sub>4</sub> atoms of the galactopyranosyl-ring via a bidentate hydrogen bond. Glu-126 may interact with the O<sub>4</sub>, O<sub>5</sub>, O<sub>6</sub> of the galactopyranoside ring via water molecules. The indole ring of Trp-151 interacts with the bottom of the galactopyranosyl ring. Glu-269 forms a salt bridge with Arg-144 [1]. A model for transport was proposed that is based on biochemical data and modeling of the outward-facing conformation by a rotation of the N-and the C-terminal helix bundle [1; 19] (Figure 3.7).

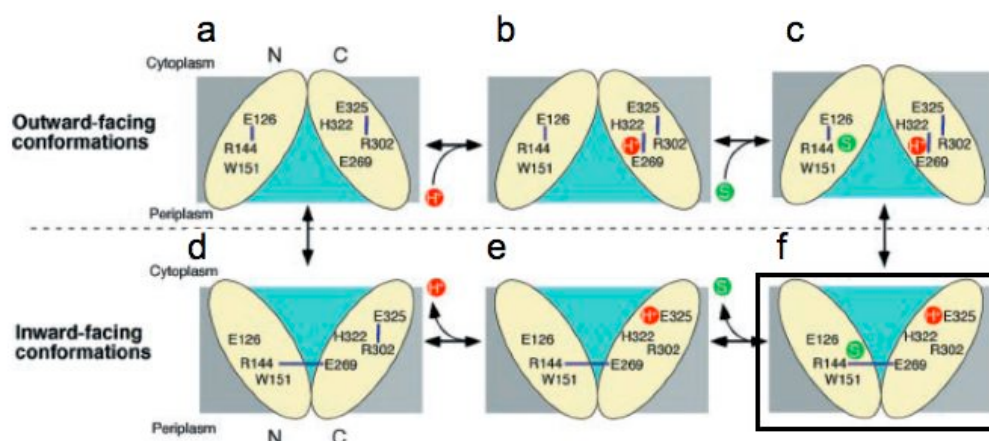


Figure 3.7: Proposed transport mechanism of LacY modified from Abramson et al. 2003; Conformation of the solved structure is marked with a rectangle.

The outward-facing form is protonated and the proton is shared between Glu-269 and His-322. A galactoside is recognized by Trp-151, Arg-144 and Glu-126, which disrupts the salt-bridge between Arg-144 and Glu-126 and brings His-322 in contact with Glu-325. Glu-269 is recruited to the binding site and forms a salt-bridge with Arg-144. This process may cause the rapid transition to the inward-facing conformation [19]. After the release of the substrate, the salt-bridge between Arg-144 and Glu-126 is formed again. The proton is released from Glu-325.

### **3.5.2 Comparison of LacY with FucP and GlpT**

Two other transporter structures belonging to the MFS family are available. The structure of the *E. coli* Glycerol-3-phosphate transporter GlpT was solved in 2003 [22] while the structure of the *E. coli* fucose/H<sup>+</sup> symporter FucP was solved recently [8].

Based on the structure of LacY in an inward-facing conformation and GlpT in an inward-facing conformation, a general mechanism for the transport mechanism of MFS family was proposed. The binding site has access to each side of the membrane in an alternating fashion based on the N-terminal and C-terminal helix bundles moving back and forth. This model is called the rocker-switch mechanism. Unlike the transport mechanism for the sodium-coupled transporters (see chapter 3.4), these transporters do not form a passageway for transport but rather regulate access of the substrate-binding site to the cytoplasm or the periplasm.

Biochemical data suggest that the formation and breakage of intra and intermolecular salt-bridges are essential for the control of helical movements in GlpT that are necessary for the inter-conversion of inward- and outward-facing conformations [29]. This formation and breakage of salt-bridges can also be seen in the substrate binding site in LacY [1]. The structure of FucP was solved in the outward-facing conformation. Again the N- and C-terminal helix bundles show a pseudosymmetrical 2-fold axis connected by a long flexible linker. The comparison of LacY and FucP suggests that rigid body rotations are the basis for the interconversion of the inward and the outward-facing conformation [8], supporting the rocker switch mechanism model (Figure 3.8).

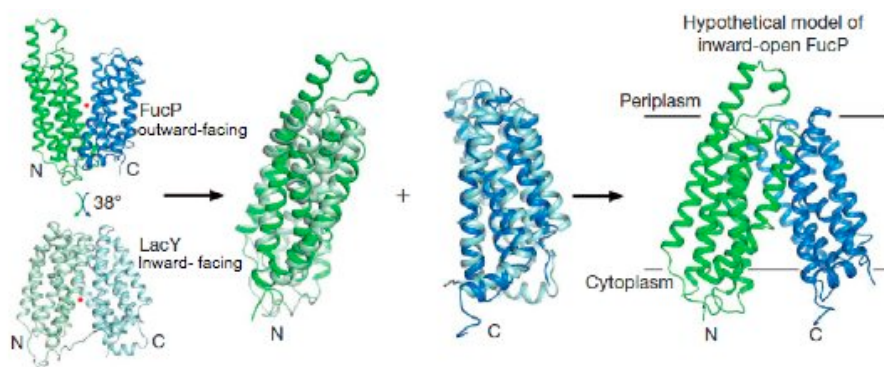


Figure 3.8: Model of inward facing FucP derived from rigid body rotation; modified from Dang, Sun et al 2010

### 3.5.3 Peptide Transporter (PTR) family

While in prokaryotes and simple eukaryotic cells transport processes are mainly driven by proton gradients, in higher eukaryotes the major driving force comes from sodium gradients [9]. Nevertheless some transport processes in higher eukaryotes are still dependent on proton force [9].

A representative proton carrier system is the Peptide Transporter (PTR) family, which transports di- and tri-peptides into the cell. The PTR family belongs to the major facilitator superfamily (MFS), together with many other proton-coupled transporters [7].

This proton-dependent group of peptide transporters can be found in organisms ranging from bacteria to humans. In mammals, two proteins of the PEPT series can be found that transport exclusively di- and tri-peptides [9]. The mammalian intestinal transporter is called PEPT1 while the renal isoform is known as PEPT2.

Although peptide transporter homologues differ greatly in sequence and size small protein stretches are highly conserved [9]. These conserved regions are called PTR motifs and two are located on the outward facing end of the first transmembrane domain (TMD) and between TMD2 and 3 respectively (Figure 3.9). The third motif in TMD5 shows absolute conservation among all members of the PTR family [9]. For PepT1 and PepT2 analysis of single point mutations suggests that TMD2 to 5 and 7 play an important role in substrate affinity and the transport mechanism [9; 26; 27] It

was also shown that a single mutation of tyrosine 167 in human PepT1 to alanine completely abolished transport activity [65].

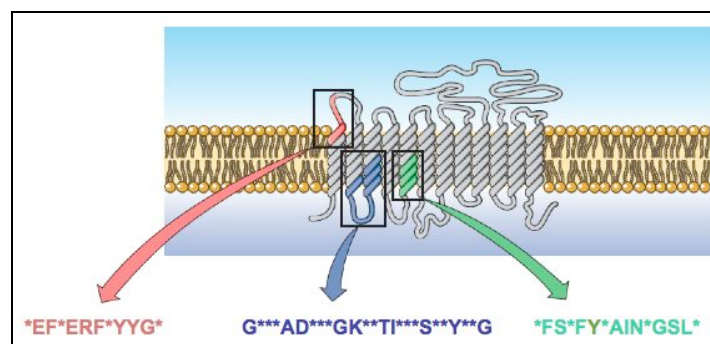


Figure 3.9: Conserved motifs in the PTR family; Y167 is marked in dark green; Figure modified from Daniel, Spanier et al 2005

Most interesting, the family of di- and tri-peptide transporters transports a variety of substrates. Despite its preference for peptides made of L- $\alpha$  amino acids and a size limitation to di- and tri-peptides, it also transports a variety of peptidomimetics such as  $\beta$ -lactam antibiotics and other drugs [9]. This special and quite unique substrate variety make this family an interesting target for further studies.

Since the homologues of the PTR family can be found from bacteria to man, prokaryotic homologues of mammalian transporters can be studied to understand the mechanisms of transport underlying the whole family of transporters.

### 3.5.4 Crystal structure of a prokaryotic homologue of mammalian PepT1 and PepT2

Recently the structure of a prokaryotic homologue of mammalian PepT1 from *Shewanella oneidensis*, called PepT<sub>so</sub>, was solved [39] in an occluded state and gave new insight into the transport mechanism of the MFS family. The transporter consists of 14 transmembrane helices that form an N- and a C- terminal six helix bundle related by a pseudo-2-fold axis (Figure 3.10 a). Two helices, namely HA and HB, are inserted into the cytoplasmic loop connecting the N- and the C-terminal halves. In the structure a central and a smaller extracellular cavity are visible. The central cavity is closed to the cytoplasm by an intracellular gate formed by side-chain interactions of residues in two helix hairpins formed by helices 4-5 (N-terminal bundle) and helices 10-11 (C-terminal bundle).

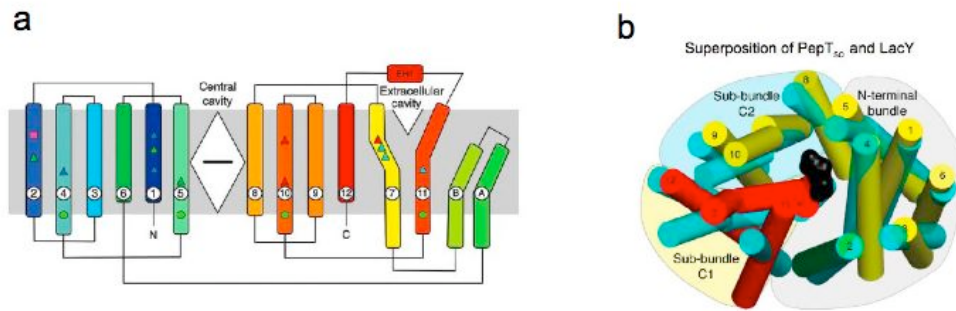


Figure 3.10: a. Topology of PepT<sub>so</sub>; b. Superposition of PepT<sub>so</sub> (green, yellow, red) and LacY (cyan) viewed from the intracellular side; modified from Newstead, Drew et al. 2001

The residues forming this intracellular gate are highly conserved in vertebrate peptide transporters including Leu327, Tyr154 and Phe150. Tyr154 and Phe150 belong to the highly conserved PTR2 motif. The central cavity is closed towards the external cavity by helix 1 (H1), H2, H7 and H8 that pack tightly together.

The central cavity has dimensions suitable to accommodate di- or tri-peptides and could include the binding site since the comparison with LacY shows the binding site at a similar location. On the surface of the central cavity several conserved residues can be found: Arg25, Arg32, Lys127, Tyr29, Tyr 68, Glu419, Ile157, Trp312, Phe315, Trp446 and Ser423. A variety of potential hydrogen-bond donors and acceptors seem to be necessary to support the transport of a diverse spectrum of substrates.

The His57 residue was shown to be the primary protonation site in human PepT1 and PepT2. The respective residue His61 is buried in the extracellular gate of the central cavity while the homologous residue is completely exposed in the inward-facing conformation in LacY.

Superposition of LacY and PepT<sub>so</sub> shows that the major changes from the occluded form to the inward-facing conformation are observed in the C-terminal bundle especially in the sub-bundle C1 (Figure 3.10b). This observation indicates that the N-terminal helix bundle is less dynamic than the C-terminal bundles, which contain the mobile gates. This would undermine the model of a rocker-switch mechanism since there would be a functional division in the helix bundles [17; 39].

### 3.5.5 The *E. coli* homologue YdgR

In addition to ABC transporter systems that transport di-peptides or oligopeptides, a tri-peptide permease (tppB) is responsible for the uptake of di- and tri-peptides in *S. typhimurium* [18]. By locus analysis the YdgR protein was identified as the corresponding tppB system in *E. coli* [9]. Electron microscopy experiments with detergent-solubilized YdgR suggest a monomeric form [61].

Substrate specificity resembles that of mammalian PepT1 [61].

Therefore studies of the *E.coli* homologue YdgR provide also insight into the transport mechanism of the PTR family in higher organisms. Since the structural information of transporters is still limited, YdgR was chosen as the target protein for crystallization trials to learn more about the underlying processes of transport. Since membrane protein crystallization is still challenging, different strategies such as co-crystallization with monoclonal antibody fragments (mFabs) and the generation of thermostable mutants was applied to facilitate crystallization.

## 3.6 Crystallization of membrane proteins

The general importance of membrane proteins is shown by the fact that more than 20-35% of all open reading frames of currently known genomes encode for membrane proteins, and more than 70% of all known pharmacological targets are membrane proteins [16]. That makes the need for more structural information concerning membrane proteins quite clear. The first structure of a membrane protein, namely the photosynthetic reaction center of the purple bacterium *Rhodospseudomonas viridis*, was solved in 1984 and showed that membrane proteins structures can be solved with general X-ray crystallography methods. Despite progress in expression, purification and crystallization techniques, structure determination of membrane proteins is still quite challenging. Structures of less than 250 unique membrane proteins are solved [5], and almost all of them are from bacteria or yeast.

The main problems of crystallizability of membrane proteins are due to their amphipathic character, which is an intrinsic property of membrane proteins. They reside in the lipid membrane with polar regions extending into the surrounding solvent. Detergent has to be used to extract them from the membrane and keep them

soluble in aqueous solvent. However, detergents directly affect protein stability and homogeneity [16].

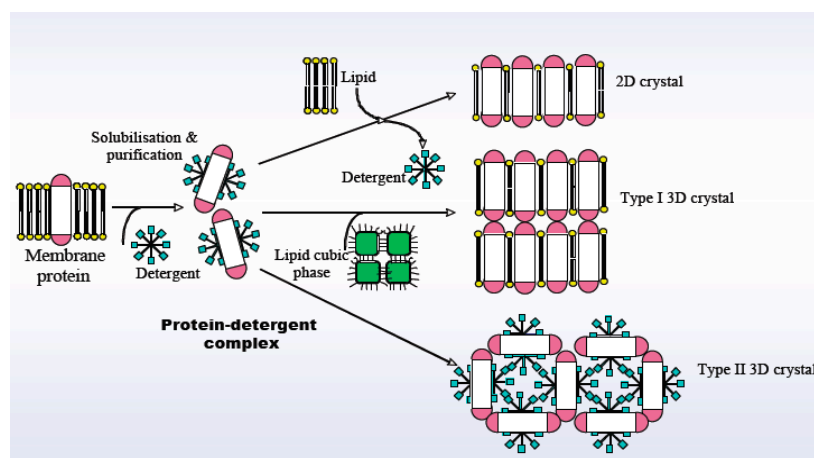


Figure 3.11: Different types of membrane protein crystals

In Figure 3.11 the different forms of membrane protein crystals are shown. While 2-dimensional (2D) crystals are prepared by the exchange of membrane proteins into lipid bilayers and are used for electron microscopy studies, 3D crystals require the protein to be extracted from the membrane via detergents. Type I 3D crystals are stacks of 2D crystals stabilized by protein-protein and protein-lipid interactions. Lipidic cubic phase, a three dimensional continuous lipid phase, can be used to generate this kind of crystal but the underlying mechanism of how crystals form in the lipidic cubic phase is not known.

Some structures, including that of rhodopsin, have been solved with this technique. However, the technique is technically difficult and most probably not suitable for the whole variety of membrane proteins.

Type 2 3D crystals form from detergent-micelle incorporated proteins. Crystal contacts are exclusively formed by polar headgroups that are not shield by the detergent micelle. Therefore they often have a high solvent content of 65-80%. Due to limited contacts in the crystal lattice, Type 2 3D crystals are often poorly ordered and may not diffract well enough to be used for determination of structural information. [16; 25]

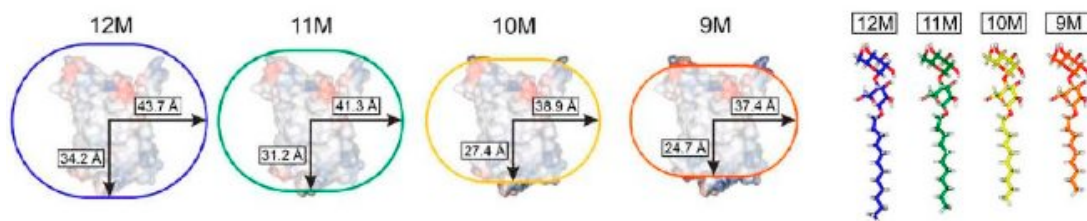


Figure 3.12: A membrane protein within micelles made of maltosides with different alkyl chain length; Modified from Kunji et al. 2008

The choice of detergent plays a main role in crystal quality. The longer the detergent molecules, the larger the crystal micelle and the less surface area is accessible for crystal contacts, resulting in poorly diffracting crystals (Figure 3.12). However, detergent with longer chain length is better tolerated by membrane proteins, since shorter detergent exposes larger parts of the protein, increasing the tendency for crystal formation.

As shown in figure X, detergents of different sizes expose varying amounts of surface accessible areas. While dodecyl maltoside (DDM, 12M) covers the whole protein leaving no exposed areas for crystal contact, nonyl maltoside (NM, 9M) may form a micelle too small to accommodate the hydrophobic parts of the protein and may promote aggregation.

To increase the tendency of membrane proteins to give rise to well-diffracting crystals, two general strategies can be used. First the surface accessible area available for crystal contact formation can be increased by co-crystallization with a monoclonal Fab fragment as described in [24] Second, the protein's intrinsic stability can be improved by mutagenesis to increase the tolerance for short chain-length detergents [52].

### 3.7 Increasing surface-accessible area with Fv or mFab fragments

Enlarging the polar surfaces of membrane protein-detergent complexes can increase the probability of obtaining well-ordered Type II 3D crystals. This can be achieved by co-crystallization with conformationally-specific monoclonal antibody (mFab) fragments [42]. Native antibodies are not suitable for this purpose since they have flexible linker regions. However, proteolytically generated mFab fragments can be

used for co-crystallization with membrane proteins. Nevertheless proteolytic cleavage can generate heterogeneous results.

Recombinant antibody fragments can be generated in the Fv or the Fab form. Generation of the Fv form includes the time-consuming cloning of encoding genes from hybridoma cell lines or direct selection for recombinant fragments from phage display libraries or by ribosomal display [24].

Screening strategies from hybridoma fusion of monoclonal antibody libraries should give rise to mAb or antibody fragments that recognize the native conformation of the target protein. Therefore the selected mAb fragment should be ELISA-positive but Western blot-negative. In the ELISA the protein-detergent complex should be presented in a native conformation, for example bound to a Nickel-chelating matrix via a His-tag [24].

The crystal structure of cytochrome *c* oxidase (COX) from *Paracoccus denitrificans* was the first example that showed the feasibility of co-crystallization of an antibody fragment with a membrane protein [42]. The yeast cytochrome *bc<sub>1</sub>* complex was also crystallized with an antibody Fv fragment [23].

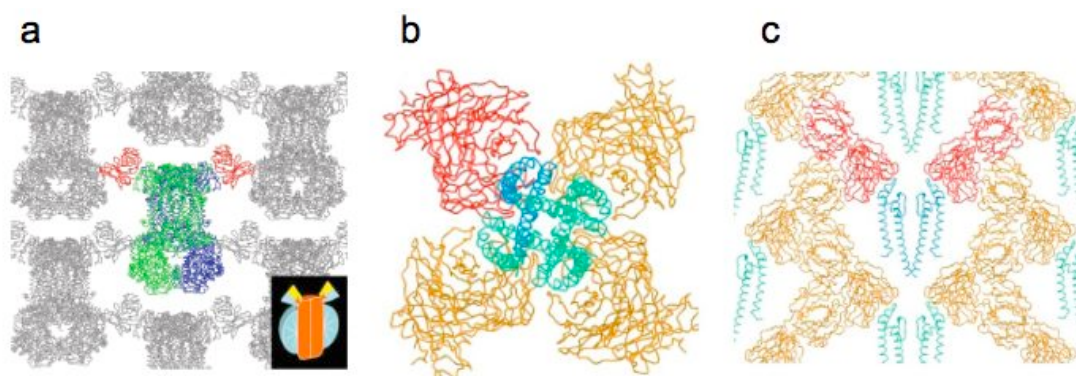


Figure 3.13: a. Crystal lattice of yeast cytochrome *bc<sub>1</sub>* (blue and green) with Fv fragment (red); Crystal lattice of KscA channel (blue and green) in complex with mFab fragment (red and yellow) top view (b) and side view (c); modified from Hunte and Michel 2002

In these structures the main crystal contacts are made by the Fv-fragments (Figure 3.13). Although the multisubunit complex cytochrome *bc<sub>1</sub>* has large hydrophilic domains on both sides of the membrane, only co-crystallization with the antibody fragment gave rise to well-diffracting crystals [24].

The structure determination of the KscA potassium channel-Fab fragment complex provided the first example of an integral membrane protein co-crystallized with a Fab fragment [68]. The crystal of the complex diffracted better than the crystals of the channel alone [12] giving more insight into the transport mechanism of KscA. Additionally the Fab fragments could be used for solving the phase via molecular replacement.

Crystal contacts of the channel-Fab-complex are exclusively made by the Fab fragment. Compared to the Fv, the size of the Fab is more suitable for co-crystallization experiments with integral membrane proteins that have no large hydrophilic domains protruding from either side of the detergent micelle [24].

### **3.8 Thermostable mutants**

A common problem of membrane proteins is their low stability in the detergent-solubilized form. After they are removed from the membrane, membrane proteins often undergo rapid unfolding and inactivation [6]. Despite the efforts to stabilize proteins with lipids or substrate, a higher stabilization effect is often required.

Studies on directed evolution and targeted mutagenesis have shown that quite a high percentage of mutations stabilize membrane proteins and that more stable membrane proteins can be generated [6; 15; 28; 66]. An additional advantage could be reduced conformational flexibility due to these stabilizing mutations. Decreased flexibility can lead to decreased protein sample heterogeneity and increase the probability of successful structure determination.

Previous publications have shown that membrane proteins can be stabilized by mutagenesis. One of the first examples was a mutation in the lactose permease LacY. The C154G mutant shows decreased conformational flexibility [54] and increased thermostability [55], both assumed to be advantageous for crystallization trials. Indeed, the structure of C154G [1] was solved before that of the native transporter LacY [20].

Another example is the determination of the structure of the  $\beta$ 1-adrenergic receptor [60]. Alanine-scanning mutagenesis identified six point mutations that stabilized the target protein by 21°C [52]. Additionally these mutations seem to decrease

conformational flexibility since the mutant has a clear preference for the antagonist. This stabilization also goes hand in hand with improved stability in a variety of detergents.

Similar effects can be observed for the adenosine A2a receptor. Several mutations were identified that stabilize the receptor in a distinct conformation, either antagonist or agonist bound [31; 34]

Therefore random mutagenesis might result in thermostable mutants. Since increased thermostability seems to go hand in hand with decreased conformational flexibility, these mutants might be promising to give well-ordered and diffracting crystals for structure determination.

### **3.9 Objectives of this study**

Beside crystal trials with native YdgR, two different strategies were tested to facilitate the formation of well-diffracting crystals. First, two monoclonal antibodies were generated to use them for co-crystallization trials with YdgR. Second, thermostable mutants could be useful for structure determination of YdgR. Our lab has identified 35 single point mutations of YdgR that result in partial or complete loss of activity serving as potential targets for crystallization [35]. 16 of these loss-of-function mutants were tested for thermostability and their tolerance for harsh detergents and finally subjected to crystallization trials.

## 4 Materials and Methods

### 4.1 Molecular Biology

#### 4.1.1 Transformation

For all cloning procedures DH5 $\alpha$  cells were used. For bacterial transformation, 100ng plasmid DNA was added to 50 ul competent cells and incubated on ice for 15 minutes. Then a heat shock at 37°C was performed for one minute followed by incubation on ice for 2 minutes. 500 ul SOC medium (Table 4.2) were added and the cells were shaken for 30 minutes at 37°C on a table shaker at 1000 rpm. The cells were spun at 2000g and the pellet was resuspended in 100 ul SOC medium and plated on LB agar plates selective for the suitable antibiotic.

Table 4.1: Bacterial strains

| Strain       | Genotype                                                                                                                                                                                                                                                |
|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| DH5 $\alpha$ | F <sup>-</sup> , supE44, $\Delta$ lacU169, [ $\Phi$ 80lacZ $\Delta$ M15], hsdR17, recA1, endA1, gyrA96, thi-1, (res-mod <sup>+</sup> ), deoR                                                                                                            |
| BL21(DE3)    | F <sup>-</sup> , ompT, gal, dcm, lon, hsdSB(rB <sup>-</sup> mB <sup>+</sup> ), $\lambda$ (DE3 [ <i>lacI</i> , <i>lacUV5</i> - T7, <i>gene1</i> , <i>ind1</i> , <i>sam7</i> , <i>nin5</i> ])                                                             |
| C43(DE3)     | F <sup>-</sup> , ompT, gal, dcm, lon, hsdSB(rB <sup>-</sup> mB <sup>+</sup> ), $\lambda$ (DE3 [ <i>lacI</i> , <i>lacUV5</i> - T7, <i>gene1</i> , <i>ind1</i> , <i>sam7</i> , <i>nin5</i> ])<br>Additionally at least two uncharacterized mutations [59] |
| XL10 GOLD    | Tetr $\Delta$ ( <i>mcrA</i> )183 $\Delta$ ( <i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i> )173<br><i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> Hte<br>[F' <i>proAB lacIqZ</i> $\Delta$ M15 Tn10 (Tetr) Amy Camr]                                        |

### 4.1.2 Agarose Gel electrophoresis

To check quantity and purity of PCR or restriction digest products, the products were loaded on a 1% (w/v) agarose gel. 1 g agarose was dissolved in 100 ml hot TAE buffer (Table 4.2). After the suspension had cooled down to ca. 40°C, Sybr® Safe DNA gel Stain (Invitrogen) was added in a ratio of 1: 10000 and the gel was poured. After polymerization the samples were loaded and the gel was run at 100 V in 1X TAE buffer.

Table 4.2: Buffers and media

| Buffer                           | Composition                                                                                                                                                                                        |
|----------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Luria Bertani (LB) medium</i> | 10 g tryptone, 5 g yeast extract, 10 g NaCl, adjusted to pH 7 with NaOH; dissolved in dH <sub>2</sub> O to 1000 mL and autoclaved                                                                  |
| <i>LB Agar</i>                   | 15 g agar dissolved in 1000 mL LB medium                                                                                                                                                           |
| <i>SOB</i>                       | 20g tryptone, 5g yeast extract, 0.5g NaCl, 2.5mg 1M KCl adjusted to pH 7 with NaOH; dissolved in dH <sub>2</sub> O to 1000ml and autoclaved; 10ml of sterile 1M MgCl <sub>2</sub> added before use |
| <i>SOC</i>                       | SOB media containing 2% glucose                                                                                                                                                                    |
| <i>10× TAE buffer</i>            | 0.1 M Tris-HCl pH 8, 10 mM EDTA-Na <sub>2</sub> -salt, 0.2 M acetic acid                                                                                                                           |
| <i>1× TE buffer</i>              | 10 mM Tris-HCl containing 1 mM EDTA-Na <sub>2</sub> -salt                                                                                                                                          |
| <i>DNA loading buffer</i>        | 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v), 0.25% (w/v) xylene cyanol                                                                                                           |

#### 4.1.3 Extraction of restriction digest after agarose gel electrophoresis

A kit (QIAquick™ Gel Extraction Kit, Qiagen) was used for this purpose. The protocol was performed as instructed by the manufacturer. Extracted DNA was dissolved in 30ul TE buffer.

#### 4.1.4 Plasmid purification

The plasmid was transformed into DH5α cells, plated on selective LB agar plates and incubated overnight on 37° C. A single colony was picked and inoculated in liquid selective LB medium and grown overnight at 37°C. The cells were harvested and a commercial kit (MiniPrep Kit, Qiagen) was used for plasmid purification. The protocol was performed as instructed by the manufacturer. The plasmid was eluted with 30 ul Elution Buffer. The DNA concentration was measured via absorbance at 260 nm with a NanoDrop (ND-100) spectrophotometer. The DNA sequence was verified using an in-house sequencing facility.

#### 4.1.5 PCR (Polymerase chain reaction)

The standard volume of the PCR reaction was 50 ul. The PCR reaction contained 100 ng template DNA, 1 unit Phusion Polymerase (Finnzymes), 2 ul of a 10 umol Primer mix, 1 ul of a 10 mM dNTP mix, 10 ul of 5X reaction buffer.

Table 4.3: PCR Protocol: Step 2-4 were repeated 25 times

| PCR step                | Temperature [°C] | Time       |
|-------------------------|------------------|------------|
| 1. Initial denaturation | 95°C             | 2 minutes  |
| 2. Denaturation         | 95°C             | 30 seconds |
| 3. Annealing            | 55°C             | 30 seconds |
| 4. Elongation           | 72°C             | 3 minutes  |
| 5. Final Elongation     | 72°C             | 10 minutes |

#### **4.1.6 Site-directed mutagenesis**

For site-directed mutagenesis, forward and reverse primers with the desired mutation were designed. The PCR set-up is comparable to a standard PCR except Phusion Polymerase was exchanged with 2.5 units of PfuTurbo® Polymerase (Stratagene). The template plasmid was digested with Dpn1 (Fermentas) for 1 hour at 37°C and transformed into DH5α cells. The cells were plated on selective LB agar plates. 10 colonies were picked and grown in liquid LB culture. The purified plasmids were sent for genotyping to an in-house sequencing facility.

#### **4.1.7 Restriction Digest**

Restriction enzymes from Fermentas and New England Biolabs were used for restriction digests. Generally a double digest was performed. Therefore restriction reactions contained 5 units of restriction enzyme A, 5 units of restriction enzyme B, 5 ul of suitable 10X restriction buffer and 2-5 ug DNA in a total volume of 50 ul. Additionally 1 unit of Alkaline Phosphatase (Fermentas) was added when vectors were digested. Alkaline Phosphatase removes 5' phosphates to prevent self-ligation

#### **4.1.8 Ligation**

T4 DNA ligase (Roche) was used for ligation. The reactions contained 100-150 ng digested vector, insert in 5 times molar excess, 2 ul of 10X ligation buffer and 1 unit T4 DNA ligase in a total volume of 20 ul. The ligation buffer contains ATP, therefore more than 2 freeze and thaw cycles were avoided. The ligation reaction was incubated overnight at 4°C. The reaction was then transformed into DH5α and plated on LB agar plates with suitable selective antibiotic.

#### 4.1.9 Cloned constructs

All YdgR constructs were cloned into the pCS19 vector (kind gift from Michael Ehrmann, University Duisburg-Essen), in which protein expression is under the control of the T5 promoter [58]. The plasmid for PepT<sub>so</sub> expression pTTQ PepT<sub>so</sub> was a kind gift from Simon Newstead (Oxford University). The lacY and lacY C154G plasmids were a kind gift from H. Ron Kaback (University of California, Los Angeles).

Table 4.4: Cloned constructs

| Construct                                     | Cloning strategy                                                                                                                                                                                                        |
|-----------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>C-term His6 YdgR</i>                       | The full length <i>ydgR</i> gene was amplified from E.coli genomic DNA and cloned into the pCS19 vector using NcoI and BglII restriction sites.                                                                         |
| <i>N-terminal His10 YdgR TEV site</i>         | The <i>ydgR</i> gene was amplified from C-term His6 YdgR plasmid and cloned into a pCS19 vector with a N-terminal His10 tag that is cleavable with TEV protease. The restriction sites for BamHI and HindIII were used. |
| <i>N-terminal His10 YdgR TEV site (6-498)</i> | Primers were designed for truncated version of YdgR and the PCR product was cloned into pCS19.                                                                                                                          |
| <i>C-term His6 S166G</i>                      | Site-directed mutagenesis                                                                                                                                                                                               |
| <i>C-term His S166G/N196K</i>                 | Site-directed mutagenesis                                                                                                                                                                                               |
| <i>N-terminal His10 S166G, TEV site</i>       | Site-directed mutagenesis                                                                                                                                                                                               |
| <i>N-terminal His10 N196K, TEV site</i>       | Site-directed mutagenesis                                                                                                                                                                                               |
| <i>N-terminal His10 S166G/N196K, TEV site</i> | Site-directed mutagenesis                                                                                                                                                                                               |

## **4.2 Protein production and purification**

### **4.2.1 Expression of YdgR**

All YdgR constructs were over-expressed in *E. coli* C43(DE3) cells. The plasmids were freshly transformed and plated on LB agar plates selective for ampicillin resistance. Three 30 ml liquid pre-cultures were set up in 100 ml baffled flasks containing LB medium with 2% glucose and the selective antibiotic ampicillin at a final concentration of 100 ug/mL. The cultures were grown with shaking (220rpm) overnight at 37°C. On the next day the cells were harvested and resuspended in fresh medium. The cells were inoculated in 6 L fresh LB medium with 2% glucose containing the selective antibiotic ampicillin (100 ug/mL). The cells were grown with shaking (190 rpm) at 37°C to an OD<sub>600</sub> of 0.4, and then the temperature was decreased to 18°C. When OD<sub>600</sub> had reached a value of 0.6, the cells were induced with 0.1 mM IPTG. The expression was carried on overnight. The cells were harvested the next day and resuspended in 50 mM Tris at pH 8 containing 300 mM NaCl, 10% glucose and 2 mM EDTA and stored at -20°C or -80°C.

### **4.2.2 Expression of LacY and C154G**

The constructs were transformed into XL-10 GOLD cells and plated on LB agar plates containing the selective antibiotic ampicillin. 30 mL pre-cultures of LB with ampicillin in a final concentration of 100 ug/mL were grown with shaking (220rpm) overnight at 37°C in 100 ml baffled flasks. The next day the cells were harvested and inoculated in 2L LB containing ampicillin (100 ug/mL) in a 5L baffled flask. The culture was grown with shaking at 180 rpm at 37°C to an OD<sub>600</sub> of 0.6 and then induced with 0.1-0.2 mM IPTG. Expression was carried out for 4 hours at 37°C.

### 4.2.3 Expression of PepT<sub>so</sub>

The construct Pept<sub>so</sub> pTTQ was transformed into *E. coli* C43 (DE3) cells. Over-expression was performed as described above for YdgR constructs except instead of LB medium containing 2% glucose TB medium was used.

### 4.2.4 Membrane Preparation of YdgR, LacY and PepT<sub>so</sub> constructs

All steps were performed at 4°C or on ice. The harvested cells were resuspended in 50 mM Tris at pH 8 containing 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM DTT and complete protease inhibitors (Roche) and were lysed with a constant cell disruptor (Constant Systems L<sub>TD</sub>) at a pressure of 2.2 kbar in two passages. To remove cell debris, the lysate was centrifuged at 15000 rpm for 30 minutes. The supernatant was then centrifuged for 42000 rpm for 1.5 hours in an ultracentrifuge. The supernatant containing the cytoplasmic fraction was discarded while the pelleted membranes were transferred to a homogenizer. The pellets were homogenized in 20 mM Tris at pH 8 containing 20 mM NaCl and 20% glycerol. The total protein concentration was measured with a DC Protein Assay (BioRad) and the homogenized membranes were diluted to 6 mg/ml. The membranes were stored on -80°C.

For LacY, all buffers contained HEPES buffer at pH 7.5 instead of Tris buffer at pH 8. The ultracentrifugation step for PepT<sub>so</sub> was elongated to 2 hours due to less stable membrane pellet consistency.

### 4.2.5 Purification of YdgR constructs

#### 4.2.5.1 Nickel affinity chromatography

Membranes were thawed and diluted 1:1 with 20 mM Tris buffer at pH 8 containing 580 mM NaCl, 2% DDM (Anatrace; final concentration 1%), 10 mM Alanlyl-alanine (Bachem; final concentration 5 mM) and 1 mM DTT (final concentration 0.5 mM). The membranes were incubated at 4°C for 1 hour on a rotator. To separate detergent-

solubilized protein from remaining membranes an ultracentrifugation step of 1 hour at 42000 rpm at 4°C was performed. The supernatant was diluted 1:1 with 20 mM Tris at pH 8 containing 300 mM NaCl, 10% glycerol, 30 mM imidazole and 5 mM Alanyl-alanine and loaded on a HisTrap FF 5 mL column (GE Healthcare) with a rate of 3 ml per minute. The column was washed with 20 mM Tris buffer at pH 8 containing 300 mM NaCl, 10% glycerol, 5 mM alanyl-alanine, 0.025 mM DTT, 30 mM imidazole and 0.03% DDM. Bound protein was washed with 20 mM Tris buffer at pH 8 containing 300 mM NaCl, 10% glycerol, 5 mM alanyl-alanine, 0.025 mM DTT, 0.015% DDM and 65 mM imidazole and eluted with 20 mM Tris buffer at pH 8 containing 300 mM NaCl, 10% glycerol, 5 mM alanyl-alanine, 0.025 mM DTT, 0.015% DDM and 155 mM imidazole. The elution fractions were concentrated (Vivaspin 100 kDa molecular weight cut-off) and exchanged to 20 mM Tris buffer at pH 8 containing 150 mM NaCl, 10% glycerol, and 0.015% DDM.

#### 4.2.5.2 *Size exclusion*

For crystallization trials, an additional purification step was performed. The elution fractions of Nickel affinity chromatography were diluted 1:1 with size exclusion buffer (20 mM Tris at pH 8 containing 150 mM NaCl, 5 mM Alanyl-alanine and 0.015% DDM) to lower the content of imidazole before concentrating with Vivaspin 100kDa. Otherwise an increased tendency for precipitation was observed. The fractions were concentrated to 5 ml and loaded on a Hi Load Superdex 200 16/60 column (GE Healthcare) pre-equilibrated with size exclusion buffer. Fractions containing the target protein were concentrated to ~3.5mg/ml with Vivaspin 100kDa and aliquots were stored at -80°C.

#### 4.2.6 **Purification of PepT<sub>so</sub>**

Membranes of cells containing over-expressed PepT<sub>so</sub> were treated as described above for YdgR but instead of loading them on a His Trap FF 5ml column, 1ml Ni-NTA Superflow (Quiagen) per 10 ml membranes was added and the sample was rotated overnight at 4°C. The sample was then transferred into a gravity flow Econo-Pac

column (BioRad) and the resin was washed with 20 column volumes of 20m M Tris buffer at pH 8 containing 300 mM NaCl, 10% glycerol, 0.03% DDM and 30 mM imidazole. PepT<sub>so</sub> was eluted with 20 mM Tris at pH 8 containing 300 mM NaCl, 10% glycerol, 0.03% DDM and 250 mM imidazole. The fractions containing Pept<sub>so</sub> were concentrated to 500 ul with Vivaspin 100 kDa and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with 20 mM Tris at pH 8 containing 150 mM NaCl and 0.02% DDM. The peak at an elution volume of ~13 ml was concentrated and aliquots were stored at -80°C.

#### **4.2.7 Purification of LacY and C154G**

Native Lac Y and the mutant C154G were purified as described above for PepT<sub>so</sub> except that all buffers included HEPES buffer at pH 7.5 instead of Tris buffer at pH 8. Additionally a second washing step was included in the purification protocol containing the same components as the standard washing step, but the imidazole concentration was increased from 30 mM to 50 mM.

### **4.3 Protein analysis and characterization**

#### **4.3.1 Measurement of protein concentration**

Protein concentration was determined by measuring absorbance at 280 nm with a NanoDrop™ Spectrophotometer (PeqLab).

#### **4.3.2 SDS- polyacrylamide gel electrophoresis (SDS- PAGE)**

Proteins were submitted to SDS-PAGE to investigate protein size and purity. The gel consists of an upper stacking and a lower separating layer. For standard procedures 12% SDS-PAGE gels were prepared (Table 4.5).

Table 4.5: Recipe for SDS-polyacrylamide gels

| Components              | Seperating gel (12%) | Stacking gel |
|-------------------------|----------------------|--------------|
| 1M Tris pH=8.8          | 12.5ml               | -            |
| 1M Tris pH=6.8          | -                    | 1.25ml       |
| 30% Acrylamide mix      | 20ml                 | 1.7ml        |
| 10% SDS                 | 0.5ml                | 100ul        |
| 10% ammonium persulfate | 0.5ml                | 100ul        |
| TEMED                   | 20ul                 | 10ul         |
| H <sub>2</sub> O        | 16.5ml               | 6.8ml        |

To estimate protein size, PageRuler™ Unstained or Prestained Protein Ladder (Fermentas) was loaded next to the protein samples. The self-made SDS-PAGE gels were run at 25 mA per gel for ~1 hour.

Also commercial SDS-PAGE gels (NuPage, Invitrogen) including suitable commercial running buffer were used. Depending on the purpose electrophoresis was performed on 12% or 4-12% gradient gels. These gels were run at 160 Volt for ~1.5 hours. Afterwards proteins were detected with Page Blue™ Protein Staining Solution (Fermentas). The procedure was performed as instructed by the manufacturer.

### 4.3.3 Silver staining

Silver staining was used to detect proteins or peptides on SDS-PAGE gels in the nanogram range. All steps were performed in glass containers. This staining procedure can be used for self-made or commercial SDS-PAGE gels.

The gels were loaded and run as described above. Then the gel was incubated for 5 minutes in solution 1. The gel was quickly rinsed and then put into distilled water for 5 minutes. After it was washed again with distilled water, it was incubated for 5 minutes in solution 2 followed by 1 minute in solution 3. Again the gel was quickly rinsed with distilled water several times and put into solution 4 for 8 minutes. The gel

was rinsed with distilled water again several times and solution 5 was added. After the appearance of protein bands the reaction is stopped with 1% (v/v) glacial acetic acid.

Table 4.6: Protocol for silver staining

| Solution          | Components                                                                                                                        |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| <i>Solution 1</i> | 60 ml 50% (v/v) acetone, 1.5 ml 50% (v/v) TCA in water, 25 ul formaldehyde solution (purchased as 37% solution)                   |
| <i>Solution 2</i> | 50% (v/v) acetone in water                                                                                                        |
| <i>Solution 3</i> | 100 ul 10% (w/v) Sodium Thiosulfate pentahydrate in 60 ml water                                                                   |
| <i>Solution 4</i> | 0.8 ml 20% (w/v) silver nitrate, 0.6 ml formaldehyde solution (purchased as 37% solution) in 60 ml water                          |
| <i>Solution 5</i> | 1.2g Na <sub>2</sub> -carbonate, 25 ul formaldehyde solution (purchased at 37% solution), 25 ul sodium thiosulfate in 60 ml water |

#### 4.3.4 Western Blot analysis

Self-made or commercial SDS-PAGE gels were run as described above with PageRuler™ Prestained Protein Ladder as a protein size marker. The protein bands were transferred electrically to a PVDF membrane (Millipore) at 400mA for 1.5 hours at 4° C. Afterwards the membrane was blocked in 20 ml blocking buffer containing TBS-T with 5% milk powder for 20 minutes. Then the membrane was incubated in TBS-T, 5% milk powder and penta-His HRP-conjugated antibody (Qiagen) at a dilution of 1:10000 for 2 hours at room temperature or overnight at 4°C. The membrane was washed 5 times for 7 minutes in TBS-T. The luminescence of antibody-bound protein bands was detected with the SuperSignal West Pico

Chemiluminescent Substrate (Thermo Scientific) on a chemiluminescent film (Hyperfilm ECL, GE Healthcare).

Table 4.7: Buffers for SDS-PAGE and Western Blot

| Buffer                       | Composition                                                                                      |
|------------------------------|--------------------------------------------------------------------------------------------------|
| <i>1× SDS running buffer</i> | 25 mM Tris/HCl pH 8.3, 200 mM glycine, 0.1% (w/v) SDS                                            |
| <i>2× SDS loading buffer</i> | 125 mM Tris pH 6.8, 30% glycerol, 2% SDS, 6 M urea, 1 M β-mercaptoethanol, 0.2% bromophenol blue |
| <i>10× Tansfer Buffer</i>    | 250 mM Tris, 1.92 M glycine                                                                      |
| <i>Transfer Buffer</i>       | 200 mL 10× TB, 200 mL methanol, 1600 mL dH <sub>2</sub> O                                        |
| <i>10× TBS</i>               | 200 mM Tris-HCl pH 7.5, 1.5 M NaCl                                                               |
| <i>TBST</i>                  | 100 mL 10×TBS, 1 mL Tween 20 in 1000 mL dH <sub>2</sub> O                                        |

#### 4.3.5 Stargazer thermostability assay

Purified native and mutant YdgR proteins were diluted to 0.1 mg/mL and 0.2 mg/mL in 50 mL of 20 mM HEPES buffer at pH 7.5 containing 150 mM NaCl, 10% glycerol, and 0.03% DDM in a black 384-well plate with a clear bottom (Nunc). The plate was centrifuged for 2 minutes at 400 rpm and 50 mL of mineral oil were added to each well and the plate was centrifuged again. The thermal aggregation ( $T_{agg}$ ) curves for each protein were determined with the Stargazer instrument (Harbinger Biotech). Regression analysis of the curves to determine the  $T_{agg}$ 's was performed with the program Bioactive (Harbinger Biotech). For testing different buffers for crystallization purposes, native YdgR was diluted to 0.1 mg/ml in a buffer screen. Additives were used in a ratio of 1:10.

#### **4.3.6 Dynamic light scattering (DLS)**

A DynaPro801 molecular sizing instrument (Protein Solutions Inc.) was used for DLS measurements. The samples were centrifuged for 10 minutes at 10000g to remove any contamination. Buffer alone was analyzed as a reference to rule out contamination of the cuvette. 15  $\mu$ L samples were measured in a quartz cuvette at 19° C. Data Analysis was performed with Dynamics V6 software (Protein Solutions Inc.)

#### **4.3.7 Analytical size exclusion YdgR, LacY and PepTso**

Purified native and mutant YdgR proteins were diluted to a concentration of 1 mg/mL. 70 mL of protein were incubated for 5 minutes at either 4° C or 50°C. The samples were centrifuged for 10 minutes at 10,000g before loading 50 mL onto a Superdex 200 PC 2.1 column (GE Healthcare) equilibrated in 20 mM Tris buffer at pH 8 containing 150 mM NaCl, 10% glycerol, and 0.03%DDM. For LacY and C154G also 50 $\mu$ g were loaded. For PepTso only 25 $\mu$ g were loaded.

#### **4.3.8 CPM fluorescence-based thermostability assay**

Purified native and mutant YdgR proteins were diluted to a concentration of 3 mg/mL in 20 mM Tris buffer at pH 7.5 containing 100 mM NaCl and 0.03% DDM. 1.5 mL protein were added to 150 mL of the same buffer containing different detergents, each at a concentration 3-fold above its CMC. CPM was dissolved in DMSO at a concentration of 4 mg/mL. The dye was diluted 1:100 in 20 mM Tris buffer at pH 7.5 containing 100 mM NaCl and 0.03% DDM, and 3 mL of the CPM dilution were added to each protein sample. The fluorescence of triplicate values of each sample (excitation 387 nm; emission 463 nm) was measured every 5 minutes over 150 minutes at a temperature of 40° C in a black 96-well plate using a Synergy 2 fluorescence plate reader (Biotek). The fraction of unfolded protein at each time point was determined as in [39].

#### **4.3.9 Lysine acetylation**

Sulfo-NHS Acetate (Thermo Scientific) was dissolved at a concentration of 2.6 mg/ml (10 mM) in purified, distilled water. Native YdgR, S166G, N196K and S166G/N196K were diluted to 0.1 mg/ml in 100 mM HEPES buffer at pH 7.5 containing 150 mM NaCl and 0.015% DDM. 3.8  $\mu$ l crosslinker were added per 100  $\mu$ l sample volume. The reaction was incubated for 1 hour at room temperature and stopped by removing the cross-linker by desalting. Proteolytic digestion and mass spectrometry analysis were performed by the in-house mass spectrometry facility.

### **4.4 *In vivo* functional assays**

#### **4.4.1 Membrane Localization**

50-100mL of *E. coli* C43(DE3) cells were grown according to the standard protocol. The cells were harvested at 4000 rpm and resuspended in 1 ml 20 mM Tris at pH 8 containing 150 mM NaCl, 10% glycerol, complete protease inhibitors (Roche), 1 mg/ml lysozyme and 25 units/ml Benzonase nuclease per measured OD<sub>600</sub>. The cells were incubated on ice for 30 minutes. To improve efficiency of lysis, ~1ml of 0.1 mm glass beads was added per ml lysate and were placed in a cell disruptor for 3 minutes at 4° C. Cell debris was removed by centrifugation at 13000 rpm at 4°C for 30 minutes. The supernatant was removed gently by pipetting with gel loading tips and membranes were pelleted in a table-top ultracentrifuge at 42000 rpm for 1.5 hours at 4°C. The supernatant was discarded and the membrane pellet was washed twice with 200  $\mu$ l buffer 20mM Tris at pH 8 containing 150 mM NaCl and 10% glycerol. Then the membranes were resuspended in 100  $\mu$ l 20 mM Tris buffer at pH 8 containing 20 mM NaCl using a small plastic homogenizer. The total protein concentration was measured with the *DC* Protein Assay (Bio Rad) and the homogenized membranes were diluted to equal concentrations.

#### **4.4.2 Alafosfalin growth assay**

The mutants were transformed into *E. coli* BL21(DE3) and individual colonies were inoculated in 1ml LB- cultures in 2 ml 96-well plates and grown overnight at 37°C with shaking at 190 rpm. These pre-cultures were transferred to transparent 96-well plates and the cells were grown under two conditions, one containing 200 µL of Luria-Bertani (LB) medium plus the antibiotic ampicillin, and one additionally containing 200 µg/mL of the antibiotic alafosfalin (Sigma-Aldrich) at 37°C at 190 rpm. The cells were induced with 0.01 mM IPTG at an OD<sub>600</sub> of 0.2-0.3. The OD<sub>600</sub> was measured at different time points with a Synergy 2 fluorescence plate reader (Biotek).

#### **4.4.3 Uptake of b-Ala-Lys(AMCA)**

Constructs were transformed into *E. Coli* BL21(DE3) cells as described above and liquid pre-cultures in LB medium containing 2% glucose and 100 µg/ml ampicillin were set up and grown overnight at 37°C with shaking at 220 rpm. 250 µl of the pre-culture were spun down and resuspended in fresh medium for inoculation in 25 ml liquid cultures in 100 ml baffled flasks. The cells were grown to an OD<sub>600</sub> of 0.6 and were then induced with 0.1 mM IPTG. Expression was carried on for 1 hour. The cultures were put on ice and the OD<sub>600</sub> was measured again. A concentration of  $3.25 \times 10^9$  cells/ml was intended for the assay. (An OD<sub>600</sub> of 1 corresponds to approximately  $8 \times 10^8$  cells/ml.) The adequate volume of culture was spun down and resuspended in 1 ml of Kreb's buffer. Triplicate samples of 40 µl of cells were added to 60 µL of buffer, substrate solution or competitor solution in a 96-well V-bottom plate. The plate was covered with an Airpore sheet (Qiagen) and put in the shaker at 37°C for 15 minutes at 180 rpm. The cultures were pelleted and washed 3 times with Krebs buffer. Then the cells were resuspended in 100 µl Krebs buffer and were transferred to a 96-well black-walled plate with a clear bottom. The fluorescence signal and OD<sub>600</sub> were measured with a Synergy 2 fluorescence plate reader (Biotek) with an excitation filter of 360/40 and an emission filter of 460/40.

Table 4.8: Solutions for b-Ala-Lys(AMCA) uptake assay

| Solution                      | Components                                                                                                               |
|-------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| <i>Kreb's buffer</i>          | 25 mM Tris buffer at pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl <sub>2</sub> , 0.8 mM MgSO <sub>4</sub> , 5 mM glucose |
| <i>β-Ala-Lys-(AMCA) stock</i> | 2.5 mM stock in DMSO freshly dissolved prior to the assay                                                                |
| <i>Substrate solution</i>     | 10ul of 2.5 mM β-Ala-Lys-(AMCA) stock in 50ul Kreb's buffer                                                              |
| <i>Competitor solution</i>    | 10ul of 2.5 mM β-Ala-Lys-(AMCA) stock with 50 ul of 20 mM competitor dissolved in Kreb's buffer                          |

## 4.5 Generation of conformationally specific F<sub>ab</sub> fragments

### 4.5.1 Purification of tag-free and N- and C-terminally truncated YdgR

N-terminal His10 YdgR 6-498 with a TEV protease cleavage site was purified via Ni-NTA affinity chromatography as described above and the His-tag was cleaved off by incubation with TEV protease overnight at 4° C. The His-tag and TEV protease were removed by re-binding to Ni-NTA agarose in batch. Then gel filtration chromatography using a HiLoad Superdex 200 16/60 column (GE Healthcare) was performed as described above.

### 4.5.2 Reconstitution of YdgR into proteoliposomes

Powdered *E. coli* polar lipid extract (AVANTI POLAR LIPIDS Inc.) was diluted in chloroform at a concentration of 20 mg/ml. 500 ul were dried in a faint nitrogen gas

stream and resuspended in 20 mM Tris at pH 7.5 containing 150 mM NaCl. 100  $\mu$ l of 10% DDM were added and the lipid-detergent mix was sonicated in a water bath for 30 min. 400  $\mu$ l of YdgR 6-498 as diluted in 20 mM Tris buffer at pH 7.5 containing 150mM NaCl, 5% Glycerol, 0.1 mM DDT and 0.06% DDM at a concentration of 0.25 mg/ml and was added to the lipids followed by incubation for 10 min on ice. 500mg Bio-Beads (Biorad) washed in 20 mM Tris at pH 7.5 containing 150mM NaCl were added and the sample was rotated at 4° C overnight. The beads were removed in the morning and 200 mg fresh washed Bio-Beads were added. After incubation for 4 hours the sample was centrifuged for 90 minutes at 42000 rpm. The pellet was resuspended in 20 mM Tris buffer at pH 7.5 containing 150 mM NaCl. To remove unfolded and/or unincorporated protein, a final spin step at 13000 rpm for 2 minutes was performed.

#### **4.5.3 Monoclonal antibody generation**

Proteoliposomes containing YdgR were used for serial immunization of three mice by the MFPL monoclonal antibody facility. The proteoliposomes were freshly prepared before each injection. Supernatants of hybridoma cell lines 5E8-D6 and 5E3-F10 were supplied by the MFPL antibody facility. Both antibodies are Western blot negative and ELISA positive.

#### **4.5.4 Large-scale production of monoclonal antibodies**

The hybridoma cells were grown in X63 Medium containing per 500 ml 435 ml DMEM (Dulbecco's Modified Eagle's Medium- high glucose; Sigma), 50 ml Fetal calf serum (Sigma), 5 ml Penicillin and Streptomycin (Sigma), 5 ml Glutamine (Sigma) and 5 ml Na-Pyruvate (Sigma). When the cells reached high density, the cells were gently removed from the bottom of the culture dish by pipetting. The cells were centrifuged at 800g for 4 minutes, were gently resuspended in new medium and were split into new culture dishes. For inoculation of the FiberCell™ Hollow Fiber Cartridge (Fiber Cell Systems Inc.),  $1-2 \times 10^8$  cell are necessary which corresponds to approximately 10 culture dishes (15cm). The Fiber cell bioreactor was washed for 2

days with 1× PBS and for 2 days with X63 Medium after which the cells were injected into the chamber. After two days the glucose consumption was measured with a Lactate Kit (Reagent 10×10mL; Trinity Biotech). Lactate values >0.6 indicate that the medium should be changed and values >0.8 indicate that antibodies could be harvested. Subsequently the medium was changed and antibodies were harvested once per day. Dead hybridoma cells were spun down at 1200g and 0.02% sodium azide was added to the supernatant containing the antibody. The supernatant was stored at 4°C.

#### 4.5.5 ELISA (Enzyme-linked immunosorbent assay)

The 96-well Nickel-coated plates (Pierce) were coated overnight at 4°C with antigen coating solution. The wells were washed twice with washing buffer. Since the plates are pre-blocked, a blocking step is not necessary. The next day, the wells were incubated with 50 ul Antisera solution per well for 1 hour at room temperature. Then the wells were washed three times with 50 ul washing buffer containing 50 mM imidazole to reduce the background of Nickel-binding proteins. Additionally the plates were washed twice with 50 ul washing buffer. The plates were incubated with 50 ul antibody solution for 1 hour at room temperature or overnight at 4°C. Then the wells were washed three times with washing buffer. 50 ul substrate solution was added. Positive wells develop a blue color that changes to yellow when the reaction is stopped with 50 ul 1N H<sub>2</sub>SO<sub>4</sub>. The color reaction was quantified by measuring the absorption at 450 nm with a Synergy 2 fluorescence plate reader (Biotek).

Table 4.9: Solutions for ELISA

| Solution                        | Components                                                                                          |
|---------------------------------|-----------------------------------------------------------------------------------------------------|
| <i>Washing buffer</i>           | 1× PBS, 0.03% DDM, 0.1% BSA                                                                         |
| <i>Antigen coating solution</i> | Washing buffer with TEV-cleaved N-terminally his-tagged YdgR 6-498 at a concentration of 5-10 ug/ml |

|                           |                                                                                                 |
|---------------------------|-------------------------------------------------------------------------------------------------|
| <i>Antisera solution</i>  | 1:1000 dilution of cell supernatant in washing buffer                                           |
| <i>Antibody solution</i>  | 1:10000 dilution of HRP conjugated rabbit anti-mouse antibody in washing buffer                 |
| <i>TMB stock solution</i> | 1mg of 3',5',5',5'-tetramethylbenzidine (Sigma) in 300 ul DMSO (stored at -20°C)                |
| <i>Substrate solution</i> | 100 ul TMB stock in 10 ml 100 mM Na-acetate pH 6 containing 0.01% H <sub>2</sub> O <sub>2</sub> |

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#### 4.5.6 Monoclonar antibody (mAb) purification

Supernatant (containing 0.1- 0.4 mg/ml mAb) was concentrated 20 times in a VIVASPIN column with a 100k molecular weight cut-off and diluted 1:5 with 20 mM sodium phosphate buffer at pH 7. The pH was adjusted to ~6.5. After loading onto a 1 mL Hi Trap Protein G column (GE Healthcare), the column was washed with 10 column volumes 20 mM sodium phosphate at pH 7. The mAb was eluted with 100 mM Glycine at pH 2.7. Tubes were pre-filled with 100 ul 1M Tris buffer at pH 8.5 per 1 ml eluate for immediate neutralization.

#### 4.5.7 Analytical size exclusion of mAb-YdgR complexes

30 ug mAb were incubated with 30 ug native or mutant YdgR for 90 min at 4°C and then loaded onto a Superose 6 PC 3.2/30 column (GE Healthcare). The running buffer contained 20 mM Tris buffer at pH 8, 150 mM NaCl, 10% glycerol and 0.015% DDM.

## **4.6 Protein crystallization**

### **4.6.1 Crystallization in detergent**

Purified native and mutant YdgR proteins were concentrated to ~3.5 mg/mL and crystallization trials were set using the sitting drop vapor diffusion method using the commercial screens Mem Gold, MemStart and MemSys (Molecular Dimensions). Initial screening was carried out with a Mosquito robot (TTP lab tech) in ratios of 100 mL protein: 100 mL reservoir and 200 mL protein: 100 mL reservoir. Optimization was performed in 96 well or 24 well screens in a grid screen manner pipetted with a Liquid handling Alchemist™ II robot.

For co-crystallization with monoclonal Fab fragments, the purified Fab was mixed with purified YdgR in a 1:2 and loaded onto a Superdex 200 10/300 GL or a Hi Load Superdex 200 16/60 column (GE Healthcare). The eluted complex was concentrated to 6.5mg/ml and used for crystal trials in commercial crystal screens.

### **4.6.2 Crystallization after relipidization**

*E. coli* Polar Lipid Extract (Avanti®, Polar Lipids Inc.) in Chloroform was transferred to small glass vials in amounts of 0, 0.25 and 0.5 mg. The chloroform was removed under a faint nitrogen stream. Three tubes of each concentration were prepared. 100 ul of the target protein at a concentration of 5-10 mg/ml were added to each tube. Subsequently, different detergent concentrations (0, 1.5 and 3 mg) were added per lipid concentration from a 100 mg/ml stock, resulting in nine samples in total. The samples were incubated with stirring at 4°C for 10-20 hours. The insoluble material was removed by ultracentrifugation at 42000 rpm for 10 minutes. The supernatant was then used for crystallization trials.

### **4.6.3 X-ray Diffraction**

Crystals were frozen in mother liquor since all described conditions were cryoprotectants themselves. The crystals were shipped to the European Synchrotron Radiation Facility (ESRF) Grenoble and tested.

## 5 Results

### 5.1 Crystallization of native YdgR

#### 5.1.1 Optimization of native YdgR purification

Initially, purification of YdgR was performed via batch Nickel affinity chromatography using an econo-column (as described Materials and Methods for PepT<sub>so</sub>) followed by size exclusion chromatography. YdgR does not run at its actual molecular weight of 55 kDa on SDS-PAGE, which commonly occurs with integral membrane proteins and is most likely due to a very high capacity for SDS binding [45]. The YdgR monomer band can be detected at a size of ~38 kDa and, as is also common for membrane proteins, a dimer band (~76 kDa) is visible in SDS-PAGE, which is most likely an artifact due to incomplete or aberrant denaturation with SDS, as no biological relevance has been shown for a dimer [61]. After batch Nickel affinity chromatography, the eluted fractions contained YdgR and impurities of various sizes (Figure 5.1 a). Even after size exclusion, impurities with a molecular weight of ~50kDa, ~30kDa and ~18kDa were detected on SDS-PAGE gels in a peak overlapping with the main fraction of YdgR. The peak containing the impurity and the pure YdgR peak could not be separated completely via size exclusion (Figure 5.1).

The 50 kDa band was identified by mass spectrometry as the subunit HslU belonging to the HslU/HslV complex. This complex contains the protease HslV and the ATPase HslU, a chaperone of the Clp/Hsp100 family. Most probably this complex binds and processes YdgR *in vitro* and therefore co-elutes with YdgR. An increase in the ~30kDa band correlates with an increased amount of the HslU/HslV complex and can most probably be regarded as partially digested YdgR.

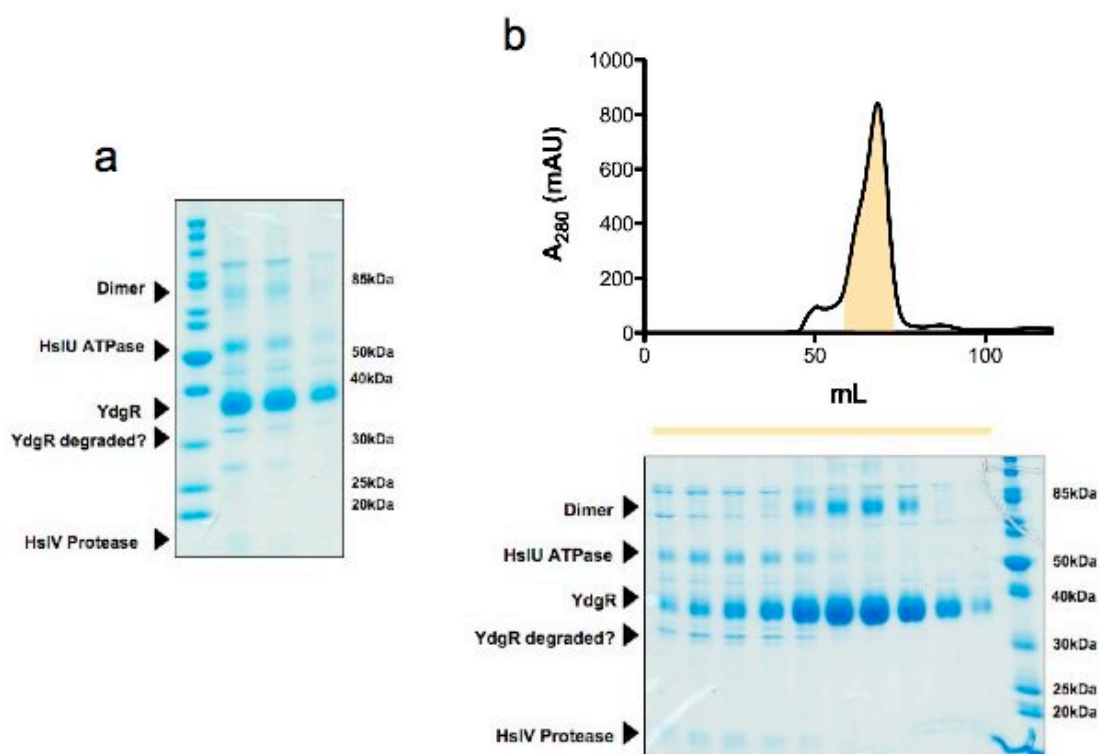


Figure 5.1: a. SDS-PAGE after batch Nickel affinity chromatography; b. Chromatogram and SDS-PAGE of size exclusion chromatography on Superdex 200 16/60

Since size exclusion was not sufficient to separate YdgR from these impurities, the Nickel affinity chromatography step was optimized. On-column binding and Stepwise elution with imidazole from a His Trap FF 5ml column improved purity dramatically as can be easily observed when fractions of batch purification and on-column purification are compared by SDS-PAGE (Figure 5.1 a and Figure 5.2 a). In the batch purification all fractions contain the HslU/HslV complex and partial degradation of YdgR can be observed. These impurities are removed on the His Trap FF 5ml column by a washing step with buffer containing 65 mM imidazole. Some YdgR was also removed in this step, but in the next step with buffer containing 155 mM imidazole pure YdgR without traces of the HslU/HslV complex is eluted (Figure 5.2 a). The profile of the subsequent gel filtration shows a sharp peak and fractions analysed via SDS-PAGE are free of HslU/HslV complex (Figure 5.2 b). Therefore on-column Nickel affinity chromatography via FPLC and subsequent size exclusion became the standard procedure for purification.

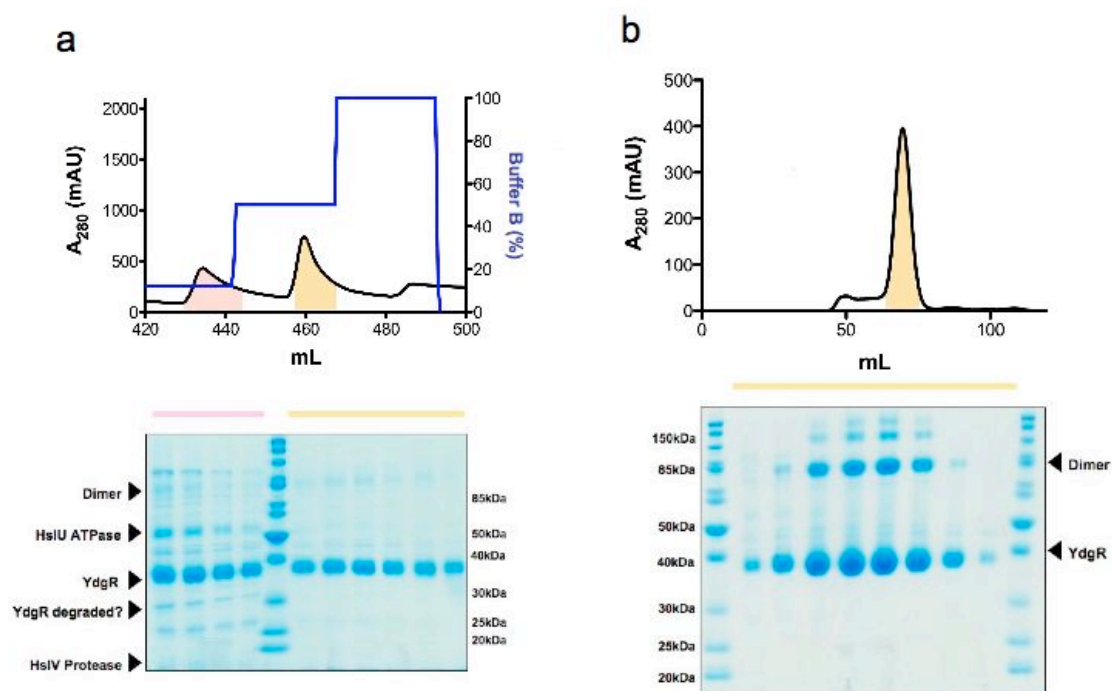


Figure 5.2: a. Chromatogram and SDS-PAGE of Nickel affinity chromatography on His Trap FF 5ml column; b. Chromatogram and SDS-PAGE of gel filtration on Superdex 200 16/60

### 5.1.2 Optimization of Protein Stability and Monodispersity

Initial crystal trials with full length C-term His6 (uncleavable) native YdgR using commercial 96-well screens were set up. Unfortunately only detergent crystals or phase separation was observed (Figure 5.3).



Figure 5.3: Result of initial crystal trials showing detergent crystals in different conditions

Since buffer conditions can affect protein stability, homogeneity, monodispersity and therefore crystallizability [13; 38] the Stargazer assay was used to test thermostability of YdgR in different buffers. The Stargazer is an instrument that measures static light scattering while the target protein is heated up continuously in a 384-well plate. An

increasing static light scattering signal indicates the thermally induced unfolding process of the target protein. The typical experimental outcome is a sigmoidal curve, whose point of inversion is identical to the aggregation temperature of the protein. Stabilizing buffers and additives should inhibit the thermally induced unfolding process and increase the aggregation temperature. Figure 5.4 shows two typical thermal unfolding curves of YdgR.

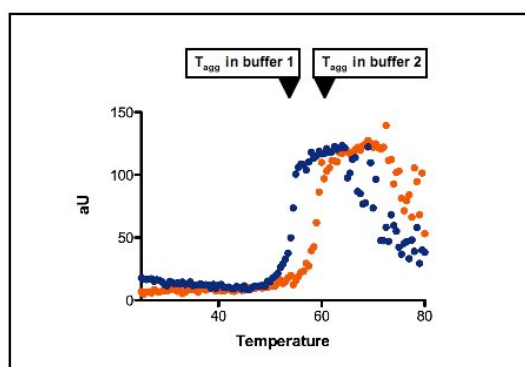


Figure 5.4: Comparison of thermal unfolding curves of YdgR in two different buffers. YdgR shows increased thermostability in the buffer indicated in orange.

To optimize buffer conditions for YdgR, a buffer screen was established that contains 24 different buffers in a pH range of 3-10 (Table 5.1). These buffers are combined with either no salt, 100 mM NaCl, 300 mM NaCl or 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , resulting in a 96-condition screen. Since Tris at pH 8 is used as a standard buffer for purification, the results were compared to the aggregation temperature ( $T_{\text{agg}}$ ) of native YdgR in this buffer. YdgR has an approximate  $T_{\text{agg}}$  of 55°C in Tris pH 8 while its  $T_{\text{agg}}$  is increased by ~3°C in buffers with a pH between 4-5.5 independent of the salt or its concentration while the other buffers do not contribute to protein stabilization.

Table 5.1 : Tested buffers

| Buffers (final conc. 50 mM) | pH  | $T_{\text{agg}}$ | Solubility Kit |
|-----------------------------|-----|------------------|----------------|
| Glycine                     | 3   | -                |                |
| Na-citrate                  | 4   | +3°C             | Precipitate    |
| Na- acetate                 | 4.5 | +3°C             | Precipitate    |
| Na- phosphate               | 5   | +3°C             | Precipitate    |
| Na-citrate                  | 5.5 | +3°C             | NO Precipitate |

| Na-phosphate           | 5.5      | +3°C         | Precipitate |
|------------------------|----------|--------------|-------------|
| MES                    | 5.8      | -            |             |
| Na/K-phosphate 6       |          | -            |             |
| Bis-Tris               | 6        | -            |             |
| MES                    | 6.2      | -            |             |
| Na-phosphate           | 6.5      | -            |             |
| Sodium cacodylate      | 6.5      | -            |             |
| MOPS                   | 7        | -            |             |
| Na/K- phosphate 7      |          | -            |             |
| HEPES                  | 7.5      | -            |             |
| Tris                   | 7.5      | -            |             |
| Imidazole              | 8        | -            |             |
| <b>Tris (Standard)</b> | <b>8</b> | <b>~55°C</b> |             |
| Bicine                 | 8        | -            |             |
| Tris                   | 8.5      | -            |             |
| Bicine                 | 8.5      | -            |             |
| CHES                   | 9        | -            |             |
| CHES                   | 9.5      | -            |             |
| CAPS                   | 10       | -            |             |

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The five potential stabilizing buffers were also tested using the JBS Solubility Kit (Jena Bioscience). This kit is normally used as a pre-screen for crystallization buffers. The buffer is mixed with the target protein in a hanging drop over a buffer reservoir. Depending on the protein's stability in the buffer, aggregation will be visible after 24 hours at room temperature. Only for Na-citrate pH 5.5 was no precipitate visible (as indicated in Table 5.1). Therefore this buffer seems to be suitable for crystallization. To determine the stabilizing effect of different additives, a commercial 96-well Additive Screen™ (Hampton Research) was used. The additives were tested in the Stargazer in combination with 50 mM Na-citrate buffer pH 5.5 as described in Materials and Methods. Figure 5.5 a shows the aggregation temperatures  $T_{agg}$  of a selection of additives. The additives in blue show a stabilizing effect while some additives such as Copper chloride (red) destabilize the target protein. Most of the additives show no effect (black). Out of 96 additives 4 seemed to have a repeatedly observed stabilizing effect. Glycerol,  $CaCl_2$  and Aminohexanoic acid increased the  $T_{agg}$  of the target protein by approximately 2-3° C while NDSB-256 raised the values

by 5-6°C. Since this is a quite strong effect compared with the other stabilizing additives, which should be confirmed unambiguously, the monodispersity of the target protein in a buffer containing NDSB-256 was tested via Dynamic Light scattering. Since the sample monodispersity increased from 16% to 20% when NDSB-256 was added to protein in a 100 mM Citrate buffer pH=5.5, this component was identified as a false positive (Figure 5.5 b). As a control the dipeptide Alanyl-alanine was added to 100 mM Citrate buffer pH 5.5. This dipeptide is a substrate of the target transporter YdgR and should therefore have a stabilizing effect [44; 50; 51]. A decrease of polydispersity from 18% in buffer alone to 12% in buffer containing the dipeptide could be detected (Figure 5.5 b). Therefore the crystallization buffer used for future crystal trials contained 20 mM Na-citrate pH 5.5, 40 mM CaCl<sub>2</sub>, 10% glycerol, 15% Aminohexanoic acid, 25mM Alanyl-alanine and 0.15% DDM.

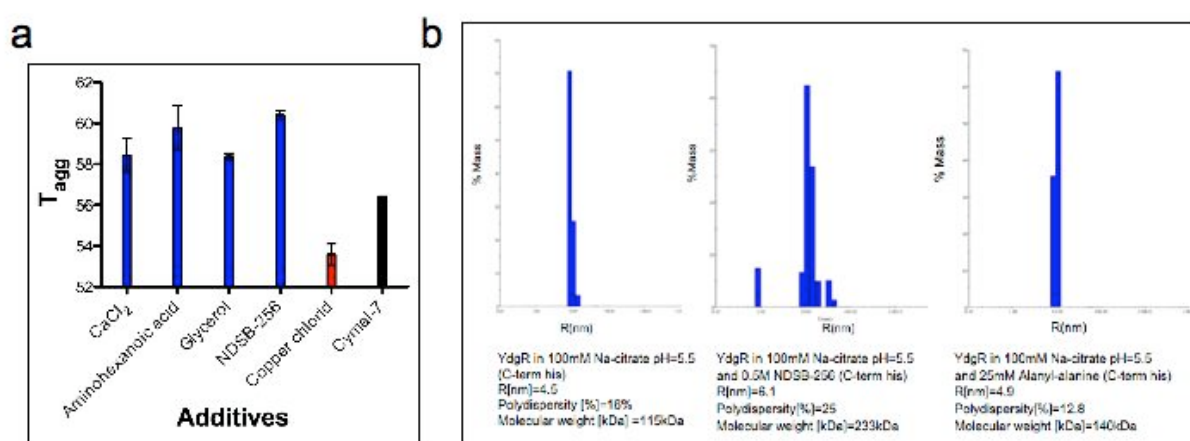


Figure 5.5: a. Aggregation temperatures of YdgR in buffers containing different additives (stabilizing additives in blue; destabilizing additives in red; additives with no effect in black); b. Results of DLS in buffers with NDSB-256 or Alanyl-alanine

### 5.1.3 Crystallization trials with native YdgR

Purified native YdgR was exchanged into the new optimized crystallization buffer and crystal trials were performed in 96-well plates as described in Materials and Methods. The focus of screening was put on commercial crystal screens designed for membrane proteins based on crystal hits of other membrane proteins, namely MemStart/MemSys and MemGold (Molecular Dimensions).

After one week small needles could be observed in condition 2.26 of the MemGold screen containing 50 mM glycine and 55% PEG 400. The needles were quite small and could not be fished for analysis and therefore the first hit had to be repeated and optimized. The optimization procedure was performed in 24-well plates in sitting-drop format. PEG 400 was varied between 40 and 60%. Different buffers at pH=9 were tested but only the pH but not the buffer identity seemed to be important, therefore 100 mM Tris at pH 9 was used for further trials. Since the Stargazer experiments suggested the positive influence of  $\text{CaCl}_2$  on target protein stability, also  $\text{CaCl}_2$  (0-40 mM) and NaCl (0-150 mM) were tested as additives in crystallization. Both salts seemed to have a positive effect on crystallizability and showers of needles and larger needles could be observed. The crystals could be optimized to rod-like structures, due to the increase or decrease of PEG 400 and the addition of  $\text{CaCl}_2$  or NaCl respectively. The largest crystals were grown in 100 mM Tris pH 8 containing 58% PEG 400 and 12 mM  $\text{CaCl}_2$  (Figure 5.6 a).

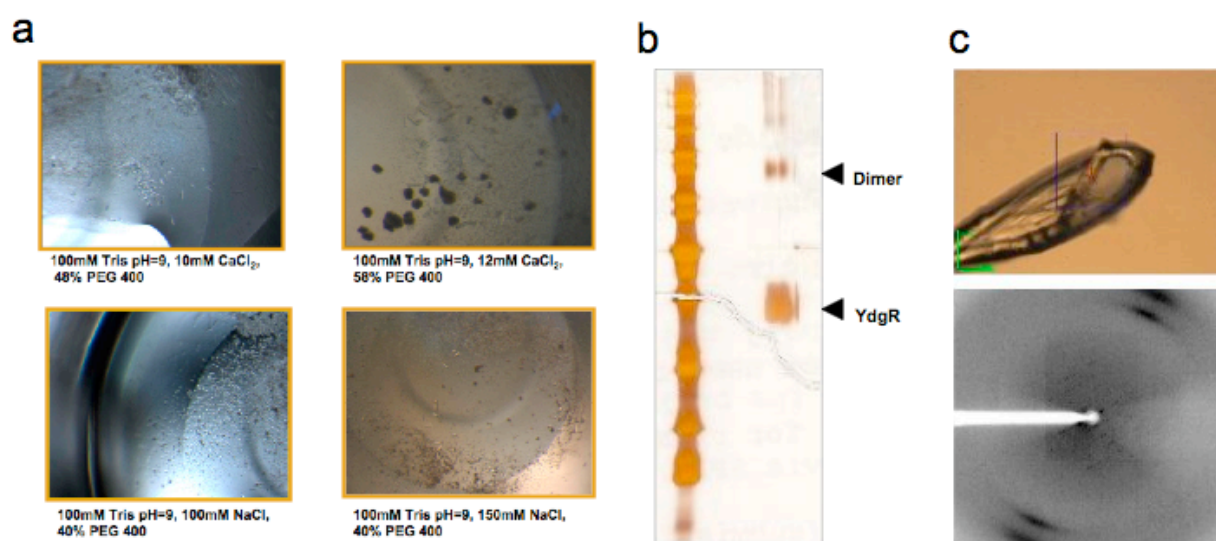


Figure 5.6: a. Optimized crystals; b. Crystals analyzed by SDS-PAGE followed by Silver staining; c. Crystal in a loop at ESRF Grenoble and the resulting diffraction pattern

Some crystals were washed in mother liquor and loaded onto an SDS gel. Since a protein band at the size corresponding to YdgR was visible after silver staining, the crystals consisted of the target protein (Figure 5.6 b). The largest crystals were fished and frozen. No cryo-protectant was necessary because the crystallization condition contained a high percentage of PEG 400. For diffraction analysis, the crystals were

sent to the European Synchrotron Radiation Facility (ESRF) in Grenoble. Unfortunately resolution was limited to 10 Å (Figure 5.6 c). Since a higher resolution is necessary for structure determination, no structural information could be gained, and there were too few reflections to index the images in order to determine the space group and size of the unit cell. Optimization of crystals was continued but no crystals could be observed in the new screens. Additionally crystals could not be reproduced in the known crystallization condition. Several screens were set up with different batches of protein but no crystals could be grown. Since delipidization due to purification conditions can affect the crystallization of membrane proteins, relipidization was performed as described in Materials and Methods, but did not result in any crystals. Also screening with commercial screens was repeated and expanded but did not give any hits.

Therefore new strategies were necessary to increase the probability of growing well-diffracting crystals that could be used for structure determination.

## **5.2 Co- crystallization with monoclonal antibodies**

### **5.2.1 Generation of hybridoma cell lines**

For antibody generation a truncated YdgR construct with a cleavable N- terminal his-tag (*N-terminal His10 YdgR TEV site 6-498*) was used to eliminate the possibility of an immune response to the His 10-tag or to the flexible N and C termini of the protein. As described above in Materials and Methods purified protein was reconstituted into proteoliposomes that were used for immunization of three mice. Additionally three mice were injected directly with purified protein solubilized in detergent alone. To check if immunization resulted in an immune response, diluted bleeds of the mice (1:1000) were analyzed by ELISA and Western Blot (Figure 5.7). Pre-immunization bleeds show low immunological reactivity against YdgR while post-immunization bleed 1 and 2 show a clear response to YdgR in both methods.

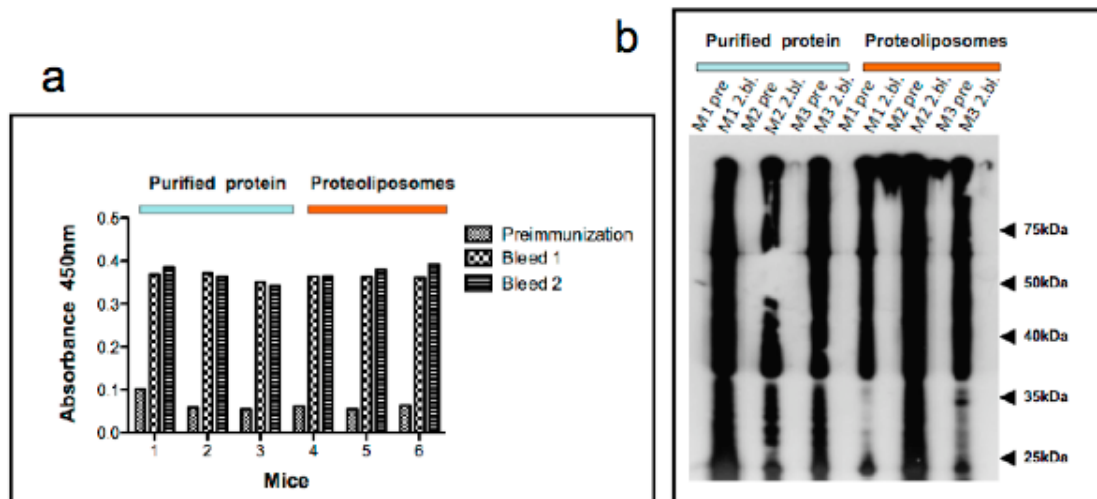


Figure 5.7: ELISA (a) and Western blot (b) analysis of pre-bleed, first, and second bleed of immunized mice

Immunization, splenectomy of mouse 3 immunized with proteoliposomes, fusion with myeloma cells and subsequent production of hybridoma cells were performed by the MFPL antibody facility. As mentioned previously, monoclonal antibodies suitable for co-crystallization with a membrane protein should only detect correctly folded proteins and therefore should not bind to unfolded protein or protein aggregate. Therefore they should be Western blot negative but ELISA positive. The supernatants of hybridoma clone mixtures were tested for their specificity for a conformational intact epitope via ELISA. Out of approximately 500 clone mixtures 23 wells showed a clear positive signal as compared to background ( $> 2$  fold above background signal in other wells). These clone mixtures were tested by Western Blot for their ability to interact with unfolded protein (Figure 5.8 a). Nine showed a clear positive signal for interaction with unfolded YdgR. These mixtures of clones could be ruled out for further processing. There was also a slight signal visible for the other clone mixtures, however, since the clones were not diluted to single clones yet, this was expected.

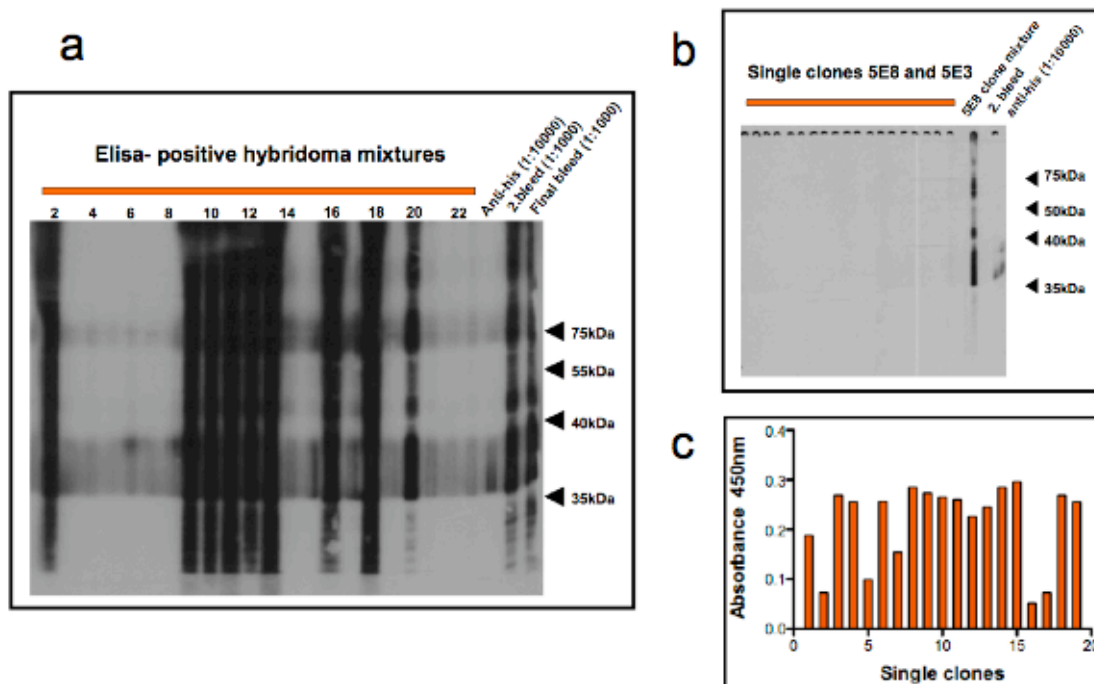


Figure 5.8: a. Western blot analysis of ELISA positive hybridoma mixtures (exposure time of 5 minutes); b. Western blot of single clones derived from hybridoma mixtures 5E8 and 5E3 (Exposure time of 5 minutes); c. ELISA of single clones derived from hybridoma mixtures 5E8 and 5E3

Mixtures 5E3 (lane 4) and 5E8 (lane 5) were selected and diluted to single clones. Nearly all of them were clearly Western blot negative (Figure 5.8 b) and ELISA positive (Figure 5.8 c) and two clones were selected. The cell lines 5E3-F10 and 5E8-D6 were derived by the MFPL antibody facility. Therefore the whole procedure successfully generated two Western-negative and ELISA-positive hybridoma cell lines.

### 5.2.2 Generation and purification of monoclonal antibodies (mAb's)

The hybridoma cell lines 5E3-F10 and 5E8-D6 were injected into two FiberCell™ Hollow Fiber Cartridges (Fiber Cell Systems Inc.). The bioreactors were operated as described in Materials and Methods. The monoclonal antibodies were purified as described in Materials and Methods with a 1 mL Hi Trap Protein G column. Cell supernatants contained high amounts of bovine serum albumin (BSA). This is usually the main impurity present during the purification of mAb's from cell supernatants. Therefore the supernatants were concentrated with VIVASPIN concentrators with a molecular weight cut-off of 100 kDa. Since BSA has a molecular

weight of 68 kDa, quite a lot of BSA could be removed this way, additionally binding of mAb to protein G is improved when sample volumes are low. Nevertheless the applied sample still contained high amounts of BSA that could be easily observed by SDS-PAGE (Figure 5.9 b). Nevertheless, binding to Protein G is highly specific and therefore very pure antibody could be eluted from the column via decrease in pH from 7 to 2.7 (Figure 5.9 a). The results of a typical purification are shown on a reducing SDS gel. Under reducing conditions the disulfide bonds between the heavy chain and the light chain are broken and both run separately on the gel. For 5E3-F10 and 5E8-D6 approximately 4-5 mg/harvest and 1-2 mg/harvest of antibody respectively could be purified from the cell supernatant.

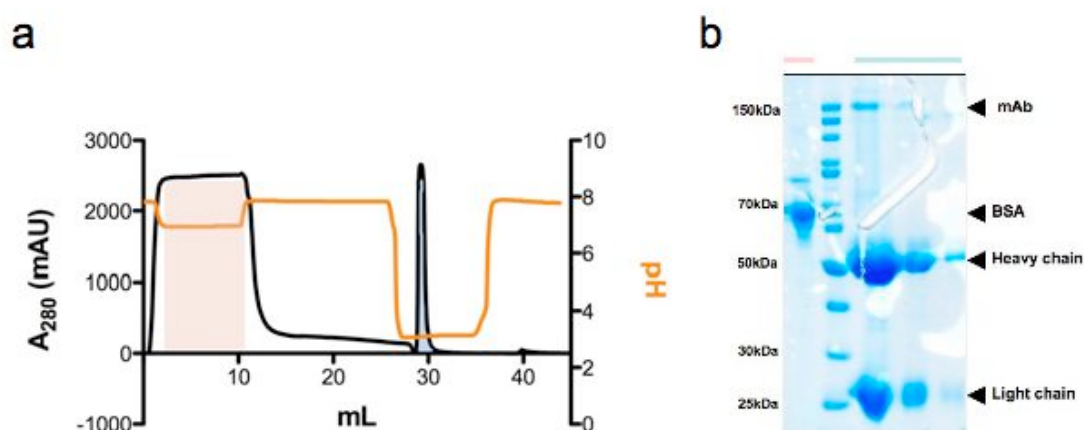


Figure 5.9: a. Typical purification profile from a 1ml Hi Trap Protein G column (loading peak in rose and elution peak in blue); b. Reducing SDS-PAGE of fractions of loading peak and elution peak

### 5.2.3 Complex formation with native YdgR and mutants

The immunization of the mice was performed with proteoliposomes derived from a truncated N-terminal his-tagged cleaved version of native YdgR. Therefore the interaction of other YdgR constructs and mutants with the two monoclonal antibodies was tested via analytical size exclusion on a Superose 6 column (GE Healthcare) before co-crystallization trials were set up.

Due to the detergent micelle surrounding YdgR, YdgR and the monoclonal antibodies show quite similar elution profiles on a Superose 6 column with peak maxima at 1.67

ml and 1.64 ml respectively. In cases where YdgR and mAb interacted and formed a complex, the complex eluted at  $\leq 1.5$  ml. (Figure 5.10 a). In the experimental set-up an excess of mAb was added. Therefore an additional peak for mAb could be observed even after complex formation.

As expected, mAb 5E8-D6 formed an antigen-antibody complex with the N-terminal his-tagged cleavable version of native YdgR (Figure 5.10 b), independent of the removal of the His-tag with TEV (data not shown) while absolutely no interaction was visible with either the C-terminal His-tagged native construct or any mutant constructs (Figure 5.10 c-g). The mutants are described and characterized in detail in chapter 5.3. This antibody seems to be specific for native YdgR. Most probably the epitope is located in a loop that is not accessible in the mutants. Additionally the C-terminal His-tag seems to block the epitope on YdgR and no interaction with 5E8-D6 can be observed.

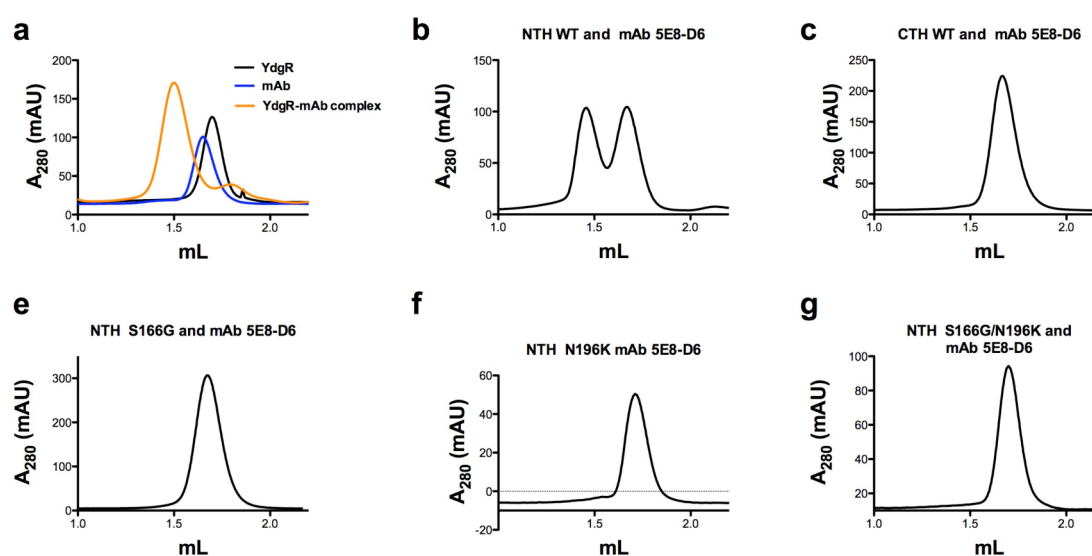


Figure 5.10: Size exclusion profiles of different constructs with mAb 5E8-D6 on a Superose 6 column

5E3-F10 was also tested with different constructs of native and mutant YdgR. Antibody-antigen interaction could be observed for all N-terminal and C-terminal His-tagged constructs (Figure 5.11 a-h). S166G, N196K as well as the double mutant were detected by the antibody, independent of His-tag location or removal (Figure 5.11 d-h). This antibody seems to interact with a more general or more generally exposed epitope on YdgR and its related mutants.

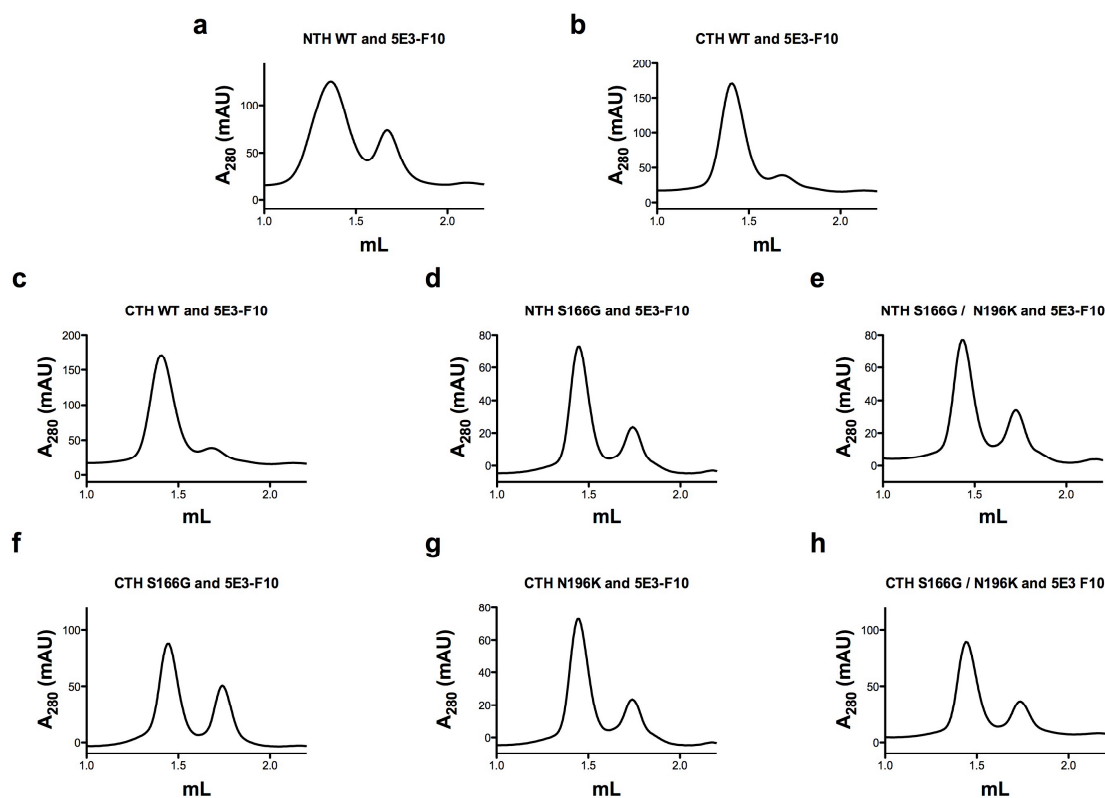


Figure 5.11: Size exclusion profiles of different YdgR constructs with mAb 5E3-F10 on a Superose 6 column

## 5.2.4 Generation and purification of monoclonal Fab fragments

For co-crystallization, the monoclonal antibodies had to be cleaved to generate monoclonal Fab (mFab) fragments. The monoclonal antibodies contain flexible regions between the Fab and the Fc region that make crystallization of a full mAb quite difficult, while monoclonal Fab fragments are ordered and compact. Normally mAb are cut for this purpose with papain endoprotease. Both antibodies were classified as IgG1 antibodies by the MFPL antibody facility. This subtype is difficult to cut with papain but ficin endoprotease normally gives good results [4]. Therefore a Mouse IgG1 Fab and F(ab')<sub>2</sub> Preparation Kit (ThermoScientific) based on ficin cleavage was used for both antibodies. The protocol was performed as described by the manufacturer, but the incubation times for the cleavage had to be optimized. Generally the mAb were cleaved for 4 hours. Longer cleavage times support further digestion of monoclonal Fab fragments even if the digest of mAb is not complete.

Due to the overdigestion, no interaction between the antigen and mAb fragments can be detected anymore.

Fc fragments and undigested mAb were separated from monoclonal Fab fragments with a Protein A Spin column. In contrast to Protein G, Protein A does not interact with Fab fragments and they can be found in the flow through as shown in Figure 5.12 using non-reducing SDS-PAGE. Since the disulfide bonds are intact, the Fab fragment runs at its actual size of ~50kDa.

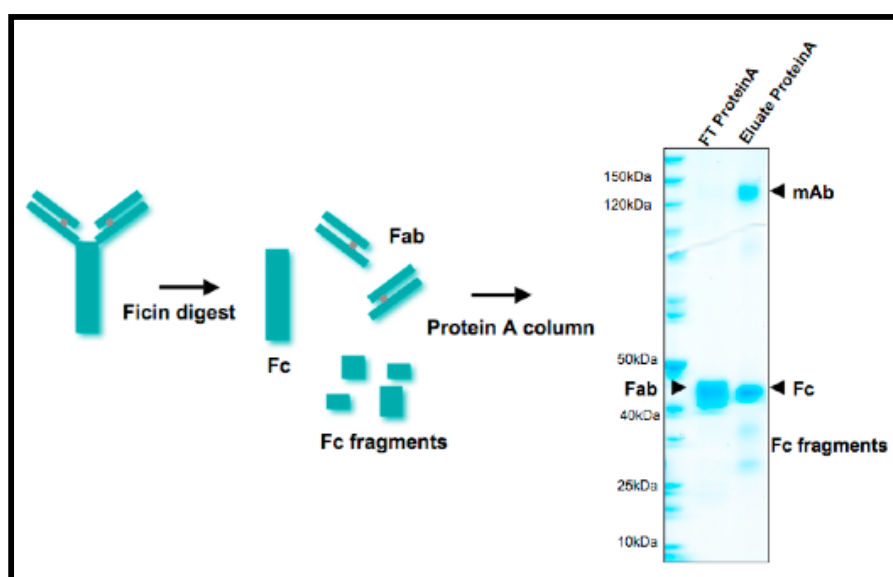


Figure 5.12: Digest of mAb and separation of Fab fragments and Fc fragments using a Protein A column (non-reducing SDS-PAGE)

### 5.2.5 Preparation of co- crystallization complex

YdgR was purified as described above and was concentrated to 3.5 mg/ml in the size exclusion buffer. Monoclonal Fab fragment was added in a ratio of 1:2. The complex and YdgR alone eluted at a quite similar elution volume, therefore an excess of mFab fragment was added to saturate the binding to YdgR. The sample was loaded on a Superdex 200 10/300GL column or a HiLoad Superdex 200 16/60 column (GE Healthcare). This rapid size exclusion step was used to create an equimolar complex and suitable crystallization sample, in which the excess of monoclonal Fab fragments was removed (Figure 5.13).

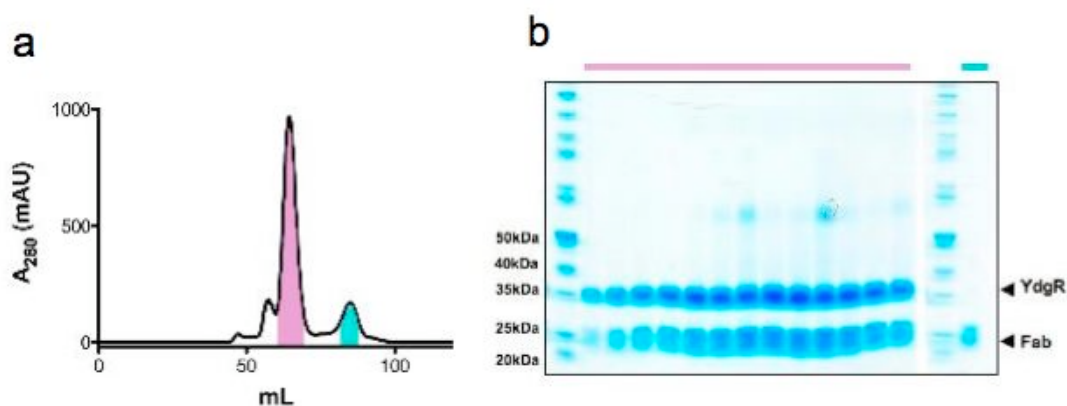


Figure 5.13: a. Size exclusion of YdgR with mFab on HiLoad Superdex 200 16/60 column (YdgR-mFab complex in rose and mFab alone in blue); b. SDS-PAGE analysis of different fractions.

### 5.2.6 Co-crystallization trials of YdgR with mFab 5E8-D6

The fractions containing the complex were concentrated to 6.5 mg/ml and used for crystal trials. A large number of different commercial 96-well crystal screens were used: JBS 1-4 (Jena Bioscience), JBS 5-9 (Jena Bioscience), Cryo 1+2 Crystallization Screen (Emerald Biosystems), Crystal Screen (Hampton Research), PGA screen (Molecular Dimensions), Index (Hampton Research), Wizard 1+2 (Emerald BioSystems), Salt Rx (Hampton Research), MemFac (Hampton Research), PEG (Hampton Research), Natrix (Hampton Research) and JBS 9-10 (Jena Bioscience).

For the complex of YdgR with 5E8-D6, two hits could be observed after 3 weeks in the Cryo 1+2 Crystallization Screen (Emerald Biosystems) in condition A6 (100 mM Cacodylate pH 6.5; 40% PEG 600; 0.2 M Na-acetate) and H10 (100 mM CAPS pH 10.5; 30% PEG 200; 200 mM Ammonium sulfate). The crystals in both hits absorbed at 280 nm under the UV lamp in a Crystal Hotel imaging system (Rigaku), which identified them as protein crystals. Both hits were sent to the ESRF synchrotron in Grenoble. The crystals derived from condition H10 did not diffract at all while the crystals grown in A6 diffracted to 8-10 Å. To improve diffraction, a 96-well format fine screen of condition A6 was set up. The grid screen screened for optimal pH (between 5.3 and 7.2) and optimal PEG 600 concentration (32-46%). Best crystals could be grown in a pH range of 5.5- 6.2 and a PEG 600 concentration of 36-42% (Figure 5.14). Higher pH increased the tendency for precipitation and higher PEG concentrations increased the probability for micro-cystals in a shower of crystals.

Unfortunately the optimized and larger crystals did not increase diffraction and therefore no data better than 8 Å could be collected.

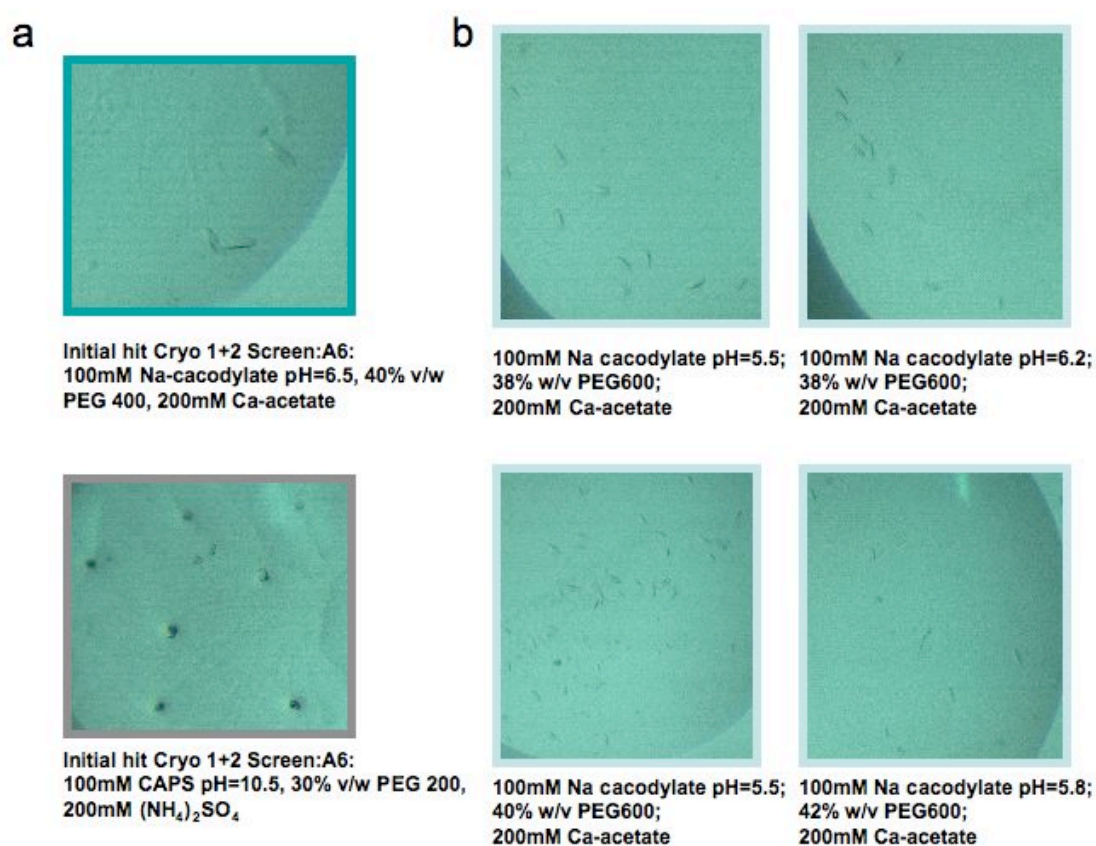


Figure 5.14: a. Initial hits of complex of mFab 5E8-D6 with YdgR; b. Optimized crystal conditions derived from Cryo1+2 Crystallization Screen

### 5.2.7 Co-crystallization trials of YdgR with mFab 5E3-F10

For 5E3-F10 different YdgR constructs were used for co-crystallization. Native YdgR with a C-terminal His-tag or N-terminal cleaved His-tag was mixed with mFab 5E3-F10. Additionally, N-terminal cleaved His-tagged S166G and N196K/S166G were used together with mFab 5E3-F10 for co-crystallization. These mutants are described further in chapter 5.3. The samples were prepared as described for co-crystallization with mFab 5E8-D6.

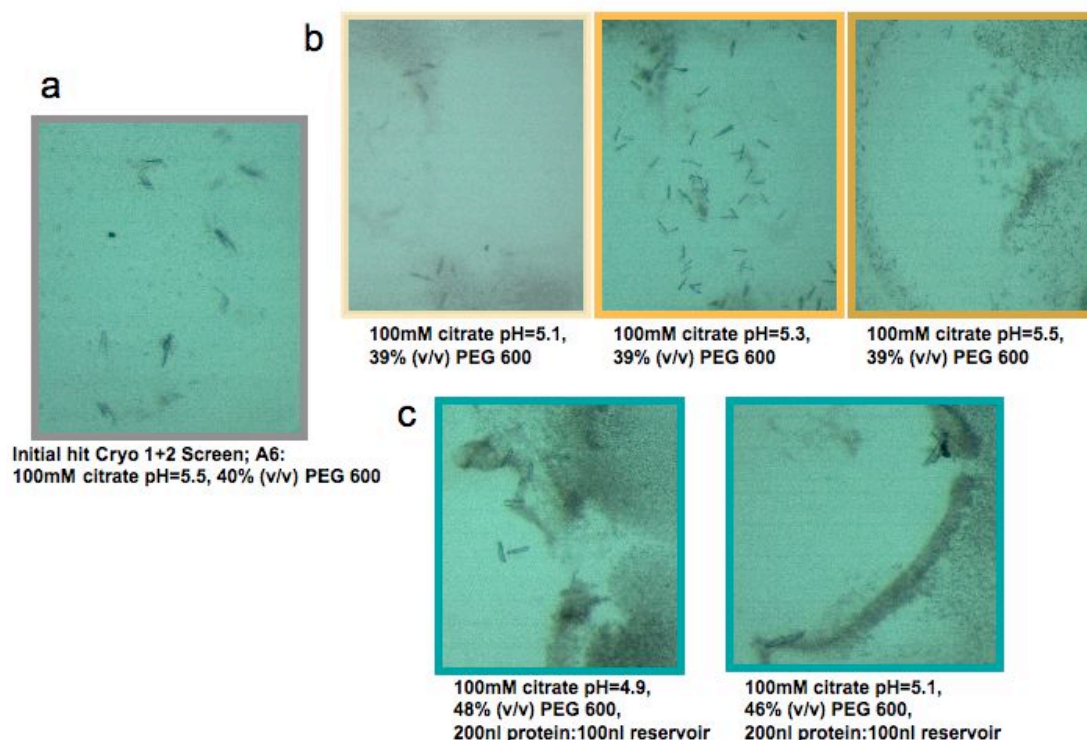


Figure 5.15: a. Initial hit Cryo 1+2 Screen; b. Change in crystal morphology due to pH change; c. Optimized crystals sent to ESRF

Crystals could be observed after approximately 2 weeks for the complex between C-terminal His-tagged native YdgR with mFab 5E3-F10 in the Cryo 1+2 Crystallization Screen (Emerald Biosystems) in condition G6 containing 100 mM citrate pH 5.5 and 40% (v/v) PEG 600 (Figure 5.15 a). Optimization was performed using a 96-well format grid screen based on variation of pH and PEG 600 concentration. Crystallization seemed to be quite dependent on pH. Slight changes in pH generated single crystals or a shower of micro-crystals (Figure 5.15 b). Additionally it seemed to improve crystal morphology to use drop ratios of protein:reservoir of 2:1 instead of 1:1. This increased the PEG 600 concentration necessary to induce the crystallization process and larger crystals could be generated (Figure 5.15 c).

The crystals were sent to the ESRF synchrotron Grenoble (Figure 5.16), but the diffraction was limited to 8-10 Å and therefore no structural information could be gained.

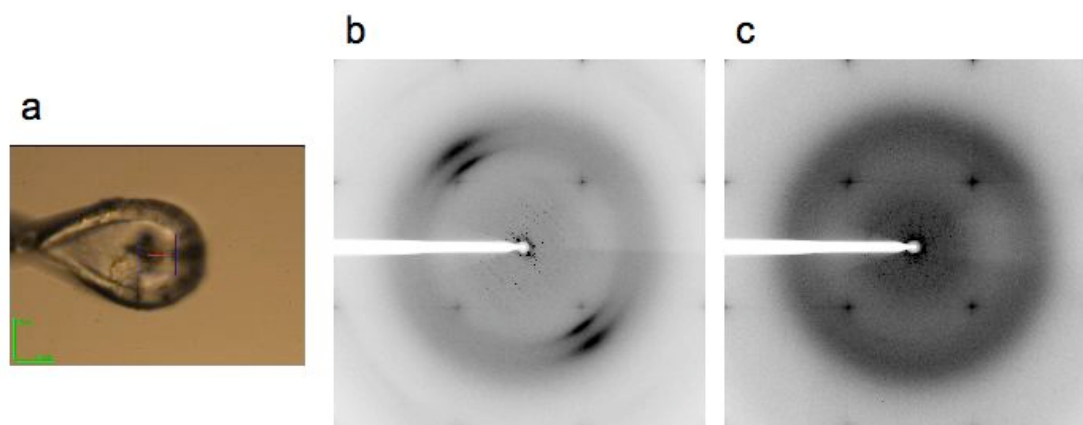


Figure 5.16: a. Crystal mounted at the ESRF; b. and c. Diffraction pattern of co-crystal of YdgR with mFab 5E3-F10 (condition 100mM citrate pH=5.1; 46% (v/v) PEG 600; 200nl protein:100nl reservoir)

## 5.3 Thermostable mutants

### 5.3.1 Analysis of thermostability of loss-of-function mutants via Stargazer

Previous work from our laboratory has identified 35 loss-of-function mutants in YdgR using random mutagenesis ([35]; see Appendix). To evaluate the YdgR mutants for thermostability we chose to measure protein aggregation by static light scattering, as employed by the Stargazer instrument. In order to determine whether the Stargazer is an appropriate method to identify mutants that will show an increase in thermostability, the native LacY protein and the thermostable LacY mutant C154G were tested as a control and the difference in  $T_{agg}$  was plotted. (Figure 5.17 a)

Sixteen of the identified 35 YdgR mutants were then purified and tested in the Stargazer at two different protein concentrations. In order to compare the  $T_{agg}$  of the mutants to that of native YdgR, the difference in  $T_{agg}$  between the mutant and native proteins was calculated (Figure 5.17 b).

Five mutants, N196K, V252E, K274I, A285V, and M295K showed an increase in  $T_{agg}$  as compared to native YdgR of more than  $+2^{\circ}\text{C}$ . Repeated testing confirmed that N196K, V252E, K274I, M295K showed a reproducible increase in  $T_{agg}$  (Figure 5.17c).

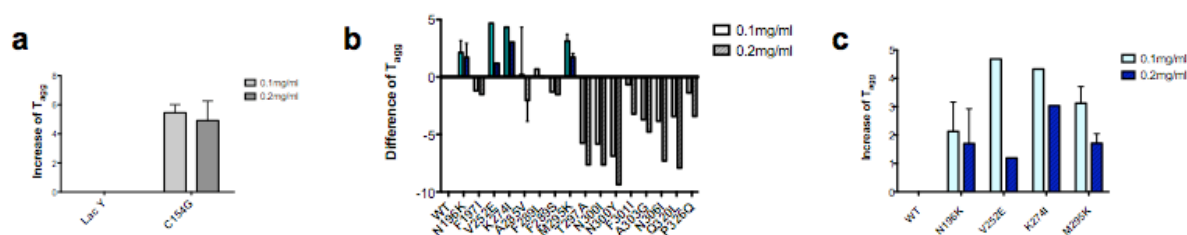


Figure 5.17: a. Differenc in  $T_{agg}$  between Lac Y and C154G b. Summary of differences of  $T_{agg}$  of all 16 mutants compared with native YdgR ; c.  $T_{agg}$  increase of thermostable mutants

### 5.3.2 Size exclusion thermostability assay

To further evaluate the thermostability of N196K, V252E, K274I and M295K, these mutants were also tested using an alternative thermostability assay. We developed a size exclusion-based assay to assess the amount of protein still folded after incubation at high temperature by comparing the size and shape of the proteins' size exclusion peak after incubation at 4° C and 50° C. The thermostable mutants are expected to show a higher amount of folded protein remaining after this treatment in comparison to the native protein. Again, LacY and the C154G mutant were used as controls in the size exclusion assay. While native LacY shows a clear decrease in the amount of folded protein remaining after incubation at 50° C, the C154G mutant is completely stable, and shows no decrease in peak size (Figure 5.18).

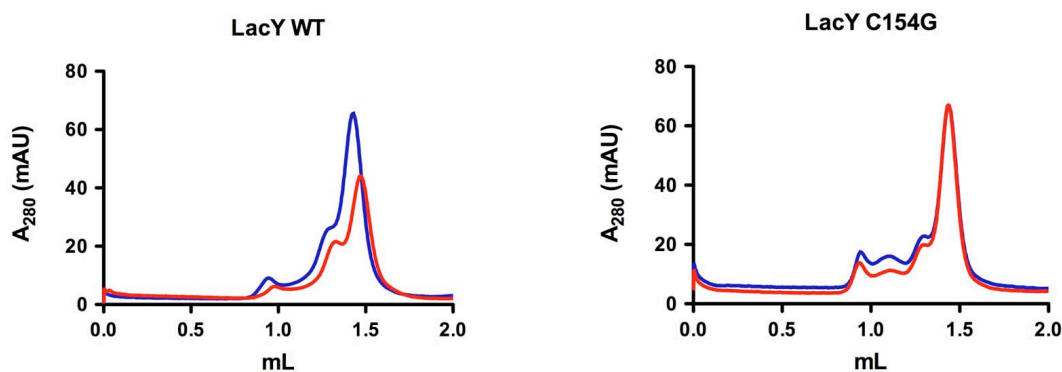
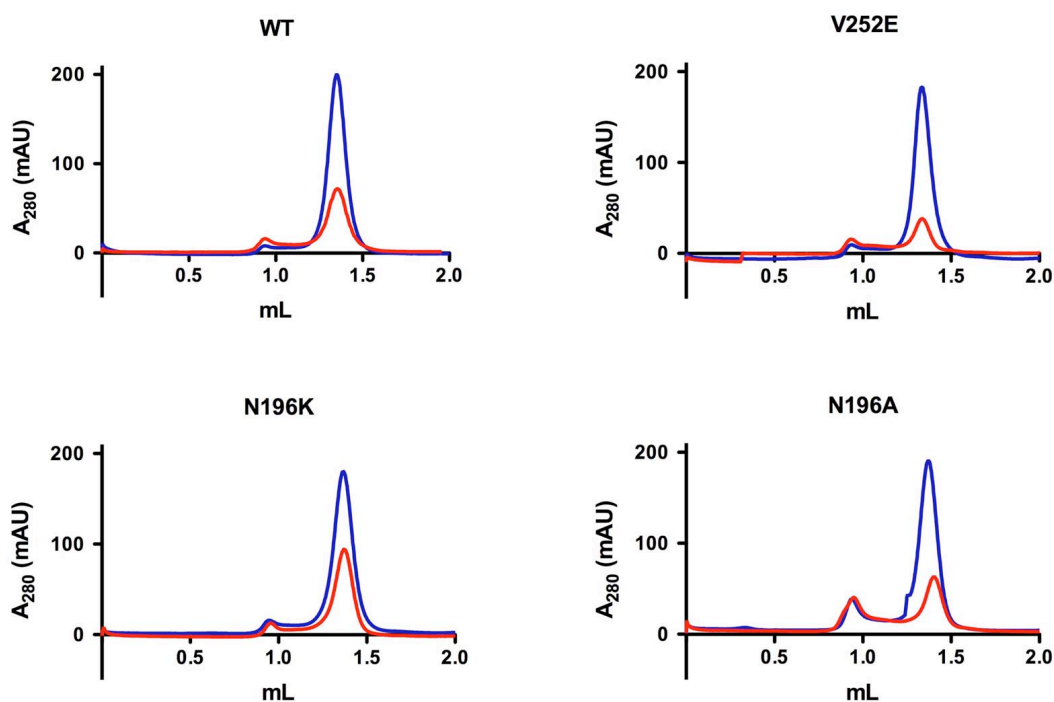


Figure 5.18: Size exclusion profile of LacY and LacY C154G incubated for 5vminutes at 4°C (dark blue) or 50°C (red)

Next, the native YdgR protein and the mutants were incubated at 4° C and 50° C and loaded onto the Superdex 200 size exclusion column. Native YdgR incubated at 4° C shows a sharp peak while the incubation at 50° C does not change the shape of the peak, but dramatically reduces the fraction of folded protein (Figure 5.19). For the mutants identified as more stable in the Stargazer assay, exclusively N196K showed increased thermostability in the size exclusion assay (Figure 5.19). The fraction of folded protein after incubation at 50°C was increased from 38% to 50% as compared to native YdgR and no aggregates were observed. The V252E mutant also shows a homogenous and sharp peak after the incubation at 4°C, but the high temperature treatment dramatically reduces the fraction of folded protein (Figure 5.19). The K274I and M295K mutants show an extensive reduction in the folded portion of protein even after incubation at 4°C (Figure 5.19). Additionally aggregates could be observed that were not detected for the native protein.

Since it has been proposed that mutations to alanine are most likely to increase thermostability [52], the N196A mutant was generated and also tested in the size exclusion assay. After incubation at 50° C the mutant shows stability comparable to native YdgR. No increase in thermostability could be observed (Figure 5.19).



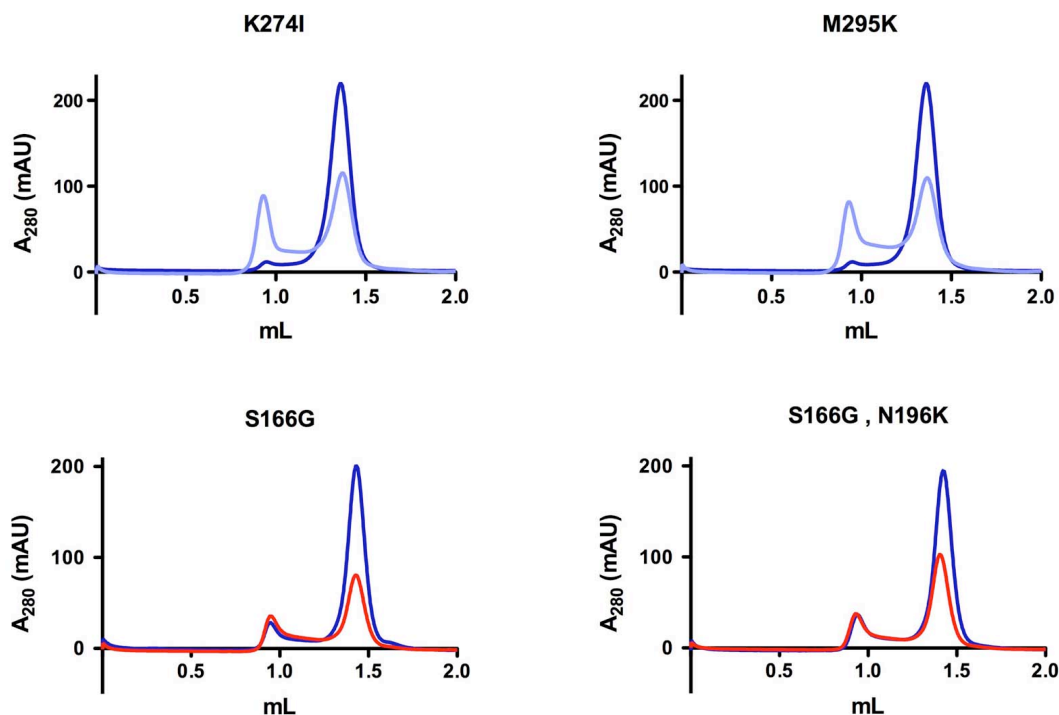


Figure 5.19: Size exclusion profiles of WT and mutants incubated for 5 minutes at 4°C (dark blue) or 50°C (red); K274I and M295K incubated for 5 minutes at 4°C (light blue) in comparison with WT after 5 minutes on 4°C (dark blue)

Generation of a homology model between LacY and YdgR [35] revealed the residue S166 to be in an equivalent position to C154 of LacY. Therefore the YdgR mutant S166G was generated in order to characterize its thermostability. The S166G mutant shows an increase in the amount of folded protein remaining after incubation at 50°C, but seems to be slightly less thermostable than N196K (Figure 5.20).

The double mutant S166G/N196K was also generated and tested to determine whether the effect of both mutations would be additive. The double mutant shows an increase in thermostability as compared with native YdgR and the single mutants (Figure 5.20).

The peak area of folded protein after incubation at 4°C and 50°C were compared and plotted in a graph as fraction of folded protein (%) after partial thermal unfolding. While the WT showed a reduction to ~38% folded protein, for the mutants S166G, N196K and the double mutant an increase to ~42-55% of folded protein could be observed. For the double mutant approximately 55% are still properly folded after 5 minutes at 50°C.

This stabilizing effect was compared with LacY and the thermostable mutant C154G. The fraction of folded LacY was reduced to ~50% by thermal stress while more or less all of C154G kept its stable and folded conformation after incubation at 50°C. Compared with S166G, which is supposed to be in an equivalent position in YdgR, C154G showed a much more dramatic improvement of thermostability (Figure 5.20).

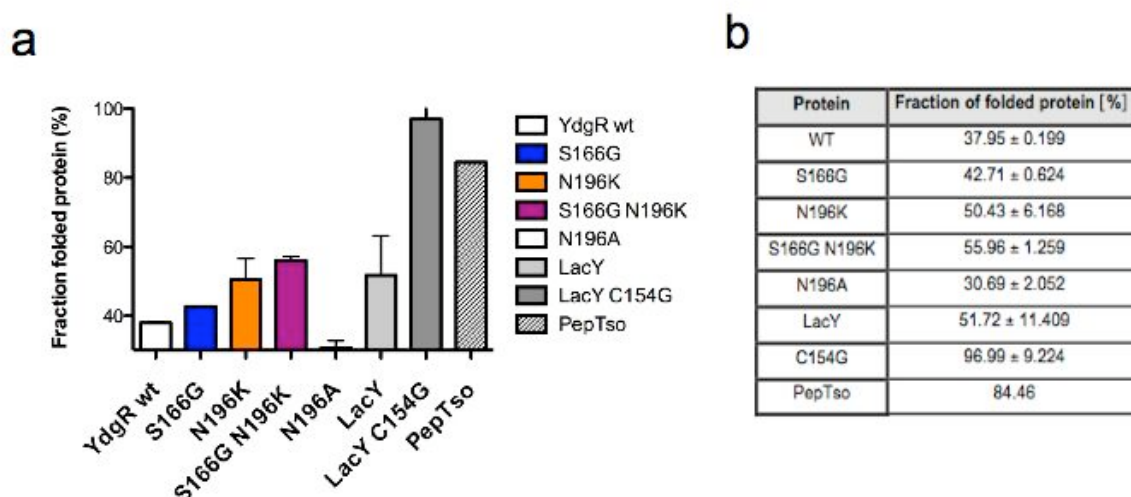


Figure 5.20: Graph (a) and Table (b) fraction of folded protein (%)

Additionally the thermostability of PepT<sub>so</sub> was tested with this assay. As mentioned above, PepT<sub>so</sub> is a prokaryotic homolog of the mammalian peptide transporters from *Shewanella oneidensis*. PepT<sub>so</sub> was crystallized and the structure was solved in 2010 [39]. This assay allowed the comparison of thermostability of the *E. coli* homolog YdgR with the *Shewanella oneidensis* homolog PepTso of mammalian peptide transporters. For PepT<sub>so</sub>, ~84% of the protein remained folded while for YdgR only ~38% of folded protein could be detected (Figure 5.20, Figure 5.21).

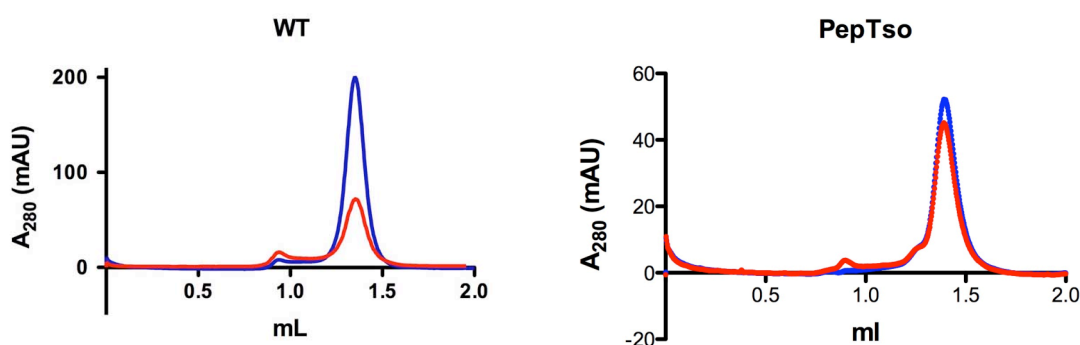


Figure 5.21: Gel filtration profile of YdgR WT and PepTso

### 5.3.3 CPM assay

A major question concerning the crystallizability of membrane proteins is the stability of membrane protein targets in different detergents, since short length detergents seem to be better able to support the formation of well diffracting crystals [36] but often are not tolerated by membrane proteins. Therefore we tested the stability of S166G, N196K and S166G/N196K in different detergents (DDM, NTM and LDAO) using a CPM fluorescence-based thermostability assay [3; 57]. This assay is based on the interaction of the thiol-reactive fluorescent dye CPM with cysteine residues, which should be deeply buried within the protein, but which become accessible during the unfolding process. The native and mutant proteins were diluted 100-fold into a new detergent and the change in fluorescence was monitored over 150 minutes during incubation at 40° C. The fraction of folded protein remaining at each time point was calculated and graphed (Figure 5.22 a).

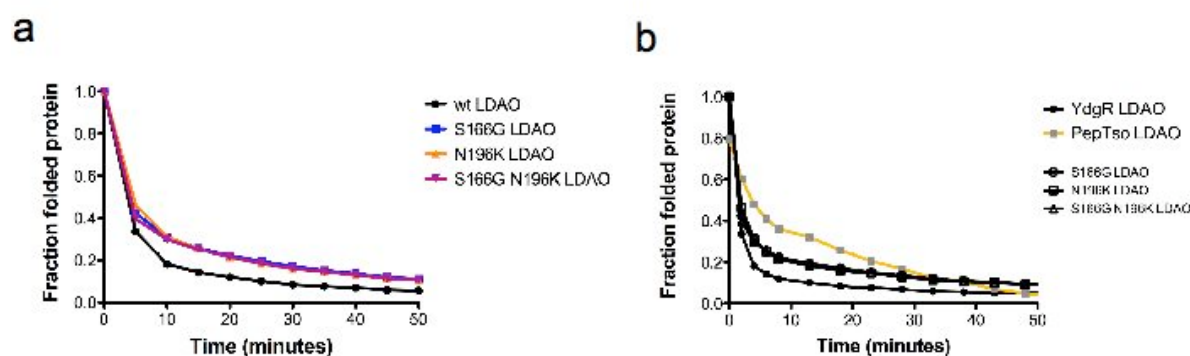


Figure 5.22: a. Fraction of folded protein of native YdgR and thermostable mutants plotted against time; b. Fraction of folded protein of native YdgR, thermostable mutants and PepTso plotted against time

For incubation in the detergent LDAO a clear difference between the native protein and the mutants is visible, and the half-life is approximately doubled. The double mutant shows a similar effect but surprisingly the degree of stabilization is comparable to the single mutants and not increased, as suggested by the gel filtration data.

When the result of the CPM assay with native YdgR and the mutants were compared with the fraction of folded PepT<sub>so</sub> in LDAO (Figure 5.22 b), it was observed that PepT<sub>so</sub> has a 9 to 10 times longer half-life in LDAO as compared with the

thermostable YdgR mutants. Both the higher thermostability and the higher tolerance to short length detergents of PepTso compared to YdgR could make it a better potential candidate for crystallization.

#### 5.3.4 Examination of transport activity of the thermostable mutants

While the N196K mutant has been previously characterized and has been shown to have a partial loss of transport activity [35], the S166G mutant has not been characterized. Therefore the mutants were characterized regarding membrane localization and transport activity using two different transport assays. Since the mutant Y156A is known to be inactive [65] it was used as a control in the transport assays. Membrane localization was tested by analysis of equal concentrations of membrane fractions by Western blot. All constructs are expressed in equal amounts and are suitable for comparison of transport activity in living cells (Figure 5.23).

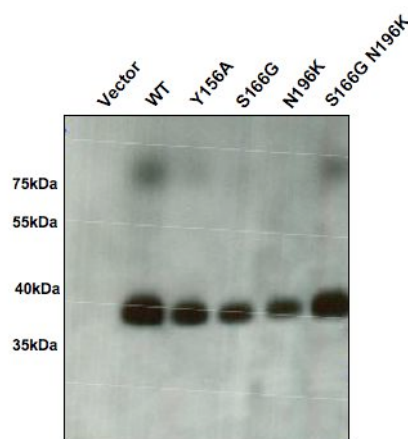


Figure 5.23: Western Blot of membrane fractions (exposure time 10 seconds)

Transport was first tested by growth in the presence of the antibiotic alafosfalin, which is a specific substrate of YdgR [9]. Bacteria expressing the functional transporter will die while those possessing an inactive transporter can grow without inhibition. When bacteria expressing each construct are grown without alafosfalin, no reduction of growth rate can be observed for native YdgR, the empty vector, or any of the mutants (Figure 5.24 a). When this growth assay is carried out in the presence of alafosfalin the growth rate of native YdgR is reduced after induction, due to expression of the functional transporter, while there is no inhibition of growth for

bacteria expressing the empty vector or the non-functional mutant Y156A. S166G, N196K as well as the double mutant S166G/N196K show a clear growth advantage as compared to native YdgR (Figure 5.24 b). Therefore all of these mutants are unable to take up alafosfalin.

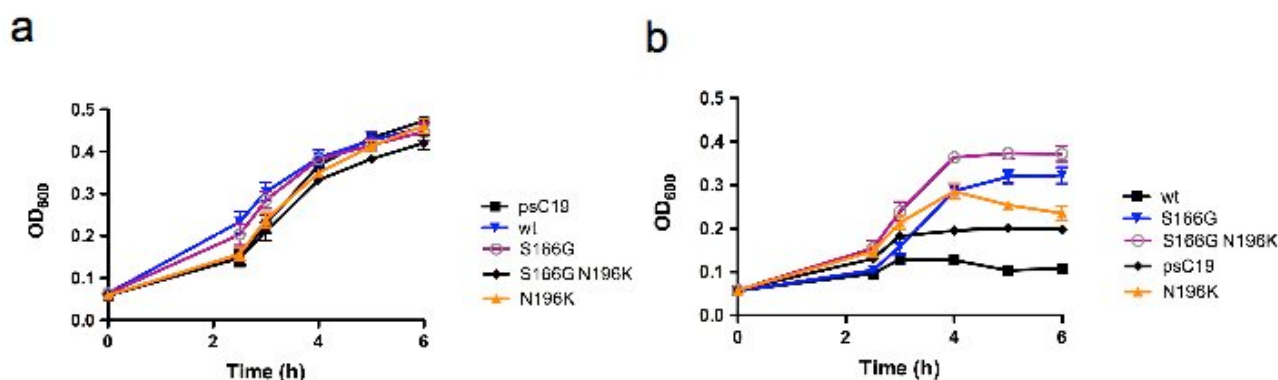


Figure 5.24: Growth assay performed without (a) or with (b) alafosfalin

Next, the uptake of  $\beta$ -Ala-Lys (AMCA), a dipeptide covalently bound to a fluorescent moiety, was tested (Figure 5.25). Native YdgR clearly shows uptake of this peptide while no increase in fluorescence is observed for bacteria expressing the empty vector or the non-functional mutant Y156A. The transport activity of S166G for  $\beta$ -Ala-Lys-AMCA was reduced dramatically while the mutant N196K shows partial transport activity. This activity is reduced in the double mutant to a value comparable to that of S166G. Data from both assays indicates that S166G and the double mutant are non-functional transporters while N196K shows some ability to transport  $\beta$ -Ala-Lys(AMCA).

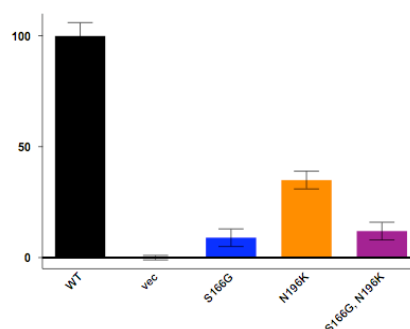


Figure 5.25: Uptake of  $\beta$ -Ala-Lys(AMCA). The transport of WT YdgR was set to 100%.

### **5.3.5 Crystallization trials with thermostable mutants**

Crystal trials of S166G, N196K and the double mutant were set up. Unfortunately no crystals could be observed.

### **5.3.6 Thermostable mutants adopt a single conformation**

As mentioned in chapter 5.2.3 one of the monoclonal antibodies generated for co-crystallization purpose is unable to recognize the mutants S166G and N196K, as shown by the lack of complex formation during gel filtration chromatography (Figure 5.10). As neither residue is predicted to be localized in an extramembrane loop, this suggests that these mutations cause the transporter to preferentially adopt a single conformation in which the antigen binding site is altered and is thereby inaccessible to the antibody.

In order to investigate this phenomenon further, we performed acetylation of accessible lysines coupled to mass spectrometry analysis to identify lysine residues in YdgR that show altered accessibility between the native transporter and the N196K and S166G mutants. This experiment was repeated several times and the digest for mass spectrometry was performed with either chymotrypsin or subtilisin to reach high sequence coverage. The reproducible differences in accessibility of lysines of native YdgR and the mutants were localized in the YdgR homology model [35] to determine their position.

Five lysine residues repeatedly show a difference in acetylation in the comparison between native YdgR and the thermostable mutants. Most interesting, only one of them, namely K177, is predicted to be located in a periplasmic loop while the others are close to the cytoplasmic side. Additionally this loop shows a dramatic difference in lysine accessibility. While the average acetylation of K177 was 0.4% for the wild-type, this value drops to zero for the thermostable mutants (Figure 5.26). That would mean that at least part of the loop between Helix 5 and 6 is not accessible for acetylation in the thermostable mutants, suggesting that the mutants are locked in the inward-facing conformation, burying the loop between Helix 5 and 6. Another lysine residue that showed a quite drastic difference in lysine acetylation pattern is K131.

While this residue is highly accessible in S166G, the wild-type and N196K proteins show a reduced average lysine acetylation. This could be due to different mechanisms of locking the mutant in one conformation. In general the lysine acetylation pattern of N196K is quite similar to the native YdgR with the exception of K177. S166G shows generally an aberrant average acetylation pattern when compared to native YdgR. Generally the lysine residues close to the cytoplasmic side show higher accessibility while the residue in the periplasmatic loop was completely buried.

In order to confirm this data and determine whether the mutants are indeed locked in the inward-facing conformation, we plan to design cysteine mutants of the potential periplasmic gating residues Glu 56 and Arg 305 in YdgR [35]. They should approach in the inward-facing conformation but their distance is increased in the outward-facing conformation. Cystein- crosslinkers of different length will be used to test the distance of these mutants in native YdgR and the mutants S166G and N196K. Cysteine cross-linking was also used to determine the gating residues of Lac Y [67; 69].

| Lysine residue | Location in model                                 | Close to         | Average acetylation (%) |       |       |
|----------------|---------------------------------------------------|------------------|-------------------------|-------|-------|
|                |                                                   |                  | WT                      | S166G | N196K |
| 8              | N- terminus                                       | Cytoplasmic side | 40                      | 80    | 40    |
| 131            | Helix 5                                           | Cytoplasmic side | 20                      | 48    | 20    |
| 177            | Loop between Helix 5 and 6                        | Periplasmic side | 40                      | 0     | 0     |
| 343/353        | Loop between Helix 8 and 9(343) and Helix 9 (353) | Cytoplasmic side | 26                      | 46    | 32    |
| 482            | C- terminus                                       | Cytoplasmic side | 31                      | 68    | 73    |

Figure 5.26: Summary of differences in lysine acetylation pattern

## 6 Discussion

YdgR is a prokaryotic member of the Peptide Transporter (PTR) family. This family is driven by a proton gradient and is widespread throughout all organisms from bacteria to man [9]. This prokaryotic transporter was chosen as a good candidate to study this family of transporters as well as to test two strategies to facilitate crystallization since formation of well-ordered crystals is still a major bottleneck in studies on integral transmembrane transporters.

### 6.1 Crystallization trials of native YdgR gave rise to unreproducible crystals

Crystallization trials of the native target protein gave rise to crystals diffracting to 10 Å. This poor diffraction is typical for 3D Type 2 crystals, especially for integral membrane proteins that do not have large soluble domains. The crystal contacts are then solely made by small accessible areas that are not covered by the detergent micelle. Contacts between the polar head groups of the detergent molecules of neighboring proteins can also support crystal formation, but contribute poorly to protein ordering in the crystal since the interactions are unspecific [43].

In addition to diffracting poorly, the crystals could not be reproduced. Since crystal formation is dependent on many different components and environmental influences, it is hard to determine the variables responsible for failed crystal formation. For the crystallization of the native lactose permease LacY, it was shown that *E. coli* phospholipid extracts were necessary for successful crystallization [20]. The solubilization of the target protein from the membrane with detergent does not result only in protein-detergent micelles, but also lipids are co-extracted from the membrane. Therefore every purification step can also be regarded as a delipidization step. Co-extracted lipids are removed to a different extent depending on the purification strategy. The effect of varying amounts of co-solubilized lipids resulting from different purification strategies and their effect on crystal quality could be observed for the glycerol-3-phosphate transporter GlpT [33].

In addition to lipid content, detergent content of the protein sample can vary from batch to batch and therefore the sample may or may not give rise to crystals [20]. The role of detergent content in the crystallization of membrane protein samples is known for quite some time. For bacteriorhodopsin it was shown that crystallization only occurred in drops where phase separation of high or low content detergent phases was prevented [36]. For crystallization of porins the high content detergent phase was necessary to give rise to crystals [36]. For other membrane proteins such as the glycerol-3-phosphate transporter GlpT, a too high content of detergent prevented crystallization [33].

Therefore slight changes in detergent or lipid content, resulting from different batches of purified protein seems to prevent crystallization. This might be true in the case of YdgR, since the crystals could only be grown and optimized with one specific batch of protein. However, relipidization and addition of E.coli lipid extract before crystallization trials of YdgR did not support crystal formation.

## **6.2 Two antibodies against YdgR were generated**

Two monoclonal antibodies against a truncated version of native YdgR were generated successfully. Both antibodies interact with a conformationally intact epitope, which was proven with positive ELISA and negative Western blot results.

Antibody 5E8-D6 interacts with an epitope that is only accessible in native YdgR. This epitope is not recognized in the thermostable mutants S166G and N196K. Additionally, this epitope seems to be blocked by a C-terminal His-tag, since no interaction with the antibody can be observed for C-terminal His-tagged YdgR while there is a clear antibody-antigen peak visible on size exclusion for N-terminal His-tagged protein (Figure 5.10) independent of tag-removal with TEV protease. Therefore the epitope is suspected to be near the C-terminal His-tag. The YdgR homology model based on the structures of LacY and PepT<sub>so</sub> [35] shows that the C-terminus and the N-terminus of YdgR are located at the cytoplasmic side of the membrane.

Since YdgR reconstituted into proteoliposomes was used for immunization, it is rather likely that the epitope is part of an easily accessible loop region rather than of a

most probably buried helical part. The epitope of 5E8-D6 is presumably located in a loop in proximity of the C-terminus. Interaction of the antibody with the loop between helix 8 and 9 seems to be rather unlikely since residues in this region were identified to be more accessible for acetylation of lysine residues 343 and 353 in the thermostable mutants (Figure 5.26). Therefore the epitope recognized by 5E8-D6 is most likely located in the connecting loops between helix HB and 7 or helix 10 and 11 (Figure 6.1b). In comparison of the LacY inward-facing structure and the PepT<sub>so</sub> inward-facing occluded structure [39], helices 7, 11 and 12 form a helix bundle that is displaced by an 11° rotation during the conversion from the inward-facing occluded state to the inward-facing conformation. This ‘C1-helix bundle’ is suspected to undergo the largest displacement during transport (Figure 6.1a). Since PepT<sub>so</sub> has over 45% homology to YdgR [35], it can be hypothesized that the role of this C1-helix bundle is conserved, and that the epitope is located within the boundaries of this area or the rest of the C-bundle. Therefore the epitope is not accessible anymore when the protein is caught in a conformation specific for the thermostable mutants, while native YdgR is still able to flip between different conformations and exposes this epitope.

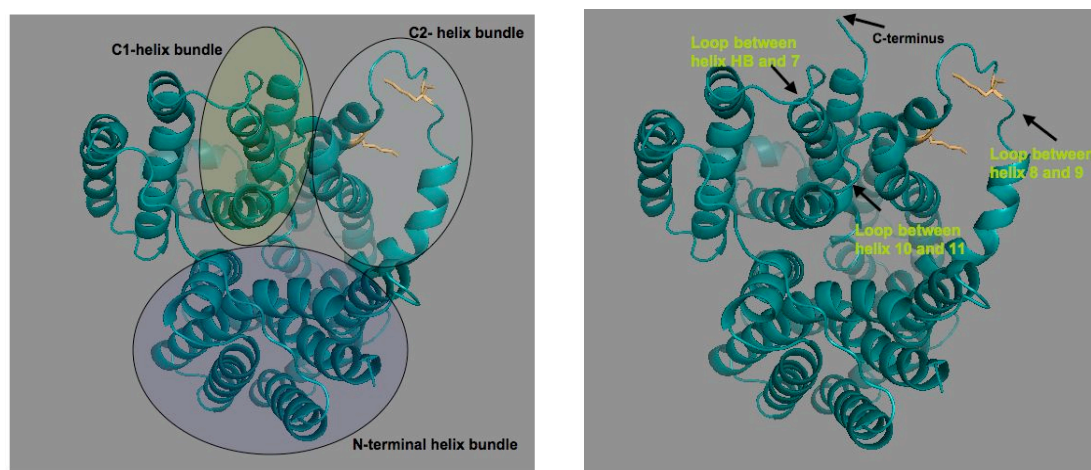


Figure 6.1: Cytoplasmic view of the YdgR homology model based on the structures of LacY and PepT<sub>so</sub> [35]; Left: N-terminal and C-terminal helix bundles as assigned for PepT<sub>so</sub> (See figure X); Right: Loops potentially containing 5E8-D6 epitope; Lysine 343 and 353 in yellow

Antibody 5E3-F10 recognizes native YdgR as well as the thermostable mutants. Therefore the epitope must be accessible in the outward and the inward-facing conformation in the native as well as in the thermostable mutants of YdgR.

### **6.3 Co-crystallization with mFab fragments resulted in crystals diffracting to 8-10 Å**

Proteolytically generated Fab fragments were used for co-crystallization trials with YdgR. Complexes of YdgR with mFab fragments of both antibodies gave rise to crystals diffracting to 8-10 Å. Optimization was performed but did not improve diffraction. The location of the epitope and the quality of the crystal contacts formed, will determine the diffraction quality of the crystals. If the interaction area of the target protein with the mFab is still rather flexible this may inhibit crystallization or give rise to crystals that are not suitable for structure determination. This cannot be tested before crystallization trials and therefore several monoclonal antibodies that are used to generate mFab fragments are required for co-crystallization trials. Unfortunately the two generated antibodies specific for YdgR did not give rise to well-diffracting crystals.

### **6.4 Two mutants with increased thermostability could be identified**

Four thermostable mutants were identified from 16 loss-of-function mutants with the Stargazer instrument. Surprisingly, only one of those mutants was confirmed to be more thermostable with a second assay. Two of the false positive mutants, namely K274I and M295K, showed heavy precipitation when incubated at 4°C and analyzed via size exclusion chromatography (Figure 5.19). The other mutant V252E shows a decreased fraction of folded protein after incubation at 50°C in comparison with native YdgR. These results suggest that initial precipitation of the target protein in the high-throughput Stargazer assay may reduce the concentration of soluble protein, which may artificially raise the measured aggregation temperatures, leading to a false positive result. We also observed that reduction of protein concentration increased the aggregation temperature (Figure 5.17). Therefore additional proof for increased thermostability is absolutely necessary as a second step after screening with Stargazer.

The only mutant resulting from the Stargazer screen confirmed to be more thermostable is N196K. Most interesting, the mutant homologous to C154G in LacY,

namely S166G, is also more thermostable than native YdgR. Therefore it seems possible to engineer mutants comparable with already described mutants, as has also been shown for G-protein coupled receptors [53]. This technique could be used for other proteins to facilitate crystallization or to lock them in one conformation.

Unfortunately, the effect of the double mutant S166G/N196K was not additive, but rather only increased very slightly in comparison with the single mutations. (Figure 5.20)

It was shown for LacY that C154 forms hydrogen bonds with the backbone of helix 1, which might be crucial for the cooperativity of the transport mechanism. Mutagenesis to glycine suggests less intimate contact between helices 1 and 5 [20]. This might be true as well for S166G in YdgR. N196 is located in helix 6, closer to the cytoplasmic side, and may also form interactions with helix 1 (Figure 6.2). The mutation to lysine may also hinder the close contact of helix 6 and helix 1 within the N-terminal helix bundle.

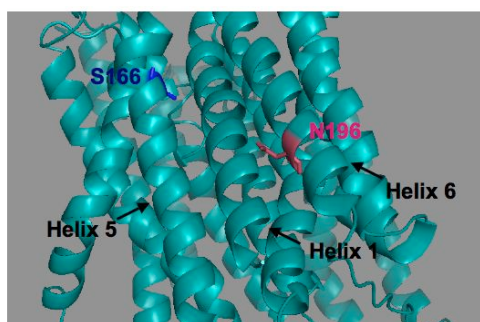


Figure 6.2: Homology model of YdgR showing the side-chains of S166 (blue) and N196 (magenta)

## 6.5 Thermostability and tolerance for harsh detergents of YdgR mutants in comparison with PepT<sub>so</sub>

As mentioned previously, the structure of PepT<sub>so</sub> from *Shewanella oneidensis* was solved in 2011 [39]. No structure of a member of the PTR family had previously been determined. Our studies on strategies to facilitate the crystallization of the *E. coli* homologue YdgR was ongoing when the PepT<sub>so</sub> structure was published. Therefore it was of high interest to determine whether PepT<sub>so</sub> shows increased resistance to thermal stress when compared to native YdgR and the thermostable mutants, since it has been proposed that increased thermostability goes hand in hand with crystallizability [34; 52]. As described in section 5.3.2, PepT<sub>so</sub> shows increased

thermostability when compared to native YdgR (Figure 5.20 and 5.21). While approximately 38% of YdgR is still folded after incubation at 50°C, 84% of PepT<sub>so</sub> remains intact and folded. Even the thermostable YdgR mutants are less thermostable than PepT<sub>so</sub>, despite their selection for thermostability, since the maximum percent of folded protein that could be reached was 55%. Additionally, PepT<sub>so</sub> shows increased tolerance for the harsh detergent LDAO, increasing the stability 10-fold in comparison with YdgR. Increased stability in LDAO was described as a ‘benchmark’ for crystallizability [57]. Our results with YdgR support this conclusion and also suggest the hypothesis that a certain threshold of thermostability and tolerance for harsh detergents is necessary for a target membrane protein to crystallize (Figure 6.3).

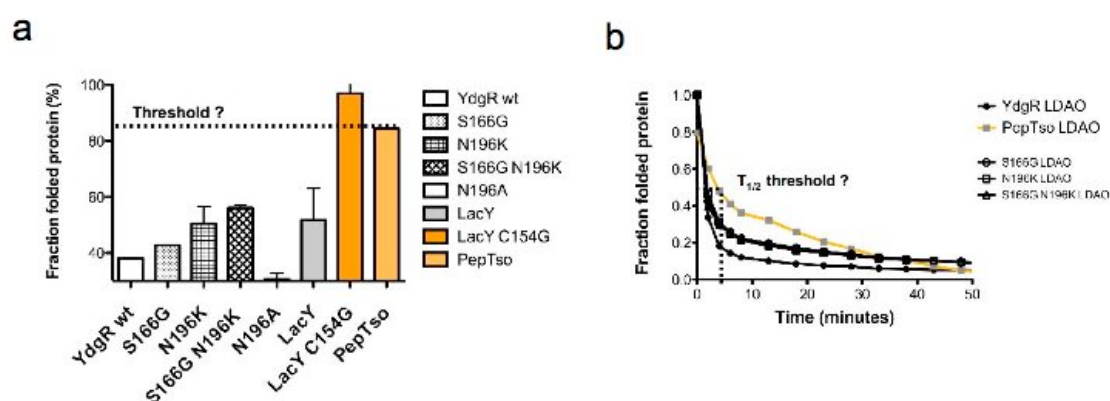


Figure 6.3: a. Result of size exclusion thermostability assay; b. Stability of YdgR and thermostable mutants in LDAO in comparison with PepT<sub>so</sub>

## 6.6 Thermostable mutants are most likely locked in one conformation

The acetylation of accessible lysine residues followed by the analysis of the acetylation pattern via mass spectrometry shows that the thermostable mutants S166G and N196K are most probably locked in the inward-facing conformation. The lysine residue K177 in the loop between helices 5 and 6 on the periplasmic side was not accessible to acetylation in the thermostable mutants, suggesting more limited accessibility on the periplasmic side. For S166G the lysine residues are generally more accessible than observed for native YdgR (Figure 5.26). The values of average acetylation for N196K are comparable with native YdgR for the lysine residues on the cytoplasmic side, except for the lysine residue 482 at the C-terminus following helix

12. This residue is more accessible in N196K as well as in S166G when compared to native YdgR. This suggests that helix 12 is rather straightened in the thermostable mutants. Since helix 12 is part of the proposed ‘C1 sub-bundle’, straightening of this helix may be an additional indication that the thermostable mutants predominantly adopt the inward-facing conformation, resulting in reduced transport activity as observed for the thermostable mutants. Nevertheless, this result has to be confirmed with additional experiments.

Especially because the structure of the LacY mutant C154G was solved in the inward-facing conformation while biochemical studies in membrane vesicles suggest that C154G is locked in the outward-facing conformation [40].

## **6.7 Lessons learned from YdgR to facilitate crystallization of integral membrane proteins**

Structure determination of YdgR failed due to poorly diffracting crystals and the low reproducibility of crystals, which made attempts at optimization difficult. Two strategies to facilitate crystallization were tested including co-crystallization with mFab fragments and identification of thermostable mutants. Co-crystallization with mFab fragments seems to be quite promising but is costly and time-consuming. Additionally several mAbs have to be cleaved and tested to identify the mFab fragment with optimal characteristics for co-crystallization and structure determination. If the epitope is too flexible, the antibody-antigen complex will still give rise to poorly diffracting crystals.

Selection for thermostable mutants appears to be a promising strategy as well. It was shown for G-protein coupled receptors that alanine-scanning mutagenesis [52] as well as random mutagenesis [10; 48] can improve receptor expression, stability in various detergents and alter binding selectivity. Resulting mutants are suitable targets for crystallization attempts as several crystal structures of engineered GPCRs have been solved [11; 32; 60]. Alanine-scanning mutagenesis is rather time-consuming while random mutagenesis seems to be a straightforward tool to generate promising mutants. The combination of random mutagenesis with a functional assay, seems to be especially suitable for selection of mutants showing high tolerance for detergents

and preference for agonist or antagonist conformations due to reduced conformational flexibility [31; 34]. It should also be possible to apply this screening method to other membrane proteins with lower binding affinities than GPCRs.

For YdgR, random mutagenesis followed by a transport assay was coupled with a thermostability and detergent screen. The thermostability screen and the detergent screen can be applied to every membrane protein target.

Single point mutations in YdgR increased thermostability and tolerance for the harsh detergent LDAO, making them a better target for crystallization experiments. But when compared to the homologue PepT<sub>so</sub> whose structure was solved, the stabilizing effect is still quite low. Therefore screening for the optimal homologue for thermostability or detergent tolerance seems to be fundamental to identify suitable targets for crystallization trials.

Additionally there seems to be a certain threshold of thermostability and tolerance for harsh detergents absolutely necessary for successful crystallization attempts. PepT<sub>so</sub> and LacY C154G show a fraction of 80-90% of folded protein after incubation at 50°C, while the thermostable YdgR mutants do not exceed values of 56%. Most probably the stabilization effect of the mutants is not high enough to promote crystallization. Additional point mutations may be necessary to increase thermostability for production of a suitable crystallization target.

Nevertheless high-throughput screening via the Stargazer or CPM assay could help to identify novel targets for crystallization of transporters and help to evaluate the necessary stabilization for engineered mutants, especially when the number of homologues is limited.

## 7 Bibliography

- [1] Abramson, J., I. Smirnova, et al. (2003). "Structure and mechanism of the lactose permease of *Escherichia coli*." Science **301**(5633): 610-5.
- [2] Alberts, J., Lewis, Raff, Roberts, Walter "Molecular biology of the cell."
- [3] Alexandrov, A. I., M. Mileni, et al. (2008). "Microscale fluorescent thermal stability assay for membrane proteins." Structure **16**(3): 351-9.
- [4] Andrew, S. M. and J. A. Titus (2001). "Fragmentation of immunoglobulin G." Curr Protoc Immunol **Chapter 2**: Unit 2 8.
- [5] Baker, M. (2010). "The gatekeepers revealed." Nature **465**.
- [6] Bowie, J. U. (2001). "Stabilizing membrane proteins." Curr Opin Struct Biol **11**(4): 397-402.
- [7] Chang, A. B., R. Lin, et al. (2004). "Phylogeny as a guide to structure and function of membrane transport proteins." Mol Membr Biol **21**(3): 171-81.
- [8] Dang, S., L. Sun, et al. "Structure of a fucose transporter in an outward-open conformation." Nature **467**(7316): 734-8.
- [9] Daniel, H., B. Spanier, et al. (2006). "From bacteria to man: archaic proton-dependent peptide transporters at work." Physiology (Bethesda) **21**: 93-102.
- [10] Dodevski, I. and A. Pluckthun (2011). "Evolution of three human GPCRs for higher expression and stability." J Mol Biol **408**(4): 599-615.
- [11] Dore, A. S., N. Robertson, et al. (2011). "Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine." Structure **19**(9): 1283-93.
- [12] Doyle, D. A., J. Morais Cabral, et al. (1998). "The structure of the potassium channel: molecular basis of K<sup>+</sup> conduction and selectivity." Science **280**(5360): 69-77.
- [13] Ericsson, U. B., B. M. Hallberg, et al. (2006). "Thermofluor-based high-throughput stability optimization of proteins for structural studies." Anal Biochem **357**(2): 289-98.
- [14] Faham, S., A. Watanabe, et al. (2008). "The crystal structure of a sodium galactose transporter reveals mechanistic insights into Na<sup>+</sup>/sugar symport." Science **321**(5890): 810-4.
- [15] Faham, S., D. Yang, et al. (2004). "Side-chain contributions to membrane protein structure and stability." J Mol Biol **335**(1): 297-305.

- [16] Fethiere "Methods in Molecular Biology; Macromolecular Crystallography Protocols."
- [17] Forrest, L. R., R. Kramer, et al. (2010). "The structural basis of secondary active transport mechanisms." Biochim Biophys Acta **1807**(2): 167-88.
- [18] Gibson, M. M., M. Price, et al. (1984). "Genetic characterization and molecular cloning of the tripeptide permease (tpp) genes of *Salmonella typhimurium*." J Bacteriol **160**(1): 122-30.
- [19] Guan, L. and H. R. Kaback (2006). "Lessons from lactose permease." Annu Rev Biophys Biomol Struct **35**: 67-91.
- [20] Guan, L., O. Mirza, et al. (2007). "Structural determination of wild-type lactose permease." Proc Natl Acad Sci U S A **104**(39): 15294-8.
- [21] Hollenstein, K., R. J. Dawson, et al. (2007). "Structure and mechanism of ABC transporter proteins." Curr Opin Struct Biol **17**(4): 412-8.
- [22] Huang, Y., M. J. Lemieux, et al. (2003). "Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*." Science **301**(5633): 616-20.
- [23] Hunte, C., J. Koepke, et al. (2000). "Structure at 2.3 Å resolution of the cytochrome bc<sub>1</sub> complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv fragment." Structure **8**(6): 669-84.
- [24] Hunte, C. and H. Michel (2002). "Crystallisation of membrane proteins mediated by antibody fragments." Curr Opin Struct Biol **12**(4): 503-8.
- [25] Iwata, S. "Methods and Results in Crystallization of Membrane Proteins."
- [26] Kulkarni, A. A., I. S. Haworth, et al. (2003). "Transmembrane segment 5 of the dipeptide transporter hPepT1 forms a part of the substrate translocation pathway." Biochem Biophys Res Commun **306**(1): 177-85.
- [27] Kulkarni, A. A., I. S. Haworth, et al. (2003). "Analysis of transmembrane segment 7 of the dipeptide transporter hPepT1 by cysteine-scanning mutagenesis." J Biol Chem **278**(51): 51833-40.
- [28] Lau, F. W., S. Nauli, et al. (1999). "Changing single side-chains can greatly enhance the resistance of a membrane protein to irreversible inactivation." J Mol Biol **290**(2): 559-64.
- [29] Law, C. J., J. Almqvist, et al. (2008). "Salt-bridge dynamics control substrate-induced conformational change in the membrane transporter GltP." J Mol Biol **378**(4): 828-39.
- [30] Law, C. J., P. C. Maloney, et al. (2008). "Ins and outs of major facilitator superfamily antiporters." Annu Rev Microbiol **62**: 289-305.

- [31] Lebon, G., K. Bennett, et al. (2011). "Thermostabilisation of an agonist-bound conformation of the human adenosine A(2A) receptor." J Mol Biol **409**(3): 298-310.
- [32] Lebon, G., T. Warne, et al. (2011). "Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation." Nature **474**(7352): 521-5.
- [33] Lemieux, M. J., J. Song, et al. (2003). "Three-dimensional crystallization of the Escherichia coli glycerol-3-phosphate transporter: a member of the major facilitator superfamily." Protein Sci **12**(12): 2748-56.
- [34] Magnani, F., Y. Shibata, et al. (2008). "Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor." Proc Natl Acad Sci U S A **105**(31): 10744-9.
- [35] Malle, E., H. Zhou, et al. (2011). "Random mutagenesis of the prokaryotic peptide transporter YdgR identifies potential periplasmic gating residues." J Biol Chem **286**(26): 23121-31.
- [36] Michel, H. (1983). "Crystallization of membrane proteins." Trends in Biochemical Sciences **8**(2): 56-59.
- [37] Morth, J. P., B. P. Pedersen, et al. (2011). "A structural overview of the plasma membrane Na<sup>+</sup>,K<sup>+</sup>-ATPase and H<sup>+</sup>-ATPase ion pumps." Nat Rev Mol Cell Biol **12**(1): 60-70.
- [38] Nettleship, J. E., J. Brown, et al. (2008). "Methods for protein characterization by mass spectrometry, thermal shift (ThermoFluor) assay, and multiangle or static light scattering." Methods Mol Biol **426**: 299-318.
- [39] Newstead, S., D. Drew, et al. (2011). "Crystal structure of a prokaryotic homologue of the mammalian oligopeptide-proton symporters, PepT1 and PepT2." Embo J **30**(2): 417-26.
- [40] Nie, Y., F. E. Sabetfard, et al. (2008). "The Cys154-->Gly mutation in LacY causes constitutive opening of the hydrophilic periplasmic pathway." J Mol Biol **379**(4): 695-703.
- [41] Olesen, C., M. Picard, et al. (2007). "The structural basis of calcium transport by the calcium pump." Nature **450**(7172): 1036-42.
- [42] Ostermeier, C., S. Iwata, et al. (1995). "Fv fragment-mediated crystallization of the membrane protein bacterial cytochrome c oxidase." Nat Struct Biol **2**(10): 842-6.
- [43] Ostermeier, C. and H. Michel (1997). "Crystallization of membrane proteins." Curr Opin Struct Biol **7**(5): 697-701.
- [44] Pantoliano, M. W., E. C. Petrella, et al. (2001). "High-density miniaturized thermal shift assays as a general strategy for drug discovery." J Biomol Screen **6**(6): 429-40.

- [45] Rath, A., M. Glibowicka, et al. (2009). "Detergent binding explains anomalous SDS-PAGE migration of membrane proteins." Proc Natl Acad Sci U S A **106**(6): 1760-5.
- [46] Ressler, S., A. C. Terwisscha van Scheltinga, et al. (2009). "Molecular basis of transport and regulation in the Na(+)/betaine symporter BetP." Nature **458**(7234): 47-52.
- [47] Reyes, N., C. Ginter, et al. (2009). "Transport mechanism of a bacterial homologue of glutamate transporters." Nature **462**(7275): 880-5.
- [48] Sarkar, C. A., I. Dodevski, et al. (2008). "Directed evolution of a G protein-coupled receptor for expression, stability, and binding selectivity." Proc Natl Acad Sci U S A **105**(39): 14808-13.
- [49] Screpanti, E. and C. Hunte (2007). "Discontinuous membrane helices in transport proteins and their correlation with function." J Struct Biol **159**(2): 261-7.
- [50] Senisterra, G. A., H. Ghanei, et al. (2010). "Assessing the stability of membrane proteins to detect ligand binding using differential static light scattering." J Biomol Screen **15**(3): 314-20.
- [51] Senisterra, G. A., E. Markin, et al. (2006). "Screening for ligands using a generic and high-throughput light-scattering-based assay." J Biomol Screen **11**(8): 940-8.
- [52] Serrano-Vega, M. J., F. Magnani, et al. (2008). "Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form." Proc Natl Acad Sci U S A **105**(3): 877-82.
- [53] Serrano-Vega, M. J. and C. G. Tate (2009). "Transferability of thermostabilizing mutations between beta-adrenergic receptors." Mol Membr Biol **26**(8): 385-96.
- [54] Smirnova, I., V. Kasho, et al. (2007). "Sugar binding induces an outward facing conformation of LacY." Proc Natl Acad Sci U S A **104**(42): 16504-9.
- [55] Smirnova, I. N. and H. R. Kaback (2003). "A mutation in the lactose permease of Escherichia coli that decreases conformational flexibility and increases protein stability." Biochemistry **42**(10): 3025-31.
- [56] Sobczak, I. and J. S. Lolkema (2005). "Structural and mechanistic diversity of secondary transporters." Curr Opin Microbiol **8**(2): 161-7.
- [57] Sonoda, Y., S. Newstead, et al. (2011). "Benchmarking membrane protein detergent stability for improving throughput of high-resolution X-ray structures." Structure **19**(1): 17-25.

- [58] Spiess, C., A. Beil, et al. (1999). "A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein." Cell **97**(3): 339-47.
- [59] Wagner, S., M. M. Klepsch, et al. (2008). "Tuning Escherichia coli for membrane protein overexpression." Proc Natl Acad Sci U S A **105**(38): 14371-6.
- [60] Warne, T., M. J. Serrano-Vega, et al. (2008). "Structure of a beta1-adrenergic G-protein-coupled receptor." Nature **454**(7203): 486-91.
- [61] Weitz, D., D. Harder, et al. (2007). "Functional and structural characterization of a prokaryotic peptide transporter with features similar to mammalian PEPT1." J Biol Chem **282**(5): 2832-9.
- [62] Weyand, S., T. Shimamura, et al. (2008). "Structure and molecular mechanism of a nucleobase-cation-symport-1 family transporter." Science **322**(5902): 709-13.
- [63] Yamashita, A., S. K. Singh, et al. (2005). "Crystal structure of a bacterial homologue of Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporters." Nature **437**(7056): 215-23.
- [64] Yernool, D., O. Boudker, et al. (2004). "Structure of a glutamate transporter homologue from Pyrococcus horikoshii." Nature **431**(7010): 811-8.
- [65] Yeung, A. K., S. K. Basu, et al. (1998). "Molecular identification of a role for tyrosine 167 in the function of the human intestinal proton- coupled dipeptide transporter (hPepT1)." Biochem Biophys Res Commun **250**(1): 103-7.
- [66] Zhou, Y. and J. U. Bowie (2000). "Building a thermostable membrane protein." J Biol Chem **275**(10): 6975-9.
- [67] Zhou, Y., L. Guan, et al. (2008). "Opening and closing of the periplasmic gate in lactose permease." Proc Natl Acad Sci U S A **105**(10): 3774-8.
- [68] Zhou, Y., J. H. Morais-Cabral, et al. (2001). "Chemistry of ion coordination and hydration revealed by a K<sup>+</sup> channel-Fab complex at 2.0 Å resolution." Nature **414**(6859): 43-8.
- [69] Zhou, Y., Y. Nie, et al. (2009). "Residues gating the periplasmic pathway of LacY." J Mol Biol **394**(2): 219-25.

Supplemental Material can be found at:  
<http://www.jbc.org/content/suppl/2011/05/10/M111.239657.DC1.html>

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 26, PP. 23121–23131, JULY 1, 2011  
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## Random Mutagenesis of the Prokaryotic Peptide Transporter YdgR Identifies Potential Periplasmic Gating Residues<sup>\*[3]</sup>

Received for publication, March 13, 2011, and in revised form, April 26, 2011. Published, JBC Papers in Press, May 10, 2011, DOI 10.1074/jbc.M111.239657.

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The peptide transporter (PTR) family represents a group of proton-coupled secondary transporters responsible for bulk uptake of amino acids in the form of di- and tripeptides, an essential process employed across species ranging from bacteria to humans. To identify amino acids critical for peptide transport in a prokaryotic PTR member, we have screened a library of mutants of the *Escherichia coli* peptide transporter YdgR using a high-throughput substrate uptake assay. We have identified 35 single point mutations that result in a full or partial loss of transport activity. Additional analysis, including homology modeling based on the crystal structure of the *Shewanella oneidensis* peptide transporter PepT<sub>so</sub>, identifies Glu<sup>36</sup> and Arg<sup>305</sup> as potential periplasmic gating residues. In addition to providing new insights into transport by members of the PTR family, these mutants provide valuable tools for further study of the mechanism of peptide transport.

Amino acid uptake provides a critical source of nitrogen, sulfur, and metabolic intermediates for many organisms. Amino acids are often transported into the cell in the form of short peptides to efficiently mediate their bulk uptake. Although several different systems mediating peptide transport exist in bacteria and fungi, only the peptide transporters (PTR),<sup>6</sup> also called proton oligopeptide transporters, are conserved in higher organisms, including humans (1, 2). Like many proton-coupled transporters, the PTR family is a subdivision of the major facilitator superfamily (MFS) (3).

Sequence alignment among members of the PTR family across species reveals three conserved motifs in the protein

sequence (4) (supplemental Fig. S1). The first such motif is found in the first predicted transmembrane helix, whereas the second is found in loop 2-3 and corresponds to the motif GXXX(D/E)(R/K)[X]G[X](R/K)(R/K), where [X] is either one or two amino acids, which is also found in many MFS transporters (5, 6). The third motif, the PTR signature motif FXXFYXX-  
INXGS, is located in the fifth predicted transmembrane helix of PTR members, and mutation of these residues leads to reduced transport activity or changes in substrate selectivity (7, 8). In addition, chimeric studies on human PTR members PEPT1 and PEPT2 have revealed that helices 2–5 as well as 7 contain residues that mediate substrate recognition (9). This is supported by cysteine scanning mutagenesis of residues in helices 5 and 7 of PEPT1, which suggests that residues in these helices line the substrate translocation pathway (8, 10).

PTR family members possess a large number of possible substrates, including essentially all 400 possible di- or 8000 tripeptides. For human PEPT1 and PEPT2, the most well characterized PTRs, transport is stereoselective for the L-enantiomers of amino acids, although D-amino acids are accepted in the N-terminal position. Substrate affinity varies depending on the sequence of the particular di- or tripeptide (11). Furthermore, these transporters are able to take up peptidomimetics such as  $\beta$ -lactam antibiotics, some angiotensin-converting enzyme inhibitors, and some antiviral pro-drugs, such as valaciclovir (12). Due to the presence of PEPT1 in the brush-border membrane of the small intestine, and PEPT2 in the kidney and blood-brain barrier, these proteins significantly influence the systemic availability of these and other pharmacological compounds (12).

The *Escherichia coli* PTR protein YdgR, also called DtpA, is one of the few prokaryotic PTR members to have been characterized and shows substrate selectivity similar to that of PEPT1 (13, 14). Recently, the crystal structure of another prokaryotic PTR family member from *Shewanella oneidensis*, referred to as PepT<sub>so</sub>, was determined and revealed a fold very similar to that of other MFS transporters, including LacY, GlpT, and EmrD (15–18). The substrate binding cavity is lined with conserved residues, many of which were previously identified as critical for function (17). However, several functional residues, including His<sup>61</sup> and Glu<sup>316</sup>, which are suggested to be essential for protonation and/or periplasmic gating, are not conserved between PepT<sub>so</sub> and other bacterial PTRs, including YdgR.

To identify additional residues critical for the function of YdgR and its homologues, we have performed random

<sup>\*</sup> This work was supported by Austrian Science Fund, SFB35 projects F3507 and F3502, the Federal Ministry of Economy, Family and Youth through "Laura Bassi Centre of Expertise" initiative project Number 253275, and Boehringer Ingelheim.

<sup>[3]</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

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<sup>6</sup> The abbreviations used are: PTR, peptide transporter; MFS, major facilitator superfamily; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; LOF, loss-of-function; PDB, Protein Data Bank.

# Mutagenesis of YdgR Identifies Gating Residues

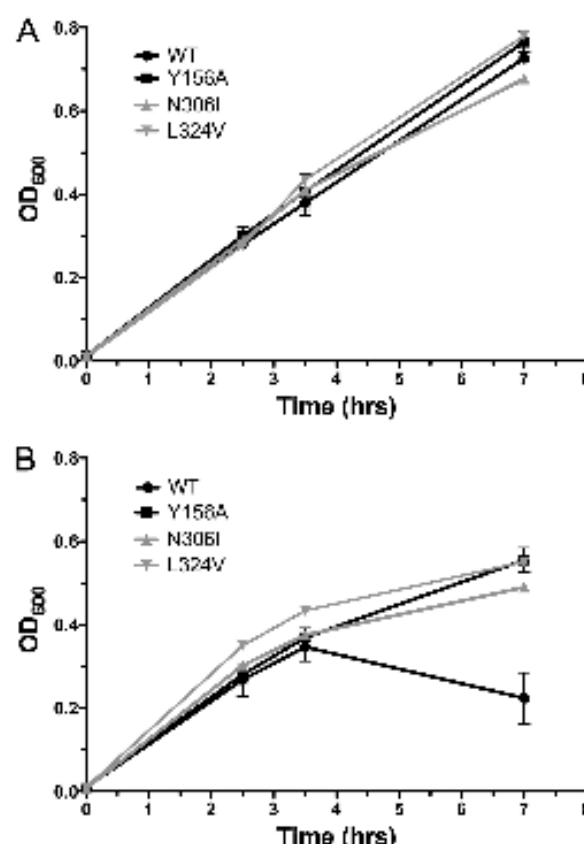


**FIGURE 1.** Selection of mutants based on the ability to survive in the presence of alafosfalin. Cells expressing individual clones from each mutant library were grown in the absence (A) and presence (B) of alafosfalin and protein expression was induced at 2.5 h.  $A_{600}$  was measured at 1 h and 4.5 h after induction. Representative primary data are shown for the native transporter (WT), the Y156A mutant (Y156A), and the clones later identified as mutants N306I and L324V. Reported  $A_{600}$  values are from measurements in 96-well plates, and are ~2.5 times lower than  $A_{600}$  determined using a 1-cm path length. The means of triplicate measurements of  $A_{600}$  for the native transporter and the Y156A loss-of-function mutant in the presence of alafosfalin from three individual experiments were compared using a paired *t* test within the Graphpad Prism 5.0 software, and were found to have a *p* value <0.0024, indicating that the difference is statistically significant.

mutagenesis on this transporter and utilized a high-throughput substrate uptake assay to identify mutants exhibiting loss-of-function phenotypes. The results of this assay combined with additional analysis allowed us to identify 15 mutations leading to a complete loss-of-function phenotype, and 12 further mutations showing partial loss-of-function. In addition, 6 mutations were identified that exhibit this phenotype only in one of the uptake assays. Mapping of these residues onto a homology model of YdgR based on the PepT<sub>sc</sub> structure and additional comparison to the known structure of the lactose permease LacY (15) allows for prediction of the potential functions of several of these residues, including Glu<sup>56</sup>, Phe<sup>289</sup>, Met<sup>295</sup>, and Arg<sup>305</sup>. Further analysis of Glu<sup>56</sup> and Arg<sup>305</sup> reveals that these residues may function as periplasmic gating residues in YdgR and other close bacterial homologues.

**TABLE 1**  
Mutants isolated in the primary screen

| Single mutants, library H2-4 | Single mutants, library H6-8 | Double mutants, library H2-4 | Double mutants, library H6-8 |
|------------------------------|------------------------------|------------------------------|------------------------------|
| E56G                         | M154K <sup>a</sup>           | L52P, K130E                  | L221R, V316L                 |
| S59P                         | L190V                        | S64Y, A99E                   | Q242H, F288L                 |
| I60N                         | N196K                        | S67T, A68P                   | G255D, N306K                 |
| A68P                         | F197I                        | V70L, L80F                   | I256F, I259F                 |
| G78C                         | V252E                        | Y71C, A75D                   | E263D, E317G                 |
| G86R                         | A264P                        | W79R, D91N                   | M275L, F279I                 |
| L98R                         | K274I                        | G86S, P133T                  | E284G, A285V                 |
| I100V                        | A285V                        | G89R, K130T                  | F289I, E317G                 |
| G101D                        | F289L                        | A95T, P133L                  | V290A, Q320H                 |
| I122N                        | F289S                        | A99V, K130I                  | Y292S, I331T                 |
| A123V                        | M295K                        | Y116C, M119K                 | T297R, A315T                 |
| G127D                        | T297A                        | M117L, S134P                 | L299P, R305H                 |
| L136R                        | N300I                        |                              | L299R, L312M                 |
| L137H                        | N300Y                        |                              | F302C, R305P                 |
|                              | F301I                        |                              | P308R, A371V                 |
|                              | A303G                        |                              | L312S, A405T                 |
|                              | R305C                        |                              | Q321L, A323P                 |
|                              | N306I                        |                              |                              |
|                              | Q320L                        |                              |                              |
|                              | L324V                        |                              |                              |
|                              | P326Q                        |                              |                              |

<sup>a</sup> Mutation occurs outside of the mutagenized region.

## EXPERIMENTAL PROCEDURES

**Molecular Cloning.**—The full-length *ydgR* gene was amplified from *E. coli* genomic DNA and cloned into the pCS19 vector (kind gift from Michael Ehrmann (19)) using the NcoI and BglII restriction sites. Site-directed mutagenesis was performed as described in Ref. 20.

**Random Mutagenesis.**—Random mutations were introduced into regions of the *ydgR* gene encompassing helices 2–4 (H2–4; base pairs 130–444) and helices 6–8 (H6–8; base pairs 535–1000) using the GeneMorph II Random Mutagenesis Kit (Stratagene). To determine the mutation frequency in each library, the resulting PCR products were inserted into the pJET cloning vector using the GeneJET Cloning Kit (Fermentas). At least 10 different clones for each library were sequenced to estimate a mutation frequency of 0.35% for library H2–4 and 0.65% for library H6–8. The PCR products were then used as megaprimers with pCS19-YdgR as a template, to create two libraries of mutants in the pCS19 background.

**Alafosfalin Growth Assay.**—The mutant libraries were transformed into *E. coli* BL21(DE3) and individual colonies were grown in 96-well plates under two conditions, one containing 200  $\mu$ l of Luria-Bertani (LB) medium plus the antibiotic ampicillin, and one additionally containing 200  $\mu$ g/ml of the antibiotic alafosfalin (Sigma).  $A_{600}$  was monitored 1 and 5 h after induction of *ydgR* expression. Clones exhibiting a growth advantage when compared with native YdgR, as determined by visual inspection of the growth curves, were sequenced within the entire open reading frame to identify the mutation(s). Several clones of each mutant identified in this manner were then re-tested in the growth assay to eliminate false positives.

**Isolation of *E. coli* Membrane Fractions.**—Cell pellets from 50-ml cultures of C43(DE3) cells transformed with mutant or control constructs were harvested and membranes were isolated as described in Ref. 21. Equal concentrations of membranes from each construct were analyzed by SDS-PAGE and Western blot using an  $\alpha$ -His-HRP-conjugated antibody (Qiagen). The intensity of each band was quantified using the ImageJ software (22).

TABLE 2

Membrane expression and transport characteristics of mutants isolated in the primary screen

| Mutant                   | Growth advantage <sup>a</sup> | Uptake of $\beta$ -Ala-Lys(AMCA) <sup>b</sup> | Membrane expression <sup>c</sup> | Corrected uptake <sup>d</sup> | Predicted location |
|--------------------------|-------------------------------|-----------------------------------------------|----------------------------------|-------------------------------|--------------------|
|                          |                               | %                                             | %                                | %                             |                    |
| Empty vector             | 2.8 $\pm$ 0.5                 | 0 $\pm$ 1                                     | 0                                | 0                             |                    |
| Wild-type                | 1.0 $\pm$ 0.2                 | 100 $\pm$ 5                                   | 100                              | 100                           |                    |
| Y156A                    | 2.5 $\pm$ 0.5                 | 2 $\pm$ 1                                     | 96                               | 2                             | Helix 5            |
| No membrane localization |                               |                                               |                                  |                               |                    |
| I100V                    | 2.6 $\pm$ 0.5                 | 3 $\pm$ 1                                     | 0                                | 0                             | Helix 3            |
| L137H                    | 2.4 $\pm$ 0.6                 | 1 $\pm$ 4                                     | 0                                | 0                             | Helix 4            |
| LOF mutants              |                               |                                               |                                  |                               |                    |
| E56G                     | 2.3 $\pm$ 0.1                 | 10 $\pm$ 4                                    | 89                               | 11                            | Helix 2            |
| S59P                     | 2.5 $\pm$ 0.3                 | -1 $\pm$ 2                                    | 100                              | -1                            | Helix 2            |
| I60N                     | 3.3 $\pm$ 0.5                 | 1 $\pm$ 2                                     | 106                              | 1                             | Helix 2            |
| A68P                     | 2.6 $\pm$ 0.3                 | 1 $\pm$ 1                                     | 105                              | 1                             | Helix 2            |
| G78C                     | 2.3 $\pm$ 0.4                 | 1 $\pm$ 3                                     | 91                               | 1                             | Helix 2            |
| L98R                     | 2.4 $\pm$ 0.4                 | 1 $\pm$ 1                                     | 114                              | 1                             | Helix 3            |
| L136R                    | 2.5 $\pm$ 0.5                 | 0 $\pm$ 1                                     | 79                               | 0                             | Helix 4            |
| A285V                    | 2.3 $\pm$ 0.2                 | 11 $\pm$ 6                                    | 105                              | 10                            | Helix 7            |
| F289L                    | 2.4 $\pm$ 0.2                 | 1 $\pm$ 1                                     | 81                               | 1                             | Helix 7            |
| F289S                    | 2.1 $\pm$ 0.3                 | -1 $\pm$ 4                                    | 67                               | -1                            | Helix 7            |
| M295K                    | 2.2 $\pm$ 0.2                 | -1 $\pm$ 2                                    | 82                               | -1                            | Helix 7            |
| T297A                    | 2.4 $\pm$ 0.1                 | 3 $\pm$ 1                                     | 103                              | 3                             | Helix 7            |
| N300I                    | 2.7 $\pm$ 0.5                 | 7 $\pm$ 1                                     | 116                              | 6                             | Helix 7            |
| F301I                    | 2.7 $\pm$ 0.2                 | 2 $\pm$ 1                                     | 75                               | 2                             | Helix 7            |
| Q320L                    | 2.2 $\pm$ 0.5                 | 7 $\pm$ 3                                     | 127                              | 6                             | Helix 8            |
| Partial LOFs             |                               |                                               |                                  |                               |                    |
| G86R                     | 1.7 $\pm$ 0.3                 | 0 $\pm$ 3                                     | 94                               | 0                             | Loop 2-3           |
| G101D                    | 1.2 $\pm$ 0.3                 | 35 $\pm$ 12                                   | 113                              | 31                            | Helix 3            |
| I122N                    | 1.5 $\pm$ 0.1                 | 17 $\pm$ 4                                    | 117                              | 15                            | Helix 4            |
| A123V                    | 1.3 $\pm$ 0.5                 | 23 $\pm$ 1                                    | 61                               | 38                            | Helix 4            |
| G127D                    | 1.3 $\pm$ 0.2                 | 60 $\pm$ 8                                    | 24                               | 240                           | Helix 4            |
| N196K                    | 1.9 $\pm$ 0.2                 | 35 $\pm$ 4                                    | 120                              | 29                            | Helix 6            |
| A264P                    | 1.7 $\pm$ 0.3                 | 39 $\pm$ 2                                    | 88                               | 44                            | Helix HB           |
| N300Y                    | 3.3 $\pm$ 0.4                 | 25 $\pm$ 8                                    | 62                               | 40                            | Helix 7            |
| A303G                    | 1.3 $\pm$ 0.2                 | 42 $\pm$ 9                                    | 94                               | 45                            | Helix 7            |
| R305C                    | 1.7 $\pm$ 0.1                 | 3 $\pm$ 1                                     | 62                               | 5                             | Helix 7            |
| N306I                    | 1.4 $\pm$ 0.2                 | 21 $\pm$ 1                                    | 100                              | 21                            | Helix 7            |
| P326Q                    | 1.6 $\pm$ 0.3                 | 2 $\pm$ 2                                     | 57                               | 4                             | Helix 8            |
| Selectivity mutants      |                               |                                               |                                  |                               |                    |
| M154K                    | 2.4 $\pm$ 0.2                 | 257 $\pm$ 70                                  | 93                               | 276                           | Helix 5            |
| L190V                    | 2.6 $\pm$ 0.5                 | 105 $\pm$ 25                                  | 89                               | 118                           | Helix 6            |
| F197I                    | 2.7 $\pm$ 0.4                 | 142 $\pm$ 26                                  | 86                               | 165                           | Helix 6            |
| V252E                    | 2.2 $\pm$ 0.2                 | 87 $\pm$ 7                                    | 84                               | 104                           | Helix HB           |
| K274I                    | 2.5 $\pm$ 0.3                 | 59 $\pm$ 21                                   | 55                               | 107                           | Helix 7            |
| L324V                    | 1.8 $\pm$ 0.5                 | 242 $\pm$ 11                                  | 80                               | 303                           | Helix 8            |

<sup>a</sup> Data are represented as the fold difference in  $A_{600}$  of mutant versus native transporter in the presence and absence of alafosfalin 5 h post-induction:  $(A_{mutant} + Alafosf) / (A_{WT} + Alafosf) / (A_{mutant} - Alafosf) / (A_{WT} - Alafosf)$ . Error values represent S.D. of triplicate measurements from at least two different experiments.

<sup>b</sup> Fluorescence values are normalized after setting the value of the overexpressed native transporter to 100%.

<sup>c</sup> % Membrane expression was determined by quantitation of the band intensity on the Western blots shown in Fig. 2 using ImageJ software (22) and normalized by setting the value of the overexpressed native transporter to 100%.

<sup>d</sup> Corrected uptake represents: (% uptake of  $\beta$ -Ala-Lys(AMCA) / % Membrane expression)  $\times$  100.

**Uptake of  $\beta$ -Ala-Lys(AMCA)**—Uptake of  $\beta$ -Ala-Lys-AMCA (Biotrend) was measured essentially as described in Ref. 14, however, a 2.5 mM  $\beta$ -Ala-Lys-AMCA stock solution was used. Uptake was measured in 25 mM Tris buffer at pH 7.5 containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM  $CaCl_2$ , 0.8 mM  $MgSO_4$ , and 5 mM glucose. Fluorescence measurements were additionally normalized to  $A_{600}$ . Competition assays were carried out in the presence of 0 to 75 mM alafosfalin or alanyl-alanine (Bachem AG). IC<sub>50</sub> values were determined by nonlinear regression analysis using the software program GraphPad Prism 5.0.

**Homology Modeling**—The HHPred-generated alignment of YdgR with PepT<sub>so</sub> (PDB code 2XUT) was used as the basis for the alignment input into the program Modeller (9 version 8) (23, 31). Evaluation of this model with PROCHECK resulted in 93.8% of the residues in the core region, 5.2% in the allowed region, 0.5% in the generously allowed region, and 0.5% disallowed (24). Figs. 5 and 6 were generated using the software package MOE (Molecular Operating Environment, version

2009.10, Chemical Computing Group, Montreal, Canada). For Fig. 6, the LacY (PDB code 2CFQ) and PepT<sub>so</sub> structures and the YdgR homology model were aligned using the Superposition tool in MOE.

## RESULTS

**Isolation of YdgR Loss-of-function Mutants**—To identify residues critical for peptide transport in the *E. coli* YdgR protein, we used PCR mutagenesis to generate mutant libraries in two regions of the *ydgR* gene. The first library, encompassing helices 2–4 (H2–4), spans amino acids 44–148 of the *ydgR* gene (base pairs 130–444), and the second library, encompassing helices 6–8 (H6–8), spans amino acids 179–334 (base pairs 535–1000) (supplemental Fig. S1).

These two libraries were then tested for functionality based on the ability to transport the phosphonopeptide antibiotic alafosfalin, which is a specific substrate of prokaryotic PTRs, transport of which leads to inhibition of cell growth (25). Growth in

## Mutagenesis of YdgR Identifies Gating Residues

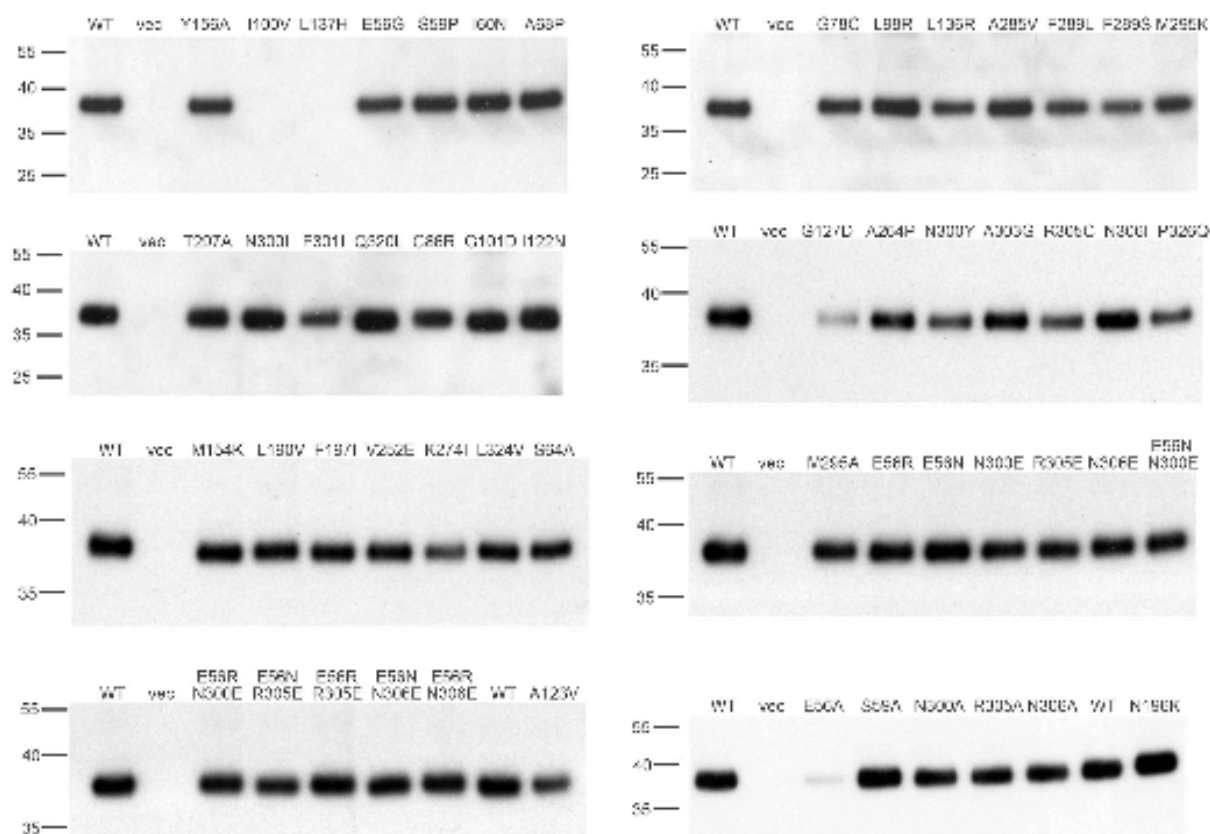


**FIGURE 2. Membrane localization of all mutants.** The localization of mutants to the plasma membrane was tested by preparation of *E. coli* membranes, analysis by SDS-PAGE and Western blot, and quantitation of the band intensity. Molecular mass markers (kDa) are indicated to the left of each Western blot. The quantitation of the expression level of each mutant is recorded in Tables 2 or 4. WT, membranes from cells overexpressing native YdgR; vec, membranes from cells expressing the empty vector.

the absence and presence of alafosfalin was monitored by measuring  $A_{600}$  at several time points after induction of *ydgR* expression (Fig. 1). Cells expressing transporters with mutations leading to a loss-of-function (LOF) phenotype are expected to grow normally in the presence of alafosfalin, whereas cells expressing the native transporter or transporters with mutations that do not affect transport ability will fail to survive, due to the toxic effect of the antibiotic. The Y156A mutant, which represents the homologous mutation of Tyr<sup>167</sup> from PepT1 and results in a non-functional transporter (26), was used as a positive control. Representative data for 2 LOF mutants isolated in this assay, the native transporter and the Y156A mutant indicates that the mutants show a clear growth advantage as compared with the native transporter in the presence of alafosfalin (Fig. 1).

Approximately 1600 mutants were screened in this assay, 600 from library H2–4 and 1000 from H6–8. From library H2–4, 101 LOF mutants were isolated. After sequencing the YdgR open reading frame, these consisted of 21 single amino acid mutations and 12 with double amino acid mutations. The remaining mutants contained single base pair deletions or insertions resulting in a frameshift, silent mutations, stop codons, or point mutations resulting in 3 or more amino acid

changes. The mutant M154K lies outside of the mutagenized region, and therefore must have arisen independently during library generation. From library H6–8, 170 LOF mutants were identified. Initial sequencing revealed the frequent occurrence of mutants containing a long insertion rather than single point mutations, most likely occurring during the megaprimer amplification step. Therefore clones were pre-screened by colony PCR to eliminate clones containing the insertion. The remaining 118 mutants were sequenced within the YdgR open reading frame to identify mutations, and consisted of 27 single point mutants (22 unique) and 17 double point mutants.

The mutants resulting in single amino acid changes were re-assayed to confirm their phenotype, resulting in the identification of 8 false positives. The remaining 35 single mutants and the 29 isolated double mutants are shown in Table 1. Comparison of  $A_{600}$  values of the native and mutant clones 4–5 h post-induction was used to calculate a numerical growth advantage for each of the 35 single mutants (Table 2).

**Functional Analysis Reveals Four Different Phenotypes**—The 35 single mutants were then further characterized. To determine whether their phenotype is simply due to loss of protein expression or membrane insertion, membrane localization was determined by isolation of the membrane fraction from small-

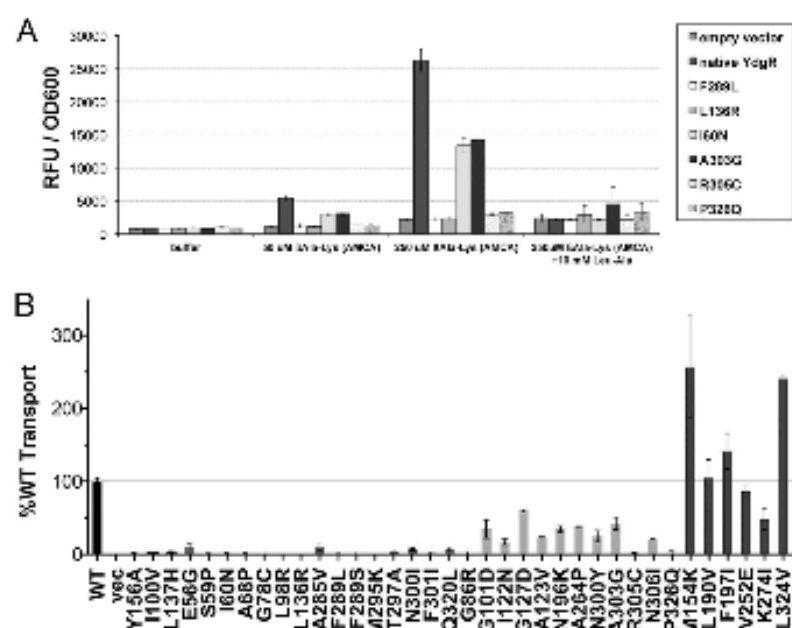


**FIGURE 3. Uptake of  $\beta$ -Ala-Lys(AMCA) reveals distinct phenotypes.** The ability of each mutant to take up the fluorescent dipeptide substrate  $\beta$ -Ala-Lys(AMCA) was measured and compared with that of the native transporter. **A**, representative data for uptake by native YdgR, empty vector, and 6 mutants are shown under conditions including either buffer alone,  $\beta$ -Ala-Lys(AMCA) alone, or  $\beta$ -Ala-Lys(AMCA) plus 10 mM of a dipeptide competitor (Leu-Ala, leucyl-alanine). Data are shown as raw fluorescence units (RFU) normalized to  $A_{600}$ . Error bars represent standard deviations from triplicate measurements. **B**, after subtraction of nonspecific transport, as measured by residual transport by cells expressing the empty vector, the transport ability of each mutant was normalized with respect to the native transporter, which was set to 100%. Error bars represent standard deviations of triplicate measurements from at least two different experiments. Wild-type (WT), vector (vec), and Y156A controls are shown as black bars, mutants are grouped and shaded according to the phenotypic categories shown in Table 2. A black line is used to indicate 100% transport.

scale cultures and verification of the presence of protein in the membrane fraction by Western blot using an  $\alpha$ -His antibody to detect the C-terminal His<sub>6</sub> tag of the protein. This analysis revealed that two mutants, H100V and L137H, show no expression in the membrane fraction, whereas G127D is present in the membrane at very low levels, and K274I is expressed at only 55% as compared with native YdgR (Table 2, Fig. 2). All other mutants show expression levels of 60% or more, and interpretation of their transport phenotype is not affected by their expression level.

To further analyze the transport activity of the individual mutants, their ability to take up the fluorescent substrate  $\beta$ -Ala-Lys(AMCA), a known substrate of YdgR (14), was assessed and compared with that of the native transporter (Fig. 3). By compiling the data from the alafosfalin growth assay and the  $\beta$ -Ala-Lys(AMCA) uptake assay, the 35 mutants can be divided into four phenotypic categories (Table 2). The first category includes the two mutants that fail to localize to the membrane. The second and largest category comprises 15 mutants that show a 2-fold or greater growth advantage in the presence of alafosfalin, and less than 10% uptake activity as compared with the native transporter. Most of these mutants represent amino acid substitutions that dramatically alter the properties of the side chain, such as hydrophobic to charged or vice versa, or mutations to glycine or proline, which may significantly alter the protein structure. However, we have also identified more conservative mutations that lead to the same phenotype, including F289L, T297A, and F301I.

The third category represents 12 mutants with a slight growth advantage in the presence of alafosfalin, and uptake activity ranging from ~20 to 60% of the native transporter. The mutants G86R, R305C, and P326Q show very little transport in the  $\beta$ -Ala-Lys(AMCA) uptake assay, however, do retain some ability to take up alafosfalin, and have therefore been included in this category. Similarly, N300Y seems unable to transport  $\beta$ -Ala-Lys(AMCA), but still retains significant ability to transport  $\beta$ -Ala-Lys(AMCA), particularly when correcting for its reduced expression level. Again, many of these mutations represent substitution of one type of amino acid for another, however, they show milder phenotypes than the complete loss-of-function mutants. Most interesting, the G127D mutant maintains significant transport activity despite its low expression level (Table 2).

A fourth intriguing group of mutants, which we refer to as selectivity mutants, are those that exhibit a loss-of-function phenotype in the alafosfalin growth assay, yet show uptake of  $\beta$ -Ala-Lys(AMCA) equivalent to or, in some cases, even greater than that of the native transporter (Table 2). This would suggest that these mutations do not affect the ability of the protein to transport substrates, but rather may affect substrate selectivity or affinity. Although the uptake assay is performed in minimal media with only one potential substrate present, the alafosfalin growth assay is performed in complex media (LB), which contains other potential di- and tripeptide substrates. Although the presence of competitors in complex media does not inhibit transport of alafosfalin by the native transporter, higher affinity

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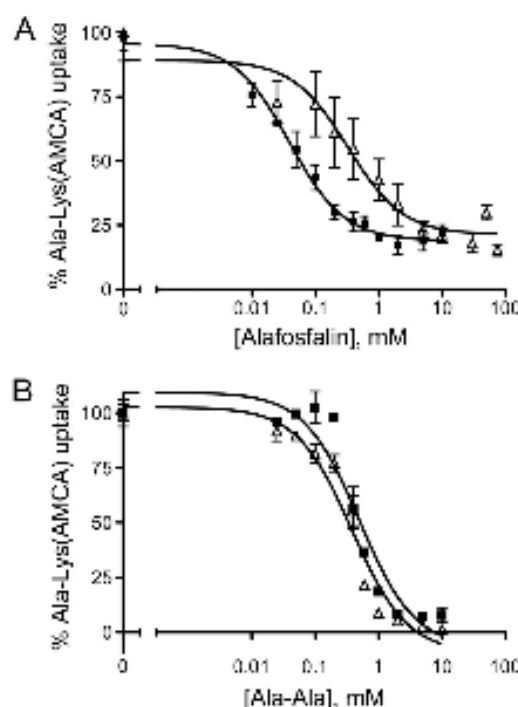


**FIGURE 4. Competitive binding data for native YdgR and the V252E mutant.** A, inhibition of  $\beta$ -Ala-Lys(AMCA) uptake by alafosfalin. Representative data for the native transporter (black squares) and the V252E mutant (white triangles) from one experiment are shown. B, inhibition of  $\beta$ -Ala-Lys(AMCA) uptake by alanyl-alanine (Ala-Ala). Representative data for the native transporter (black squares) and the V252E mutant (white triangles) are shown. Data for all mutants are summarized in Table 3.

**TABLE 3**

Apparent affinities of the selectivity mutants for alafosfalin and alanyl-alanine (Ala-Ala)

| YdgR mutant | Alafosfalin                   |                             | Ala-Ala                       |                             |
|-------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|
|             | IC <sub>50</sub> <sup>a</sup> | K <sub>i</sub> <sup>b</sup> | IC <sub>50</sub> <sup>a</sup> | K <sub>i</sub> <sup>b</sup> |
| Wild-type   | 0.11 ± 0.07                   | 0.07 ± 0.08                 | 0.33 ± 0.16                   | 0.21 ± 0.2                  |
| M154K       | 0.39 ± 0.12                   | 0.25 ± 0.16                 | 0.12 ± 0.01                   | 0.08 ± 0.01                 |
| L190V       | 0.59 ± 0.03                   | 0.38 ± 0.04                 | 0.32 ± 0.06                   | 0.20 ± 0.08                 |
| F197I       | 0.71 ± 0.02                   | 0.45 ± 0.03                 | 0.48 ± 0.01                   | 0.31 ± 0.01                 |
| V252E       | 0.44 ± 0.21                   | 0.28 ± 0.27                 | 0.33 ± 0.05                   | 0.21 ± 0.06                 |
| K274I       | 0.08 ± 0.04                   | 0.05 ± 0.05                 | 0.31 ± 0.07                   | 0.20 ± 0.09                 |
| L324V       | 1.5 ± 0.07                    | 0.96 ± 0.09                 | 0.56 ± 0.10                   | 0.36 ± 0.13                 |

<sup>a</sup> IC<sub>50</sub> values represent averages from at least two experiments with error calculated as S.D.

<sup>b</sup> K<sub>i</sub> values were determined using the equation:  $K_i = IC_{50}/(1 + [substrate]/K_D)$ . The K<sub>D</sub> value used was 0.44 ± 0.05 mM, which is the apparent affinity of YdgR for  $\beta$ -Ala-Lys(AMCA) as determined in Ref. 14.

interaction with competitors, and/or lower affinity interaction with alafosfalin, may prevent alafosfalin uptake in these mutants.

**Selectivity Mutants Show Lower Apparent Affinity for Alafosfalin**—To test the hypothesis that these mutants have a lower apparent affinity for alafosfalin than the native transporter, we determined the IC<sub>50</sub> and K<sub>i</sub> values for alafosfalin for these six mutants by performing the  $\beta$ -Ala-Lys(AMCA) uptake assay in the presence of increasing concentrations of alafosfalin (Fig. 4A, Table 3). This experiment reveals that mutants M154K, L190V, F197I, V252E, and L324V have K<sub>i</sub>

values 3.5–13.5-fold higher than the native transporter, indicating weaker interaction with alafosfalin. In contrast, the mutant K274I has a K<sub>i</sub> value equal to that of the native transporter.

The structure of alafosfalin mimics that of the dipeptide alanyl-alanine, however, in alafosfalin (L-alanyl-L-1-aminoethylphosphonic acid) the carboxyl group on the C-terminal moiety is replaced by phosphonic acid. To determine whether these mutants show lower apparent affinity for dipeptide substrates with an unmodified carboxyl terminus, the IC<sub>50</sub> and K<sub>i</sub> values for alanyl-alanine were also determined (Fig. 4B, Table 3). For alanyl-alanine, the K<sub>i</sub> values for five mutants are within  $\pm 2$ -fold of that of the native transporter, and for one mutant, M154K, the apparent affinity is higher than that of the native transporter. This suggests that the lower apparent affinity for alafosfalin for 5 of these mutants is not a general property of substrate binding, but rather may be specific for the substrate alafosfalin. We conclude that the loss of apparent affinity for alafosfalin is related to the presence of the additional C-terminal charged hydroxyl, either through direct steric hindrance of binding or unfavorable charge-charge interactions. As the K274I mutant shows apparent affinities similar to those of the native transporter for both alafosfalin and alanyl-alanine, it is not clear from these experiments why this mutant shows a LOF phenotype in the alafosfalin growth assay.

**Localizing Mutations Based on Homology Modeling**—To attempt to correlate the phenotype of individual mutations with their location in the transporter, we generated a homology model of YdgR based on the structure of PepT<sub>sc</sub> (17). PepT<sub>sc</sub> shares 26% identity and 45% similarity with YdgR (supplemental Fig. S1). Almost all of the residues in the peptide binding site of PepT<sub>sc</sub> are conserved in YdgR, and also localize to the substrate cavity in our model (supplemental Fig. S1, Fig. 5, A and B).

The majority of LOF mutants isolated in our screen are located in helices 2, 4, and 7 of our homology model, with the majority in helix 7 (Table 2, supplemental Fig. S1). Significantly fewer mutants were found in helices 3, 6, and 8. This correlates well with data from other MFS family members, as based on the structures of LacY, GlpT, EmrD, and PepT<sub>sc</sub>, helices 2, 4, and 7 are located in the hydrophilic cavity representing the substrate translocation pathway and contain many critical functional residues (15, 16–18). Residues that when mutated result in a partial LOF phenotype are located primarily in helix 4 (Ile<sup>122</sup>, Ala<sup>123</sup>, Gly<sup>127</sup>) or close to the end of helix 7 (Asn<sup>300</sup>, Ala<sup>303</sup>, Arg<sup>305</sup>, Asn<sup>306</sup>), and appear to be more distant from the substrate binding site (17) (Fig. 5, supplemental Fig. S1).

Most of the LOF or partial LOF mutations isolated in our screen are located in the transmembrane helices, and, as mentioned previously, represent quite severe mutations. Therefore, many of these mutations most probably lead to dramatic changes in helix packing (I60N, L98R, I122N, L136R) or introduce kinks or bends in the helices (S59P, A68P, G78C, G86R, G101D, G127D, A264P, P326Q). However, several mutations are more conservative (A285V, F289L, F301I) yet also result in complete loss of function. Phe<sup>289</sup> is of particular interest as in our model this residue is in a position equivalent to that of

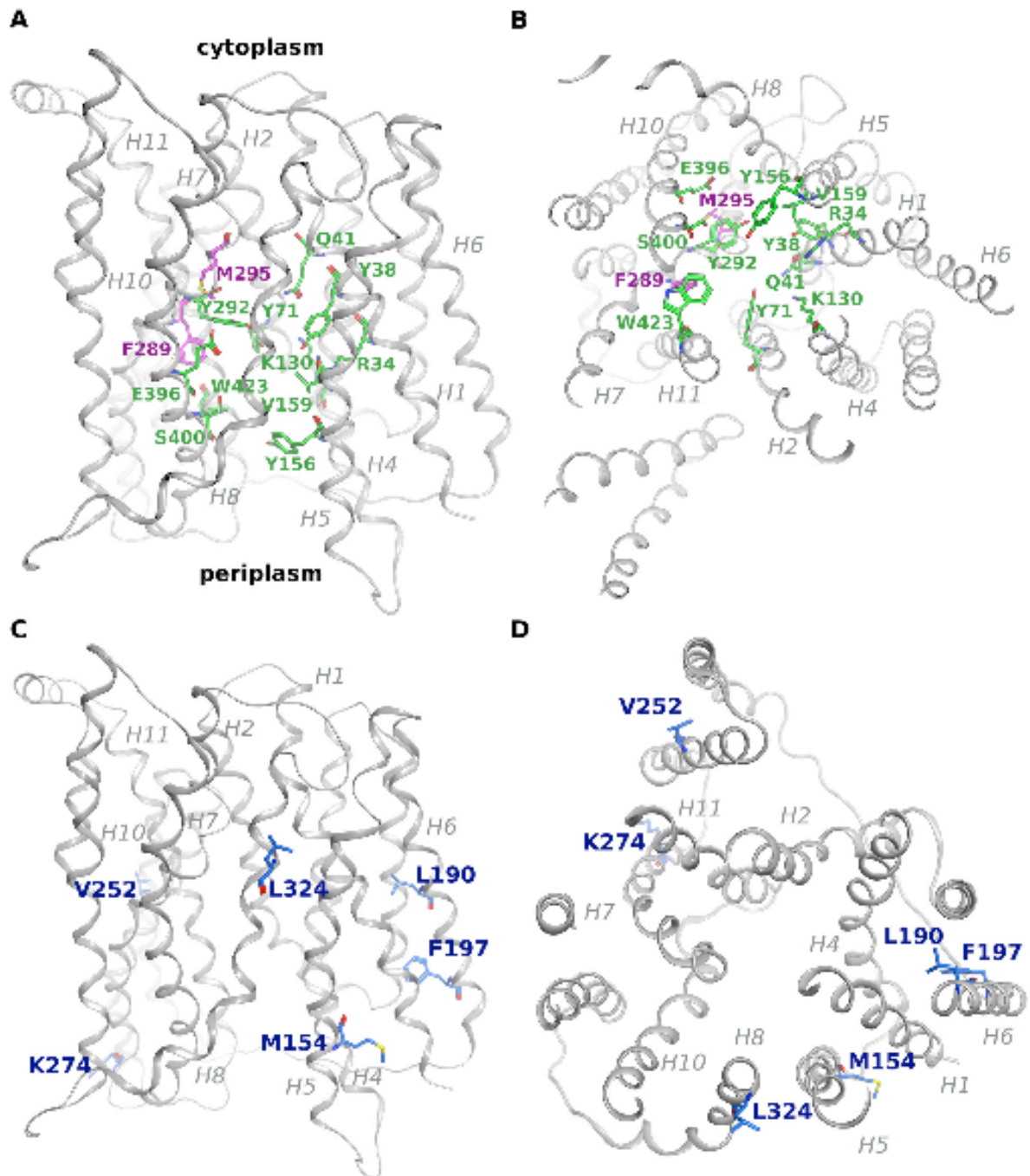


**FIGURE 5. Homology modeling reveals putative functional residues.** A homology model of YdgR was generated based on the structure of the PepT<sub>sc</sub> transporter (PDB code 2XUT). The YdgR backbone is colored gray and shown in ribbon representation with amino acids shown in stick representation. The transmembrane helices are labeled in gray. A, view perpendicular to the membrane. Conserved amino acids lining the substrate binding cavity are colored green, CPK. Phe<sup>289</sup> and Met<sup>295</sup> are shown in magenta. B, view parallel to the membrane from the cytoplasmic side. Colored as in A. C, view perpendicular to the membrane. Residues resulting in selectivity against alafosfalin are colored blue, CPK. D, view parallel to the membrane from the periplasmic side, colored as in C.

Trp<sup>312</sup> in PepT<sub>sc</sub>, one of the few residues lining the substrate cavity that is not strictly conserved in YdgR (Fig. 5, A and B, supplemental Fig. S1).

Another residue of interest is Met<sup>295</sup>, as it is located in the substrate binding cavity of our homology model, and the mutant M295K shows a complete loss of transport function

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TABLE 4

Membrane expression and transport characteristics of alanine and charge-swap mutants

| Mutant      | Growth advantage <sup>a</sup> | Uptake of $\beta$ -Ala-Lys(AMCA) <sup>b</sup> |     | Membrane expression <sup>c</sup> | Corrected uptake <sup>d</sup> |
|-------------|-------------------------------|-----------------------------------------------|-----|----------------------------------|-------------------------------|
|             |                               | %                                             | %   | %                                | %                             |
| E56A        | 2.6 $\pm$ 0.5                 | 9 $\pm$ 1                                     | 7   | 129                              |                               |
| E56N        |                               | 15 $\pm$ 5                                    | 106 | 14                               |                               |
| E56R        |                               | 4 $\pm$ 3                                     | 95  | 4                                |                               |
| S59A        | 0.8 $\pm$ 0.2                 | 149 $\pm$ 36                                  | 111 | 134                              |                               |
| S64A        |                               | 95 $\pm$ 19                                   | 72  | 132                              |                               |
| M295A       | 2.3 $\pm$ 0.1                 | 72 $\pm$ 8                                    | 81  | 89                               |                               |
| N300A       | 2.6 $\pm$ 0.1                 | 24 $\pm$ 13                                   | 88  | 27                               |                               |
| N300E       |                               | 9 $\pm$ 4                                     | 85  | 11                               |                               |
| R305A       | 2.4 $\pm$ 0.6                 | 7 $\pm$ 3                                     | 85  | 8                                |                               |
| R305E       |                               | 9 $\pm$ 4                                     | 79  | 11                               |                               |
| N306A       | 1.2 $\pm$ 0.1                 | 72 $\pm$ 8                                    | 87  | 8                                |                               |
| N306E       |                               | 8 $\pm$ 3                                     | 87  | 9                                |                               |
| E56N, N300E |                               | -2 $\pm$ 2                                    | 77  | -3                               |                               |
| E56R, N300E |                               | -2 $\pm$ 1                                    | 92  | -2                               |                               |
| E56N, R305E |                               | 35 $\pm$ 15                                   | 81  | 43                               |                               |
| E56R, R305E |                               | 220 $\pm$ 66                                  | 101 | 218                              |                               |
| E56N, N306E |                               | -2 $\pm$ 2                                    | 93  | -2                               |                               |
| E56R, N306E |                               | -2 $\pm$ 2                                    | 96  | -2                               |                               |

<sup>a</sup> Data are represented as the fold-difference in  $A_{600}$  of mutant versus native transporter in the presence and absence of alafosfalin 5 h post-induction:  $(A_{600}^{\text{mutant}} + A_{600}^{\text{alafosfalin}}) / (A_{600}^{\text{mutant}} + A_{600}^{\text{alafosfalin}}) / (A_{600}^{\text{WT}} + A_{600}^{\text{alafosfalin}})$ . Error values represent S.D. of triplicate measurements from at least two different experiments.

<sup>b</sup> Fluorescence values are normalized after setting the value of the overexpressed native transporter to 100%.

<sup>c</sup> % Membrane expression was determined by quantitation of the band intensity on the Western blots shown in Fig. 2 using ImageJ software (22) and normalized by setting the value of the overexpressed native transporter to 100%.

<sup>d</sup> Corrected uptake represents: (% uptake of  $\beta$ -Ala-Lys(AMCA)) / (% membrane expression)  $\times$  100.

(Table 2, Fig. 5, A and B). To determine whether the side chain of Met<sup>295</sup> plays a role in substrate binding, we created mutant M295A and measured its ability to transport substrates in both the alafosfalin growth assay and the  $\beta$ -Ala-Lys(AMCA) uptake assay. M295A is unable to transport alafosfalin, with a growth advantage of  $2.3 \pm 0.1$  over native YdgR (Table 4). However, M295A shows 70%  $\beta$ -Ala-Lys(AMCA) uptake activity as compared with native YdgR, which increases to 89% when accounting for the reduced expression level of the mutant (Fig. 7A, Table 4). Therefore we conclude that this residue does not play a significant role in mediating substrate interaction in general, but may modulate affinity toward certain substrates, as it is selective against alafosfalin.

The residues that when mutated show selectivity against alafosfalin are, perhaps surprisingly, located distant from the substrate binding cavity (Fig. 5, C and D). Residues Leu<sup>190</sup> and Phe<sup>197</sup> are located in helix 6 and most likely affect packing with the adjacent helices 1, 3, and 4. Val<sup>252</sup> is located in helix HB of the membrane hairpin, which is not thought to play a role in transport, but may also affect packing of adjacent helices. Met<sup>154</sup> is located at the cytoplasmic end of helix 5, whereas Lys<sup>274</sup> is located at the cytoplasmic end of helix 7. Leu<sup>324</sup> is located in helix 8, within the strictly conserved "LNP" motif, and its mutation may affect the interface with helices 10 and 7 (Fig. 5, C and D, supplemental Fig. S1).

Several polar or charged residues that were isolated as LOF mutants (Glu<sup>56</sup>, Ser<sup>59</sup>, Asn<sup>300</sup>, Arg<sup>305</sup>, Asn<sup>306</sup>) are located in the periplasmic halves of helices 2 and 7, and may form part of a hydrogen bonding network critical for closing of the periplasmic cavity, as seen in LacY and GlpT (27, 28). As the putative gating residues of the peptide transporter PepT<sub>ec</sub>, His<sup>61</sup> and Glu<sup>316</sup>, are not conserved in YdgR and many other bacterial

PTRs (17) (supplemental Fig. S1), we hypothesized that one or more of these residues are potential candidates for the periplasmic gating residues of YdgR. To compare the positions of these residues to the putative gating residues of PepT<sub>ec</sub> and the previously characterized gating residues of LacY, we aligned the structures of PepT<sub>ec</sub>, LacY, and the YdgR homology model. Glu<sup>56</sup> and Arg<sup>305</sup> are within close proximity in our homology model, and are located in positions similar to the LacY gating residues Ile<sup>40</sup> and Asn<sup>245</sup> (28) (Fig. 6).

**Glu<sup>56</sup> and Arg<sup>305</sup> Are Potential Gating Residues**—To determine whether any of the charged residues near the periplasmic ends of helix 2 and 7 isolated in our screen may be responsible for periplasmic gating we first mutated Glu<sup>56</sup>, Ser<sup>59</sup>, Asn<sup>300</sup>, Arg<sup>305</sup>, and Asn<sup>306</sup> to alanine. If the side chains of any of these residues are involved in periplasmic gating, we would expect mutation to alanine to result in a LOF phenotype similar to that of the original mutation.

We tested these new mutants in the alafosfalin growth assay and  $\beta$ -Ala-Lys(AMCA) uptake assay. This analysis reveals that mutants N300A and R305A have phenotypes identical to those of the original mutation isolated in the screen, suggesting a functional role for the side chains of these amino acids (Fig. 7A, Table 4). E56A also shows a loss-of-function phenotype, however, this is due to a dramatic loss in membrane expression (Table 4, Fig. 2). However, because E56G also displays a LOF phenotype and localizes to the membrane, we hypothesized that the Glu<sup>56</sup> side chain is also critical for function. In contrast, as Ser<sup>59</sup> and Asn<sup>306</sup> show an increase in transport activity as compared with the original mutation, we conclude that these residues are unlikely to be critical gating residues. Additionally, we mutated Ser<sup>64</sup> to alanine, as this residue is in a position equivalent to that of His<sup>61</sup> in PepT<sub>ec</sub> (supplemental Fig. S1) and tested this mutant in the  $\beta$ -Ala-Lys(AMCA) uptake assay, where it displays activity equal to that of the native transporter, making this side chain unlikely to play a role in gating (Fig. 7A, Table 4).

To test whether E56 interacts with Asn<sup>300</sup> or Arg<sup>305</sup> we created several "charge-swapped" transporters, in which Glu<sup>56</sup> is mutated to either asparagine or arginine, and Asn<sup>300</sup> or Arg<sup>305</sup> is mutated to glutamate. We also included Asn<sup>306</sup> in this study due to its proximity to Arg<sup>305</sup>. We expected that transporters containing the single mutations would be inactive, whereas if the side chains of these residues form a hydrogen bond or charge-based interaction, a double mutant in which the residues are swapped may rescue activity. When tested, the single mutants all show no activity in the  $\beta$ -Ala-Lys(AMCA) uptake assay (Fig. 7B, Table 4). The double mutants tested are also inactive, with the exception of the E56N/R305E double mutant, in which transport activity is restored to 35% of the native transporter, and the E56R/R305E double mutant, in which transport activity is dramatically increased to 220% of the native transporter (Fig. 7B, Table 4). We therefore propose that Glu<sup>56</sup> and Arg<sup>305</sup> are the periplasmic gating residues in YdgR, fulfilling the roles of His<sup>61</sup> and Glu<sup>316</sup> in PepT<sub>ec</sub>.

## DISCUSSION

We have identified residues in the *E. coli* YdgR peptide transporter that compromise transport ability when

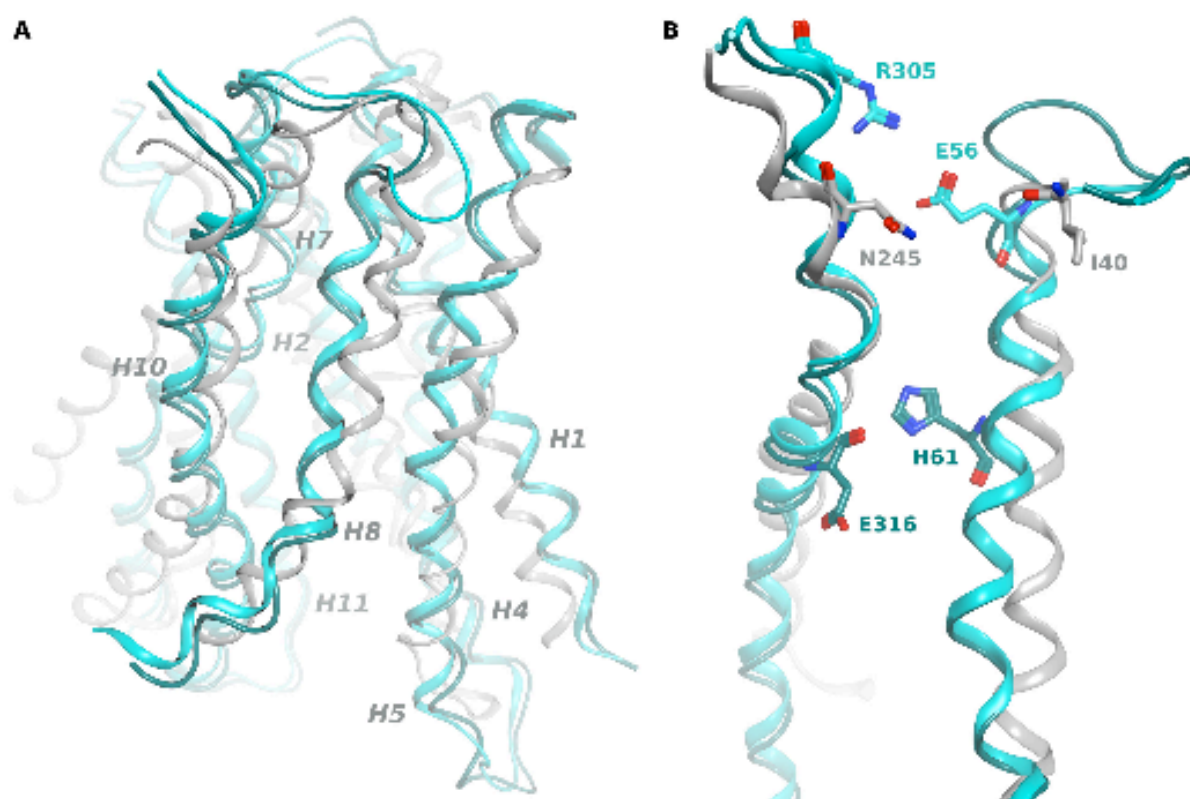


**FIGURE 6. Alignment of the YdgR model with LacY and PepT<sub>so</sub> identifies potential periplasmic gating residues.** The YdgR homology model was aligned with the crystal structures of LacY (PDB code 2CFQ) and PepT<sub>so</sub> (PDB code 2XUT) to compare the positions of the periplasmic gating residues from LacY (Ile<sup>40</sup>, Asn<sup>245</sup>) and PepT<sub>so</sub> (His<sup>61</sup>, Glu<sup>316</sup>) with Glu<sup>56</sup> and Arg<sup>305</sup>. A, view perpendicular to the membrane. The backbone of the YdgR homology model (cyan) is aligned with that of the LacY structure (light gray) and that of PepT<sub>so</sub> (dark gray). For clarity only helices lining the substrate cavity are shown, and are labeled in gray. B, the isolated helices 2 and 7 of the aligned structures, colored as in A. Gating residues from each protein are shown in stick conformation.

mutated, based on random mutagenesis and selection for mutants with a loss-of-function phenotype. By generating a homology model of YdgR based on the structure of PepT<sub>so</sub>, we have localized these mutations to specific transmembrane helices with proximity to the substrate translocation pathway. Based on our homology model, our functional data, and studies of PepT<sub>so</sub>, LacY, GlpT, and PepT1 several interesting insights emerge regarding individual residues as well as YdgR function in general.

The phenotypes of several mutants correlate well with the results of mutagenesis of other PTR or MFS members. Two of the mutants isolated in our study, Gly<sup>78</sup> and Gly<sup>86</sup>, confirm the importance of the conserved MFS motif in loop 2–3, and are both conserved in prokaryotic homologues of YdgR (supplemental Fig. S1). Gly<sup>78</sup> is equivalent to Gly<sup>64</sup> in LacY, which seems critical for maintaining conformational flexibility, as only mutation to alanine is tolerated at this position (29). In agreement with studies on LacY, mutation of Gly<sup>78</sup> shows a more severe phenotype than mutation of Gly<sup>86</sup> (6).

We were also able to identify Phe<sup>289</sup> as the likely equivalent of Trp<sup>312</sup> of PepT<sub>so</sub>, one of the few residues in the substrate binding pocket that is not strictly conserved between YdgR and PepT<sub>so</sub>. Both phenotypes of the Phe<sup>289</sup> mutants, two of which

were isolated in our screen, and our homology model point to a role for Phe<sup>289</sup> equivalent to that of Trp<sup>312</sup>. In PepT<sub>so</sub>, Trp<sup>312</sup> is forming hydrophobic interactions with Trp<sup>446</sup> (Trp<sup>423</sup> in YdgR) in the substrate binding cavity. In human PEPT1, mutation of the Trp<sup>312</sup> equivalent Trp<sup>294</sup> to alanine also results in reduced transport activity (26), providing additional support for the role of this residue in substrate transport.

We also propose Met<sup>295</sup> as an additional residue in the substrate binding pocket that may mediate substrate affinity. This residue is conserved in many bacterial species, although not in PepT<sub>so</sub>. As mutation of Met<sup>295</sup> to alanine creates a selectivity mutant, it is clear that even changes to less conserved residues in the substrate binding pocket or in residues influencing packing of the helices lining the substrate cavity may affect substrate affinity and selectivity.

Because even conservative mutations distant from the substrate translocation pathway, such as L190V or L324V, lead to changes in the apparent affinity for selected substrates, we propose that YdgR undergoes large and dynamic conformational transitions, as has been shown for LacY and GlpT (27, 30). Due to the large number of mutations we isolated in helix 7, it seems that in particular the interhelical interface of this helix is critical for proper protein function. This is in agreement

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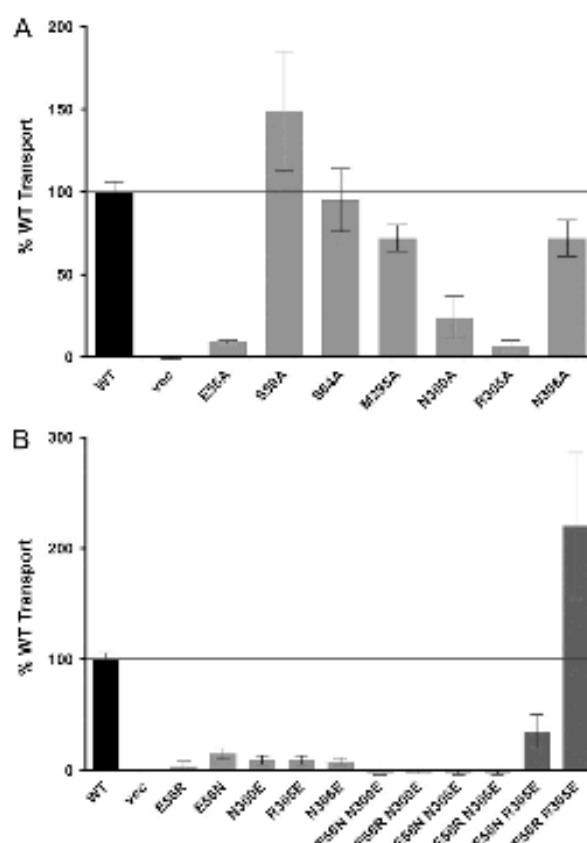


**FIGURE 7. Glu<sup>56</sup> and Arg<sup>305</sup> are potential periplasmic gating residues.** The ability of each mutant to take up the fluorescent dipeptide substrate  $\beta$ -Ala-Lys(AMCA) was measured and compared with that of the native transporter. Error bars represent standard deviations of triplicate measurements from at least two different experiments. Wild-type (WT) and vector (vec) controls are shown as black bars, single mutants are shaded in light gray, and double mutants in dark gray. A black line is used to indicate 100% transport. A, the ability of E56A, S59A, S64A, M295A, N300A, R305A, and N306A to take up  $\beta$ -Ala-Lys(AMCA) is compared with wild-type (WT) and vector (vec) controls. B, the ability of single and double charge-swap mutations of Glu<sup>56</sup>, Arg<sup>305</sup>, and Asn<sup>306</sup> to take up  $\beta$ -Ala-Lys(AMCA) is compared with wild-type (WT) and vector (vec) controls.

with structural work on PepT<sub>so</sub>. Comparison between LacY in the open-in conformation and PepT<sub>so</sub> in the occluded conformation shows the greatest displacement in helix 7 (17). This underscores the similarity between YdgR and MFS transporters.

Although the crystal structure of PepT<sub>so</sub> identified the potential periplasmic gating residues His<sup>61</sup> and Glu<sup>316</sup>, these residues are not conserved in most other prokaryotic PTR family members. Through our mutagenesis screen, we identified several candidate amino acids that may play a role in periplasmic gating, and we were able to confirm that two of these residues, Glu<sup>56</sup> and Arg<sup>305</sup>, are critical for transport function, most likely through formation of a charge-based interaction that may regulate gating. As these residues are located on the periplasmic termini of helices 2 and 7 in our homology model, they would also be positioned close to the small extracellular cavity seen in the PepT<sub>so</sub> structure, formed by the periplasmic halves of heli-

ces 2, 7, 11, and 12 (17). As this cavity has been proposed as the entry site for peptide substrates (17), these residues are likely to be properly positioned for coupling of gating and substrate binding.

Comparison of the locations of the putative periplasmic gating residues of PepT<sub>so</sub> and YdgR with those of LacY indicates that the position of the periplasmic gate of YdgR seems to be much closer to that of LacY than that of PepT<sub>so</sub> (Fig. 7B). The putative gating residues of YdgR show higher conservation within bacterial PTR family members than His<sup>61</sup> and Glu<sup>316</sup> of PepT<sub>so</sub>, however, they are also not conserved in more distant homologues (supplemental Fig. S1). This suggests that periplasmic gating may not be strictly conserved within the PTR family, and more than one periplasmic gating mechanism may have evolved. Taken together, the results of our analysis suggest that YdgR shares many functional similarities with both PepT<sub>so</sub> and LacY, thus adding support to the hypothesis that members of the MFS superfamily, and perhaps other proton-coupled transporter families, share a common fold and mechanism, despite transporting a wide range of substrates.

**Acknowledgments**—We thank Stefan Westermann and Tim Clausen for critical reading of the manuscript, and Subhdeep Sarker for assistance with GraphPad Prism 5.0. We are grateful to Daniel Harder and Hannelore Daniel for helpful discussions at the start of this project and for providing us with initial amounts of  $\beta$ -Ala-Lys(AMCA).

## REFERENCES

- Paulsen, I. T., and Skurray, R. A. (1994) *Trends Biochem. Sci.* **19**, 404
- Steiner, H. Y., Naider, F., and Becker, J. M. (1995) *Mol. Microbiol.* **16**, 825–834
- Chang, A. B., Lin, R., Keith Studley, W., Tran, C. V., and Saier, M. H., Jr. (2004) *Mol. Membr. Biol.* **21**, 171–181
- Daniel, H., Spanier, B., Kottra, G., and Weitz, D. (2006) *Physiology* **21**, 93–102
- Henderson, P. J., and Maiden, M. C. (1990) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **326**, 391–410
- Jessen-Marshall, A. E., Paul, N. J., and Brooker, R. J. (1995) *J. Biol. Chem.* **270**, 16251–16257
- Hauser, M., Kauffman, S., Naider, F., and Becker, J. M. (2005) *Mol. Membr. Biol.* **22**, 215–227
- Kulkarni, A. A., Haworth, I. S., and Lee, V. H. (2003) *Biochem. Biophys. Res. Commun.* **306**, 177–185
- Fei, Y. J., Liu, J. C., Fujita, T., Liang, R., Ganapathy, V., and Leibach, F. H. (1998) *Biochem. Biophys. Res. Commun.* **246**, 39–44
- Kulkarni, A. A., Haworth, I. S., Uchiyama, T., and Lee, V. H. (2003) *J. Biol. Chem.* **278**, 51833–51840
- Daniel, H., Morse, E. L., and Adibi, S. A. (1992) *J. Biol. Chem.* **267**, 9565–9573
- Brandsch, M., Knütter, L., and Bosse-Doenecke, E. (2008) *J. Pharm. Pharmacol.* **60**, 543–585
- Harder, D., Stolz, J., Casagrande, F., Obrdlik, P., Weitz, D., Fotiadis, D., and Daniel, H. (2008) *FEBS J.* **275**, 3290–3298
- Weitz, D., Harder, D., Casagrande, F., Fotiadis, D., Obrdlik, P., Kelety, B., and Daniel, H. (2007) *J. Biol. Chem.* **282**, 2832–2839
- Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) *Science* **301**, 610–615
- Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) *Science* **301**, 616–620
- Newstead, S., Drew, D., Cameron, A. D., Postis, V. L., Xia, X., Fowler,

- P. W., Ingram, J. C., Carpenter, E. P., Sansom, M. S., McPherson, M. J., Baldwin, S. A., and Iwata, S. (2011) *EMBO J.* **30**, 417–426
18. Yin, Y., He, X., Szewczyk, P., Nguyen, T., and Chang, G. (2006) *Science* **312**, 741–744
19. Spiess, C., Beil, A., and Ehrmann, M. (1999) *Cell* **97**, 339–347
20. Liu, H., and Naismith, J. H. (2008) *BMC Biotechnol.* **8**, 91
21. Surade, S., Klein, M., Stolt-Bergner, P. C., Muenke, C., Roy, A., and Michel, H. (2006) *Protein Sci.* **15**, 2178–2189
22. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) *Biophotonics Int.* **11**, 36–42
23. Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234**, 779–815
24. Laskowski, R., MacArthur, M., Moss, D., and Thornton, J. (1993) *J. Appl. Crystallogr.* **26**, 283–291
25. Gibson, M. M., Price, M., and Higgins, C. F. (1984) *J. Bacteriol.* **160**, 122–130
26. Bolger, M. B., Haworth, I. S., Yeung, A. K., Ann, D., von Grafenstein, H., Hamm-Alvarez, S., Okamoto, C. T., Kim, K. J., Basu, S. K., Wu, S., and Lee, V. H. (1998) *J. Pharm. Sci.* **87**, 1286–1291
27. Law, C. J., Almqvist, J., Bernstein, A., Goetz, R. M., Huang, Y., Soudant, C., Laaksonen, A., Hovmöller, S., and Wang, D. N. (2008) *J. Mol. Biol.* **378**, 828–839
28. Zhou, Y., Nie, Y., and Kaback, H. R. (2009) *J. Mol. Biol.* **394**, 219–225
29. Jessen-Marshall, A. E., Parker, N. J., and Brooker, R. J. (1997) *J. Bacteriol.* **179**, 2616–2622
30. Guan, L., and Kaback, H. R. (2006) *Annu. Rev. Biophys. Biomol. Struct.* **35**, 67–91
31. Söding, J., Biegert, A., and Lupas, A. N. (2005) *Nucleic Acids Res.* **33**, W244–W248 (Web Server issue)

## 9 Curriculum vitae

### Personal Data

Full Name: Bettina Spitzenberger  
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### Academic Career

2008-present: PhD student in the group of Peggy Stolt- Bergner, PhD as part of the Vienna Biocenter PhD program at the Institute of Molecular Pathology (IMP), Vienna (Austria)

2006-2007: Diploma thesis at the Department of Biomolecular Structural Chemistry in the group of Kristina Djinovic-Carugo on “Structural insight into the interaction of human carbonic anhydrase II and human anion exchanger 1 C-terminal region”

2000-2006: Graduate studies in “Molecular Biology” at the University of Vienna

### Publications

*“Random Mutagenesis of the Prokaryotic Peptide Transporter YdgR Identifies Potential Periplasmic Gating Residues”*

Elisabeth Malle, Hongwen Zhou, Jana Neuhold, **Bettina Spitzenberger**, Frey Klepsch, Thomas Pollak, Oliver Bergner, Gerhard F. Ecker, and Peggy C. Stolt-Bergner