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Using Cys-Scanning Analysis Data in the Study of Membrane Transport Proteins

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1. Introduction

Membrane transport proteins represent a core group of gene products in all known genomes and play crucial roles in either human physiology and disease or diverse environmental adaptations of microorganisms. Despite their importance, analyses of such proteins have long been hindered by a lack of high-resolution models, which reflects the inherent problems of studying membrane transport proteins outside the membrane. Recently, however, progress with crystallography and structural modeling of membrane transport proteins and expanding information on new sequence entries from genome analyses have set the stage for more systematic mutagenesis approaches to link high-resolution structural data with functional evidence.

The current structural insight on secondary transporters and their classification raises new fundamental questions on the relationships of structure with function. A crucial aspect is that many of the functionally divergent homologs, or even separate families of secondary transporters, are evolutionarily and structurally related. Thus, the majority of known structures fall in only two common folds: lactose permease (LacY) and the neurotransmitter-sodium symporter prototype (LeuT). Other transporters with different folds still display the core feature of organization in structural repeats that coordinate to form the dynamic binding site (Boudker & Verdon, 2010). The binding site operates through the commonly accepted alternating access mechanism, which is now explained as involving both rocking movements of domains pivoting at the binding site and local motions of outside and inside gates flanking the binding site, leading to alterations between outward-facing, inward-facing and intermediate substrate-occluded conformation states (Forrest et al., 2011). Apart from the structural knowledge, systematic insight on the secondary transport mechanisms requires the concerted use of structural and functional approaches, as was achieved in the seminal case of lactose permease (Kaback et al., 2011). This is important to note, since the X-ray structures represent static snapshots of highly dynamic proteins outside their native membrane environment; and, with few exceptions (Weyand et al., 2011), interpretations have been based on compilations of different snapshots from different structural homologs.

In the context of the recent structural evidence, it is striking that transport protein families tend to display high evolutionary conservation in sequence and overall structure; but they also display high functional variations between homologs, implying that relatively few side-chain changes may account for key local effects on active-site conformation and function.

However, mutations responsible for such evolutionary plasticity are rarely discernible in the background of many optimizing, permissive or near-neutral mutations that have accumulated in the present-day sequences over evolutionary time (Harms & Thornton, 2010). Traditional structure-function analysis often fails to annotate such important residues because the mutations may be uninformative or lead to complete loss-of-function/structure phenotypes in the native background. Therefore, a rationally designed mutagenesis study of one particular homolog may yield important information on the overall active-site architecture and mechanism; but it is not enough to reveal the spectrum of molecular determinants dictating the different specificity trends within the family.

In practice, it is common that an evolutionarily-broad transporter family consists of several hundreds or even thousands of related members. They share the same structural fold and binding-site architecture based on X-ray crystallography of one, usually distal, homolog. Few of the members might have been characterized extensively with respect to function, and only one or two might have been studied rigorously with site-directed mutagenesis approaches. How then can we explore the whole spectrum of specificities in such families and derive more essential information on the molecular basis of the different functional profiles?

One approach to this research problem is based on data derived from Cys-scanning analysis. Cys-scanning mutagenesis has already proven to be an essential tool (often referred to as the "gold standard") for the study of structure-function relationships in membrane proteins (Frillingos et al., 1998). In several separate examples of membrane transporters, it has provided valuable insight on active site conformation and function, even before an X-ray structure has become available (Chen & Rudnick, 2000; Kaback et al., 2001; Sorgen et al., 2002; Zomot et al., 2002). Revisiting Cys-scanning evidence after elucidation of a corresponding high-resolution structure has yielded important novel implications on the mechanism (Crisman et al., 2009; Forrest et al., 2008; Guan & Kaback, 2006; Kaback et al., 2007, 2011). The site-specific knowledge derived from Cys-scanning analysis of a specific transporter can also be used, in principle, to design rapid and cost-effective mutagenesis strategies for the functional analysis of different, structurally-related homologs.

The rationale of this approach is to use data from a systematic Cys-scanning analysis of one homolog (the study prototype) in combination, when applicable, with homology-modeling to select new homologs for targeted mutagenesis studies. This approach is based on differences and the extent of conservation, not in the overall sequence, but in the subset of residues delineated as putatively important from the Cys-scanning data. These putatively important residues correspond to positions of the study prototype where a native amino acid is irreplaceable or replaceable with only few side chains or is sensitive to site-specific alkylation of a substituted Cys leading to inactivation. In several experimental paradigms, such positions have been shown to be (a) relatively few (less than 15% of the total number of residues, in general); (b) much more highly conserved among homologs than in the rest of the protein and often mapping within conserved motif sequences (for example, see Georgopoulou et al., 2010); and (c) linked either directly or indirectly with the substrate binding site conformation and function (Kaback et al., 2007). This latter property allows the use of this set of positions as targets of rationalized mutagenesis in new homologs, in order not only to provide a measure of the functional conservation between different homologs, but also to delineate determinants responsible for particular switches in substrate preference or specificity.

In this chapter, I first analytically explain the rationale, discrete steps and aims of the Cys-scanning-based approach. I then describe applications in two families of ion gradient-driven membrane transporters. The first is the oligosaccharide-proton symporter family (OHS), which includes the lactose permease LacY and other closely related sugar transporters. The second is the nucleobase-ascorbate transporter family (NAT/NCS2), which is evolutionarily ubiquitous and encompasses more distantly related homologs and specificities. It is important to emphasize that such an experimental approach, although conceptually sound, has seen limited applications in the field of membrane transport proteins to date. A more systematic and generalized application of this approach is expected to have a major impact on the field, since it should allow rapid and effective mutagenesis designs. The impact will occur even in the absence of a high-resolution model, provided only that Cys-scanning analysis has been performed for one of the transporter homologs.

2. Rationale, discrete steps, and aims of the approach

Cys-scanning mutagenesis is a well-established strategy for structure-function analysis of proteins. It has proven particularly useful and provided valuable insight for the analysis of polytopic membrane proteins and, in particular, membrane transporters. Cys-scanning protocols rely on the engineering and availability of functional protein variants that are devoid of all or part of the native Cys residues (Cys-less or Cys-depleted versions, respectively) and the use of these Cys-less or Cys-depleted versions as a background for site-specific mutagenesis to introduce new single-Cys replacements at selected positions. The term *scanning* derives from the common application of this strategy to individually replace each amino acid residue in a contiguous sequence portion or even in the whole sequence of a protein with Cys and create an extensive library of single-Cys replacement mutants for this protein. In addition, a battery of different site-directed techniques can be applied to probe specific features of each Cys-substituted position (accessibility to solvent, relevance to substrate binding, sensitivity to the conformational changes of turnover, proximity to other sites in the protein) with appropriate sulfhydryl-specific reagents. Thus, Cys-scanning analysis often yields a wealth of data that are used to build comprehensive structure-mechanistic models for the protein under study, even in the absence of high-resolution crystallographic evidence (Frillingos et al., 1998; Sorgen et al., 2002; Tamura et al., 2003).

Nevertheless, the research potential of Cys-scanning analysis is not limited to the systematic study of structure-function relationships of individual proteins. The evidence derived from Cys-scanning analysis of a study prototype can serve as a basis to materialize rapid and effective mutagenesis designs in new, previously unknown or unstudied proteins that are related to the study prototype by sequence or structure homology. In this way, evolutionarily broad families of related proteins can be studied with respect to their active site consensus architecture and function, the spectrum of different specificity trends and mechanistic deviations, and the molecular determinants responsible for these differences. New homologs and mutagenesis targets must be selected appropriately, on the basis of the prototypic Cys-scanning evidence. Such a Cys-scanning-based approach may prove extremely contributory in the field of membrane transporters. It can increase the low representation of experimentally characterized homologs in most transporter families and reduce the paucity of crystallographic structural models, as described in the *Introduction* section.

Five discrete steps of the approach are analyzed below, with emphasis on the rationale referring to membrane transport proteins. The probable outcomes from application of this strategy (as delineated in 2.5) are substantiated further by two research paradigms presented in section 3. The two paradigms are rather seen as pilot experimental studies which demonstrate the applicability and importance of such an approach for dissecting substrate recognition and selectivity determinants in new, *ab initio* studied transporters.

2.1 Capitalizing on the Cys-scanning mutagenesis legacy

Cys-scanning mutagenesis and site-directed cysteine modification has been widely used to elucidate structure-function relationships in membrane transport proteins. The reasons for this broad application are both practical and conceptual.

Practical reasons include:

a. the feasibility of engineering of many bacterial transporters devoid of native Cys residues (Cys-less versions) that are functionally equivalent to wild type (for example, Culham et al., 2003; Jung, H. et al., 1998; Sahin-Tóth et al., 2000; Slotboom et al., 2001; van Iwaarden et al., 1991; Weissborn et al., 1997) and represent an ideal substrate for Cys-scanning analyses, as well as the robust evidence that the vast majority of single-Cys replacement mutants do not affect dramatically the transporter expression, structural integrity or function (see Frillingos et al., 1998; Tamura et al., 2003);
b. the availability of a diverse compendium of thiol-specific reagents from established companies such as Molecular Probes, Toronto Research Chemicals, and others;
c. the range of thiol-specific reagents and strategies developed for membrane proteins, such as substituted-cysteine accessibility method (SCAM) using hydrophilic methanethiosulfonate (MTS) derivatives (Akabas et al., 1992) or other reagents (Yan & Maloney, 1993), cysteine-cysteine cross linking protocols (Wu & Kaback, 1996), site-directed fluorescence spectroscopy (Jung, K. et al., 1993; Wu et al., 1995), site-directed spin labeling (SDSL) (Voss et al., 1995), and site-directed alkylation in situ with radioactive (Frillingos & Kaback, 1996; Guan & Kaback, 2007) or fluorescent probes (Georgopoulou et al., 2010; Jiang et al., 2011);
d. the obvious advantage of Cys-scanning technologies over strategies, like Ala-scanning mutagenesis, which do not allow further site-specific derivatization of the substituted amino acid;
e. the fact that high-resolution crystallographic models did not appear for membrane transport proteins until the last two decades, *e.g.*, the ion gradient-driven transporters, for which the first X-ray structure appeared less than a decade ago (see Abramson et al., 2003), due to inherent difficulties with these hydrophobic, integral in the membrane and conformationally dynamic proteins. This delay allowed sufficient time for Cys-scanning applications to expand and provide alternative low-resolution approaches to the study of structure and mechanism (see Kaback & Wu, 1997).

Conceptual reasons include:

a. the success of systematic Cys-scanning analyses in revealing important residues of membrane transport proteins, including irreplaceable residues, binding-site residues or residues that are important conformationally for the mechanism of energy coupling. This is based on two parameters: (i) the utility of using single-Cys mutants in indicating

positions of low significance for the mechanism (active and alkylation-insensitive Cys mutants) and, at the same time, delineating the relatively few residues of potentially major significance (inactive or alkylation-sensitive Cys mutants) for more extensive study with site-directed mutagenesis; (ii) the diverse array of specific Cys modification reagents and protocols that have been developed and used to probe accessibility to solvent, relevance to substrate binding, sensitivity to the conformational changes of turnover, proximity to other sites or other functional properties for each Cys-substituted position (Frillingos et al., 1998);

b. the fact that low-resolution models derived for membrane transport proteins with Cys-scanning approaches continue to provide insight for this class of proteins, even in the post-crystallization era of research (see Kaback et al., 2007, 2011). Most characteristically, the information on the conformational dynamics of an active transport protein deduced from appropriate site-directed Cys modification assays is a valuable complement to the static crystal-structure images. Such information is always needed for an integrated insight on the transport mechanism (Kaback et al., 2011).

The wealth of data derived from the library of single-Cys, paired-Cys and other site-directed mutants produced for a particular transporter in the course of a Cys-scanning mutagenesis study can be used to design new approaches for the analysis of other homologs that might be poorly studied or even not characterized previously with respect to function. In many cases, the availability of at least one high-resolution structural prototype (from a solved X-ray structure for one homolog representing an evolutionarily broad family or group of families with structurally related transporters) might provide valuable additional information and allow the formulation of preliminary structural models. However, even in the absence of such models, the information from Cys-scanning analysis of a prototypic homolog *per se* is sufficient to guide selection of new homologs for study and of amino acid targets for effective site-directed mutagenesis designs in these homologs. The selection of new homologs depends, of course, on the research question asked. However, a common theme is to interrogate what is the structure-functional basis of particular differences in substrate selectivity or in the specificity profile for the recognition of ligands. These questions are highly relevant to the current state of the art in the field of membrane transporters, since many different transport proteins appear to be evolutionarily and structurally related; and rather small sequence changes at key residues are expected to dictate major functional differences (Boudker & Verdon, 2010; Forrest et al., 2011; Lu et al., 2011; Weyand et al., 2011; Yousef & Guan, 2009).

2.2 Selecting new homologs and mutagenesis targets to study

The first and "rate-determining" step in the Cys-scanning-based approach for the analysis of new transporter homologs is the selection of new homologs and mutagenesis targets to study. This selection is based essentially on the set of residues delineated as important from the Cys-scanning analysis of the prototypic homolog and depends, as a consequence, on the extent and results of analysis of the study prototype (Figure 1).

In principle, the Cys-scanning data refer to the functional properties of single-Cys, paired-Cys and other site-directed mutants engineered in the course of the relevant studies. A scanning experiment has two parts at minimum:

1. Analysis of Cys-replacement mutants: the scope is to delineate positions where a mutant is inactive, of very low activity or sensitive to inactivation by specific alkylating agents such as the relatively small and membrane permeable *N*-ethylmaleimide (NEM), which is commonly used to scan for alkylation-sensitive cysteines. Selected mutants are analyzed for site-directed alkylation in the presence or absence of substrate or in other conditions pertinent to appropriate mechanistic questions;
2. Further site-directed mutagenesis at positions where a single-Cys mutant presents with very low or negligible activity or high sensitivity to inactivation upon alkylation with NEM: the scope is to delineate positions where several replacements yield very low activity or different kinetics or specificity than wild type, and define a pattern of permissive and non-permissive replacements (taking into account the bulk, hydrophobicity, polarity, geometry or other properties of the side chain changes).

Overall, the two lines of experiments are expected to delineate a set of residues which are crucial for the transport mechanism of the study prototype in various respects. For example, positions where bulky replacements or alkylation of a substituted cysteine with the maleimidyl adduct lead to inactivation may reflect important conformational constraints and interactions with other parts of the protein that are essential for the permease turnover (Jiang et al., 2011; Tavoulari & Frillingos, 2008). On the other hand, residues which are replaceable with few or no other side chains and, at the same time, accommodate site-specific mutants of impaired affinity or distorted specificity for substrate may be crucial for substrate recognition and binding, while positions of Cys replacements which are protected from alkylation in the presence of substrate may be at the vicinity of the binding site. It is generally true that such important residues fall in one of the following three categories: (a) irreplaceable; (b) replaceable with few other side chains; (c) sensitive to inactivation of the Cys replacement by NEM. These three potential properties can be used to define the set of important residues of the study prototype, as deduced from Cys-scanning analysis data (Karena & Frillingos, 2011; Papakostas & Frillingos, 2012). It is also unequivocally true that this set of residues represents positions with a higher degree of side chain conservation than the rest of the protein and, in cases of transporters that have been studied thoroughly, correspond to a small percentage of the total amino acids in the sequence, usually 10-15% (Frillingos et al., 1998; Georgopoulou et al., 2010; Mermelekas et al., 2010; Tamura et al., 2003). These features make this clearly defined set of residues suitable for use as a basis to select (i) new homologs for *ab intio* study and (ii) amino acid targets for site-specific mutagenesis in these homologs (Figure 1). Homology modeling is also of great value in selecting mutagenesis targets for the new homologs provided that a prototypic crystal structure is available (for examples, see section 3). For reasons explained in the previous section, the Cys-scanning approaches yield additional dynamic information on the role of specific residues that cannot be provided by the structural models *per se*.

More explicitly, the first step of the approach involves a homology search referring not to the whole coding sequence but to the set of the important residues of the study prototype (as defined above). The aim of this search is to select new homologs (from the unknown or poorly-characterized pool of sequence entries) on the basis of specific differences in sequence, implying distinct conserved patterns that might correlate with shifts in specificity. This process resembles the search for characteristic sequence motifs that are conserved as a consensus throughout a transporter family. However, it is more effective in practice, as it is reinforced with experimental data (see Georgopoulou et al., 2010; Karatza et al., 2006; Kasho

et al., 2006; Papageorgiou et al., 2008). If the homolog under investigation is characterized functionally and turns out to be different in specificity from the prototype in an assayable manner, amino acid targets for site-directed mutagenesis of the new homolog are selected from the set of important residues of the prototype. The method is as follows:

1. Residues that are invariant between the new homolog and the study prototype are used as targets of conservative replacements or more extensive mutagenesis to provide a measure of the functional conservation for key conserved residues between the two transporters
2. Residues that differ between the new homolog and the study prototype are used as targets of replacements of each relevant position of the new homolog with the corresponding amino acid found in the prototype, in an attempt to modulate specificity or other substrate-recognition properties and draw comprehensive conclusions on the determinants of the different transporter preferences. Such mutagenesis designs may need to include combinatorial replacements and/or construction of cross-homolog chimeras, as described in the next section.

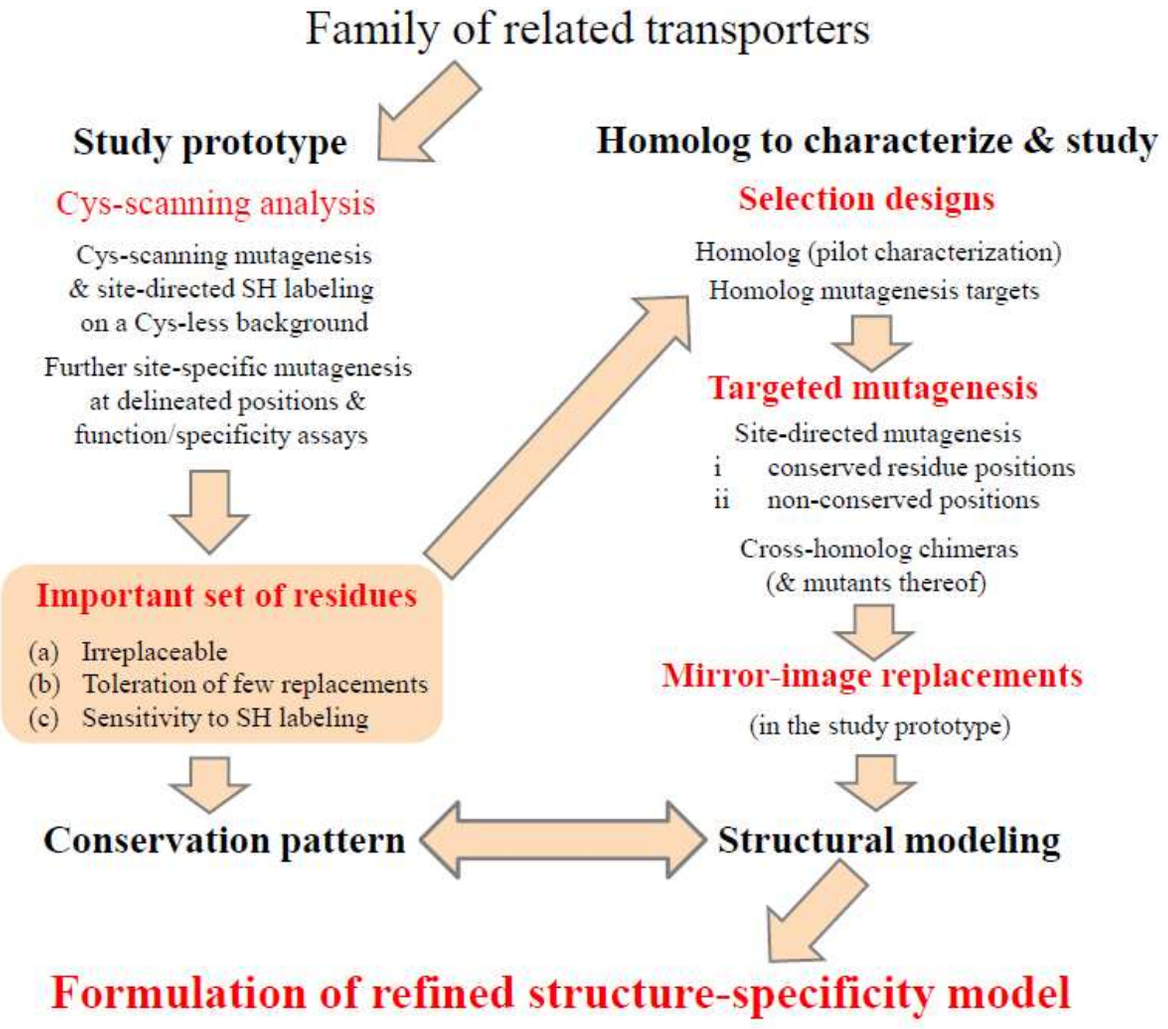

Fig. 1. Flowchart of the Cys-scanning analysis-based strategy for *ab initio* study of new transporter homologs. A detailed account of the individual steps and aims of this strategy is presented in section 2.

2.3 Exploring the use of cross-homolog chimeras and mutants

To dissect the molecular basis of different substrate selectivity trends between closely related transporters, the study of individual site-directed replacements is usually not sufficient; and more combinatorial approaches, involving multiple mutagenesis targets, are needed. The reason is that small contributions from relatively low-effect side chain changes may be crucial for the functional profile outcome, depending on the molecular background used for *in vitro* mutagenesis. In other words, the same replacements may have different effects when combined with different pre-existing mutations on the same transporter background.

Clearly, therefore, combinatorial replacements are important; and both site-specific replacement mutants and cross-homolog chimeras (replacing larger sequence regions and motifs that contain the single-amino acid targets with the homologous ones of the study prototype) can be used in this respect. In particular, the engineering of cross-homolog chimeras between transporters with different substrate profiles often yields variants that are relatively unstable or promiscuous with respect to recognition of substrates. The properties of a chimera may represent an advantageous starting point for exploring a series of mutational events leading to the evolution of new specificities (Tokuriki & Tawfik, 2009). For example, the engineered chimeras might lead to low expression, low activity or promiscuous specificity profiles (Papageorgiou et al., 2008; Papakostas et al., 2008). Chimeras with such properties can be subjected to further site-directed mutagenesis to re-introduce amino acids of the original set of important residues at specified positions. This further mutagenesis might reveal important determinants of uptake and specificity that are not evident in the native transporter backgrounds. The hypothesis is based on two pieces of evidence. (a) Mutations responsible for key functional switches in proteins often yield uninformative phenotypes. This is thought to be due to the effects of other, less important, mutations (a phenomenon known as conformational epistasis) (Harms & Thornton, 2010). (b) More promiscuous and conformationally dynamic proteins exhibit greater evolvability, or potential for divergence of new functions, as supported recently by studies involving molecular evolution, ancient gene resurrection and directed evolution (Tokuriki & Tawfik, 2009). The applicability of such combinatorial and evolution-directed studies is rather limited, at present, and has not been explored extensively in membrane transport proteins. However, this field is rapidly progressing and will probably have a significant impact on the rationale of related site-directed mutagenesis designs in the near future (Morange, 2010).

2.4 Mirror-image replacements in the prototypic transporter

Depending on the results from functional and specificity-profile analysis of the new homolog mutants and/or cross-homolog chimeras, mirror-image replacements can be designed and engineered at the corresponding positions of the study prototype to test whether a particular shift in specificity of the new homolog can be achieved in the inverse direction by replacements of the study prototype at the same residue sites. This line of experimentation is important because it reveals the extent to which a particular side chain influences the substrate recognition profile and may distinguish residues that have a major role on substrate preference from less influential ones. In addition, the comparison between mutants bearing replacements at the same site, but in different native (or chimeric) backgrounds, provides information on the functionally significant interactions of a key specificity mutation with other positions in the different related transporters.

2.5 Formulation of structure-specificity homology models

The sum of data from the comparative analysis of mutants and chimeras between the two related transporters with different specificities (*i.e.*, the new homolog and the initial study prototype) can be used to formulate refined structure-function models highlighting particular aspects of specificity. In addition, the conservation pattern of the residues involved in specificity can be taken into account to draw more generalized structure-specificity conclusions on a group of homologous transporters of the family. This process is facilitated most appropriately when an X-ray structure is available for at least one structural homolog of the transporter family under study, as is the case with the two research paradigms described in the following section.

3. Applications of the Cys-scanning-based approach

The use of a Cys-scanning analysis-based approach (as outlined in Figure 1) has seen limited application to membrane transport proteins to date. In this section, we present two paradigms of use of such an approach to address research questions on the differential substrate preference between closely related transporter homologs. The first paradigm (3.1, MelY) refers to homologs of the well known and thoroughly-studied lactose permease from *Escherichia coli* (LacY), which is a reference protein for all secondary (ion gradient-driven) active transporters. The lessons derived from the detailed site-directed analysis of LacY, which refers to frontline research contributions over almost three decades, are essential for understanding any transporter of this class (Guan & Kaback, 2006; Jiang et al., 2011). The X-ray structures solved for LacY and related homologs are also seminal, as the LacY structural fold typifies the Major Facilitator Superfamily (MFS), encompassing one fourth of all transporters, as well as other more distantly related families (Kaback et al., 2011). The second paradigm (3.2, UacT) refers to the evolutionarily ubiquitous family of nucleobase transporters NAT/NCS2, which has been studied with respect to structure-function relationships only in two members, UapA (Amillis et al., 2011) and XanQ (Karena & Frillingos, 2011). This family of transporters is modeled on a newly described, rather unusual, structural fold (Lu et al., 2011), which has important implications for the binding-site architecture and mechanisms of active transporters. It is also important with respect to potential biomedical applications concerning the development of pathogen-selective cytotoxic nucleobase analogs for targeted antimicrobial therapies (Köse & Schiedel, 2009).

3.1 Substrate selectivity of MelY (in the oligosaccharide-proton symporter family OHS)

The lactose permease from *Escherichia coli* (LacY) is a prototypic example for the study of secondary active transporters. It has been analyzed extensively with cysteine-scanning and site-directed mutagenesis leading to the delineation of residues that are crucial for the mechanism of β-galactoside:H^+ symport. X-ray structures of LacY solved in the presence or absence of substrate (Abramson et al., 2003; Chaptal et al., 2011; Guan et al., 2007; Mirza et al., 2006) have confirmed many of the conclusions derived from the biochemical and biophysical studies. The protein is composed of two domains, one N-terminal and one C-terminal, each representing a bundle of six transmembrane alpha-helices and designated N6 and C6, respectively (Abramson et al., 2003). Symmetrical movements between the two domains are associated with alternating opening and closure of the binding site to either

side of the membrane during the mechanism of active transport (the alternating access model) (Kaback et al., 2007). High-resolution structural evidence from three homologous transporters crystallized in the inward-facing conformation and one (the fucose permease FucP) captured in an outward-facing conformation provided additional strong support for the mechanism of alternating access in lactose permease and related transport proteins (Dang et al., 2010).

LacY belongs to the Oligosaccharide:H^+ Symporter (OHS) family, a member of the Major Facilitator Superfamily (MFS) (Kasho et al., 2006). The OHS family includes several functionally characterized bacterial proteins, such as the lactose permeases of *Citrobacter freundii* and of *Klebsiella pneumoniae*, the melibiose permease (MelY) of *Enterobacter cloacae*, the raffinose permease (RafB) of *E. coli*, and the sucrose permease (CscB) of *E. coli* (Kasho et al., 2006; Vadyvaloo et al., 2006). Although specificity profiles between members of this family are often closely related, mutagenesis studies to examine the basis of substrate selectivity in members other than LacY are rare. One such example refers to *E. cloacae* MelY, a symporter that had not been analyzed for structure-function relationships prior to application of a Cys-scanning based approach (Tavoulari & Frillingos, 2008).

3.1.1 The research question: subtle selectivity difference between LacY and MelY

MelY (GenBank BAA19154) exhibits 57% identity and 75% similarity in sequence with *E. coli* LacY (UniProtKB P02920). Both proteins transport lactose or melibiose or the monosaccharide galactose (with K_m values ranging from 0.2 mM to 0.6 mM), but MelY is unable to transport the analog methyl-1-thio-*β*,D-galactopyranoside (TMG), that is a very efficient substrate for LacY (K_m 0.54 mM). However, MelY recognizes TMG as a ligand and conserves Cys148 (of helix TM5) in the sugar binding site as a TMG-binding residue (Tavoulari & Frillingos, 2008). Therefore, there is a subtle difference in specificity between the two galactoside transporters. The difference concerns the inability of MelY to catalyze the active transport of TMG, although it can bind this substrate analog with high affinity, comparable to LacY. (The K_i values for competitive inhibition of the lactose uptake by TMG are in the range of 1-2 mM for both transporters.)

Homology alignment and threading of MelY into the known structure of LacY (Protein Data Bank ID: 1PV7) shows that the organization of residues in the putative MelY sugar-binding site is the same as in LacY, and residues irreplaceable for the symport mechanism are conserved. Moreover, MelY differs from LacY in only 15% of the subset of residues at which either a single-Cys mutant is inactivated by site-directed alkylation or few amino acid replacements are tolerated (Figure 2). These observations provide a basis for a systematic site-directed mutagenesis study of MelY aiming at identifying subtle-selectivity determinants within the set of important LacY residues (Frillingos et al., 1998; Kaback et al., 2001), which differ in MelY. Such an approach was taken recently (Tavoulari & Frillingos, 2008) for the dissection of side chain determinants of the substrate profile in MelY relative to the well known LacY.

The difference between the two transporters concerns the uptake of one particular analog (TMG). Since the difference is clearly not in binding per se but in the subsequent translocation reactions, that inability of MelY is probably a substrate-specific impairment of binding from the conformational changes needed to complete turnover. In other words, it

appears that, with TMG as a substrate, the conformational movements of MelY permease are inefficient. Thus, TMG binds but is not transported by wild-type MelY to any significant extent. To elucidate the structure-functional basis of this property in detail, both individual and combinatorial replacements were employed, involving rationally designed mutagenesis and analysis of several cross-ortholog chimeras, as described in the next sections.

3.1.2 Selection of mutagenesis targets

The initial targets for site-specific mutagenesis included residues of the important set of LacY (Figure 2) that are conserved or not conserved in MelY. Mutagenesis of MelY at conserved positions showed that irreplaceable residues of LacY are also irreplaceable in MelY (Asp-131, Arg-149, Glu-274, Arg-307, His-327, Glu-330), while Cys-153 (corresponding to Cys-148, which has been extensively utilized as a binding-site reporter in LacY) displays similar properties to those of Cys-148. Most notable is that Cys-153 of MelY, like Cys-148 of LacY, is highly sensitive to alkylation by *N*-ethylmaleimide (NEM) leading to inactivation of the single-Cys-153 mutant. Like in LacY, the presence of substrate fully reverses this inactivation. These initial observations established that the mechanism of galactoside transport is very similar between MelY and LacY and, in particular, that key residues of the binding site (like Cys-148/Cys-153) have the same functional role in both transporters. In addition, it was established that, although non-transportable, the analog TMG is specifically recognized as a ligand and is bound by MelY and that this binding involves the same functionally conserved residues as in LacY (Tavoulari & Frillingos, 2008).

In a second round of mutagenesis, non-conserved residues of the important set of LacY were replaced with the corresponding amino acid found in MelY or vice versa, aiming at modulating specificity to the counter homolog direction. These non-conserved positions are Leu-65/Val-70, Gly-96/Ala-101, Ala-122/Ser-127, Val-264/Als-269, Ala-279/Ser-284, Cys-355/Gln-360 and Val-367/Ala-372 (Figure 2). In the progress of studies, which involved functional analysis of mutants with respect to their efficiency for active transport of lactose, melibiose and TMG (Tavoulari & Frillingos, 2008), both combinatorial mutagenesis and chimera engineering were employed in an attempt to fully convert the one selectivity type (TMG-permissive or TMG-abortive) to the other (TMG-abortive or TMG-permissive, respectively) by replacing multiple selectivity-related targets simultaneously or larger sequence regions or domains of the transporters.

3.1.3 Cross-homolog chimeras

An interesting feature of transporters of the MFS superfamily, which highlights the conformational autonomy of the two domains (Figure 3), is that *in vivo* expression of the gene in two segments (after splitting the sequence at the central cytoplasmic loop between N6 domain and C6 domain) leads to functional complementation (Bibi & Kaback, 1990). Such functional complementation has also been observed with LacY splits at other loop sites, as depicted in Figure 2 (Kaback & Wu, 1997; Kaback et al., 2001). It was then not surprising that many of cross-homolog chimeras engineered between LacY and MelY at sites corresponding to active split junctions were also active with respect to galactoside transport (Figure 2; S. Frillingos, unpublished information). These observations emphasize the conformational flexibility allowed between the two domain repeats for establishing the dynamic binding site in LacY-type transporters. These observations are consistent with

structural and modeling information as well (Radestock & Forrest, 2011). In the course of the Cys-scanning analysis-based approach for studying the selectivity profile of MelY (Tavoulari & Frillingos, 2008), active MelY/LacY chimeras were used as a background for mutagenesis and proved crucial for implementing selectivity switches from the one profile to the other, as explained in the next section.

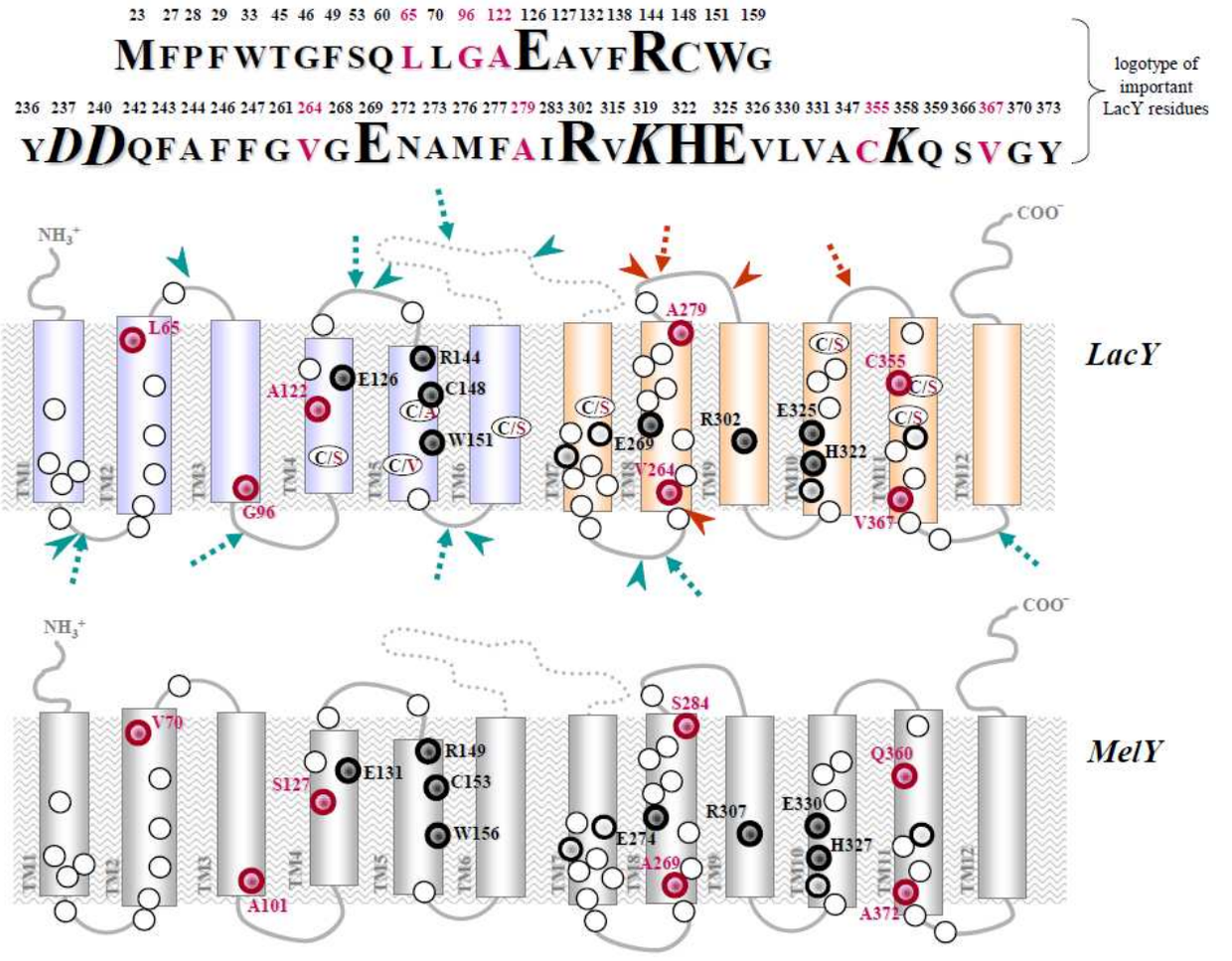

Fig. 2. **Topology models of LacY and MelY and the important set of residues in LacY**. A logotype of the important residues deduced from the Cys-scanning analysis of LacY is shown on top, with larger-size letters indicating functionally irreplaceable residues, medium size indicating residues that are replaceable with few alternative side chains and involved in binding, smaller size indicating residues where a Cys replacement is sensitive to inactivation by *N*-ethylmaleimide and *italics* denoting two pairs of Asp-Lys which are irreplaceable with respect to the charge-pair balance and/or orientation in the membrane (Abramson et al., 2003; Frillingos et al., 1998). Topology models are derived from the X-ray structure of LacY (PDB 1PV7) in combination with prediction algorithms and experimental data on the accessibility of loops to reagents or sequence insertions/deletions (Kaback et al., 2001) and homology threading of MelY (Tavoulari & Frillingos, 2008). The α-helical segments are indicated in rectangles (*blue* and *orange* in LacY denote helices of the N6 and the C6 domain, respectively) and the large intracellular loop between N6 and C6 is shown with a dashed line. *Arrowheads* and *broken arrows* in the LacY model denote positions of splits (the permease gene is split in two coding sequences which are expressed separately in

the same cell to test for functional complementation) or junctions of LacY/MelY chimeras, respectively; splits or chimeras are shown in *teal* (active constructs) and in *red* (inactive). The positions of the important LacY residues are shown in both models with *circles* and mutagenesis targets are *bolded* and shown in *black* (conserved residues) or in *red* (residues that differ in MelY). The eight native Cys residues of LacY and the amino acid replacing each Cys in the Cys-less permease version are shown in ellipses.

3.1.4 Site-directed mutagenesis of LacY and mirror-image replacements in MelY

The practical aim of mutagenesis studies targeted at positions of the non-conserved set of important LacY residues (Figure 2) was to establish side-chain requirements for converting the one transporter profile to the other, with focus on the criterion of whether a transporter variant can take up TMG. The switch between the two selectivity profiles was accomplished, to a major extent, by using combinations of site-specific replacements with cross-homolog chimeras interchanging the N6 and C6 domains of the two transporters (Tavoulari & Frillingos, 2008).

More analytically, the experimental strategy was applied as follows:

1. Switch from LacY to the transport profile of MelY
   - Engineering and analysis of site-specific replacements of LacY residues:

     Site-directed mutagenesis was performed to replace each one of the important LacY residues that are not conserved in MelY (Figure 2) with the corresponding amino acid found in MelY and assay the mutants for active transport of lactose, melibiose and TMG. One of these mutants (V367A), as well as a double-replacement combining this mutation with another important-site mutation in TM11 (V367A/C355Q), showed negligible TMG uptake and a transport profile that resembles the one of MelY. The remaining six mutants (L65V, G96A, A122S, V264A, A279S and C355Q) transport lactose, melibiose or TMG at high rates and show no deviation from the LacY profile (Tavoulari & Frillingos, 2008).

   - Analysis of chimeras that exchange domains N6 and C6 between LacY and MelY:

     The alternating access mechanism in LacY entails dynamic movements of domains N6 and C6 relative to each other to implement the conformational changes of turnover. N6-C6 chimeras, which exchange these two domains between LacY and its closely related homolog MelY, are highly active, implying a considerable flexibility in promoting such conformational changes between the two domains. Detailed functional analysis, however, revealed that both N6-C6 chimeras (with N6 from LacY and C6 from MelY or vice versa) were incapable of transporting TMG although they recognized TMG as a lactose-competitive ligand; on the other hand, the two chimeras were equally efficient to transport lactose or melibiose as LacY and MelY (Tavoulari & Frillingos, 2008). Thus, the interchange of the two domains in these chimeras represents a mutagenesis strategy different from the single-replacement mutations in helix TM11 to convert the LacY selectivity profile to that of MelY.

2. Switch from the transport profile of MelY to the profile of LacY
   - Mirror-image replacements of residues in MelY:

Focusing on position Ala-372/Val-367 (see 1a), site-directed replacements involving mutation A372V in combination or not with Q360C (TM11) were made in the background of MelY. These mirror-image replacements failed to restore significant TMG uptake and showed no deviation from the transport profile of MelY, implying that a single change at position Ala-372/Val-367 is insufficient to yield the TMG-permissive phenotype.

- Mutagenesis in chimeric N6-C6 backgrounds:

  Combination of the mutations V367A (1a) or A372V (2a) with the corresponding N6-C6 background was examined to see whether the TMG-uptake activity can be restored by manipulating flexibility between the two domains. Strikingly, the N6(LacY)-C6(MelY/A372V) mutant showed high affinity and capacity for TMG uptake and a selectivity profile that is indistinguishable from the profile of LacY (Tavoulari & Frillingos, 2008), implying that the TMG transport cycle is restored by the interaction of Val-372(367) with residues of the N6 domain (Figure 3). The reverse mutant, N6(MelY)-C6(LacY/V369A), was indistinguishable in substrate selectivity from MelY and the parental N6-C6 chimeras.

3.1.5 The refined structure-function-selectivity model

An obvious conclusion from the above results would be that efficient transport of TMG requires fine-tuned coordination between the N6 domain and TM11 in C6 domain; this interaction is probably mediated through interactions of an alkylation-sensitive and solvent-accessible face of TM11 (Jiang et al., 2011) with residues of the N6 domain and Val-367 at the periplasmic end of TM11 might have an important contribution in this respect (Tavoulari & Frillingos, 2008).

Homology modeling of MelY in comparison with LacY (Figure 3) shows that Val-367 (TM11) is close to Ala-50 (TM2) in LacY and forms a hydrophobic network that might contribute to the functional inward-facing conformation along with other side chains from TM1, TM2 and TM5 of the N6 domain (Figure 3F). Such a network might be more crucial for TMG than for lactose or melibiose; the non-galactosyl moiety of TMG (which is small, hydrophobic and aglycon) is oriented differently in the binding pocket of LacY than are the non-galactosyl moieties of the disaccharides lactose or melibiose. The non-galactosyl moiety of TMG may promote a slightly different inward-facing conformation, in which hydrophobic interactions might play a pivotal role. On the other hand, orientation of the galactosyl moiety, which determines specificity and is bound by highly conserved and irreplaceable residues (Met-23, Glu-126, Arg-144, Cys-148, Trp-151, Glu-269), is the same for all substrates (Abramson et al., 2003). In MelY all galactosyl-binding residues are conserved; but the putative hydrophobic network at the periplasmic side is disrupted, with less bulky and/or less hydrophobic residues (V367A, A50S, I48V, F30L, A25T, V158G, I157T) (Figure 3E). This difference may account for the fact that MelY binds TMG, but fails to couple this binding to any significant transport. Interactions of the methyl group of TMG in the binding pocket of MelY might promote a tight closure of helices to the periplasmic side incompatible with active transport.

Formation of an efficient hydrophobic network will depend on the side chain contributions from the N6 half. Thus, despite the presence of a Val at position 367, packing of the helices

at the periplasmic side of mutant MelY(A367V) or chimera N6(MelY)-C6(LacY), which contribute less bulky and less hydrophobic side chains from the N6 domain, might still be refractory for an efficient coupling with TMG transport. On the other hand, optimal contacts between helices allow proper formation of the inward-facing conformation and progress of turnover for the TMG. Uptake appears to be restored when Ala-367 is replaced with Val in the N6(LacY)-C6(MelY) chimera, which reintroduces the side chains of LacY in TM1, TM2 and TM5 of the N6 domain. This might be due to reestablishment of hydrophobic interaction of Val-367 with residue(s) of the N6 domain at the periplasmic side and reconstitution of an efficient hydrophobic network.

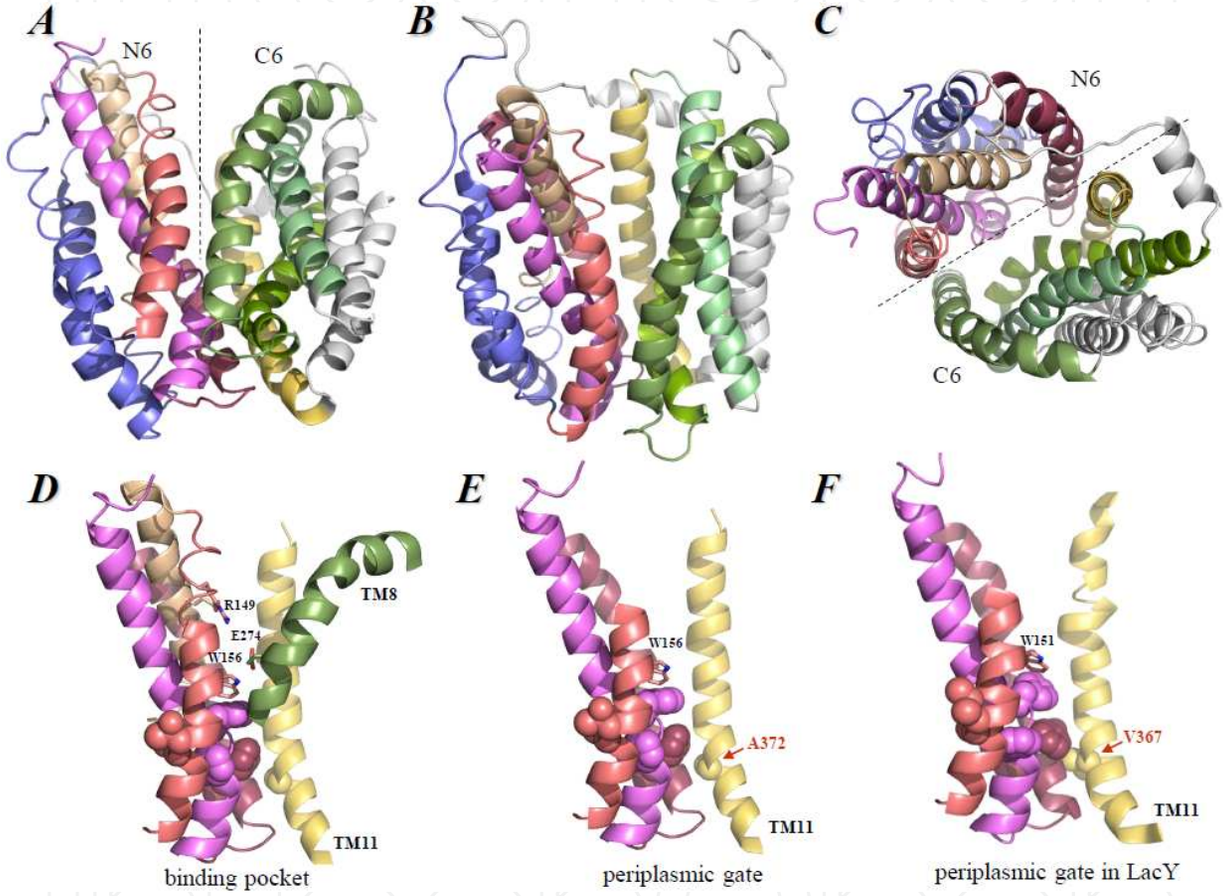

Fig. 3. **Structural models of MelY and comparison with the prototypic homolog (LacY)**. The sequence of MelY was threaded on the known X-ray structure of LacY (PDB 1PV7) (Abramson et al., 2003) using the SWISSPROT modeling server. The structural models were displayed with PyMOL v1.4. The overall helix packing model is shown in three different views (***A-C***). Views ***A*** (from the side of the membrane) and ***C*** (from the periplasm) highlight the axis of pseudosymmetry (broken line), which defines the two domains (N6 and C6). Domain N6 contains the bundle of transmembrane helices TM1 (*violet*), TM2 (*raspberry*), TM4 (*wheat*), TM5 (*salmon*) and two peripheral ones, TM3 and TM6 (*blue*). Domain C6 contains the bundle of helices TM7 (*split pea green*), TM8 (*smudge green*), TM10 (*pale green*), TM11 (*yellow orange*) and the peripheral TM9 and TM12 (*grey*). View ***B*** highlights the central position of TM11 at the interface between the N6 and C6 bundles. The arrangement of TM11 with respect to TM8 and to the N6 bundle of TM1, TM2, TM4 and TM5 is shown more clearly in ***D-F***. Panel ***D*** highlights key galactoside-binding residues, which are invariant

between LacY and MelY. Panel ***E*** (and ***F***, showing LacY) highlights the cluster of residues (at TM11, TM5, TM2 and TM1), which gate the periplasmic substrate pathway and differ between MelY and LacY (see text). The residue implicated in the change of specificity (Ala-372/Val-367) is indicated with an *arrow* and *red label*.

3.2 Ab initio analysis of UacT (in the nucleobase-cation symporter-2 family NAT/NCS2)

The Nucleobase-Ascorbate Transporter (NAT) or Nucleobase-Cation Symporter-2 (NCS2) family is evolutionarily ubiquitous and includes more than 2,000 putative members in all major taxa of organisms. Despite their relevance to the recognition and uptake of several frontline purine-related drugs, only 16 members have been characterized experimentally to date. These are specific for the cellular uptake of uracil, xanthine or uric acid (microbial, plant and non-primate mammalian genomes) or vitamin C (mammalian genomes) (Gournas et al., 2008; Yamamoto et al., 2010).

The NAT/NCS2 family is of particular interest in two respects. First, in an evolutionary perspective, it encompasses transporters with largely different substrate profiles that model on a novel, unprecedented structural fold, as revealed recently (Lu et al., 2011). Second, in a biomedical perspective, it offers important possibilities for translation of the structure-function knowledge to the rational design of targeted antimicrobial drugs, based on the fact that the human homologs do not recognize nucleobases or related cytotoxic compounds (Yamamoto et al., 2010). An additional research challenge is that only 15 of the ~2000 predicted members have been identified functionally and only two members have been studied rigorously with respect to analysis of structure-function relationships, namely the xanthine permease XanQ of *E. coli* (Georgopoulou et al., 2010; Karena & Frillingos, 2011) and the uric acid/xanthine permease UapA of *A. nidulans* (Amillis et al., 2011; Papageorgiou et al., 2008). It is notable that mutagenesis data from both lines of study have revealed striking similarities between the two transporters, reinforcing the idea that few residues conserved throughout the family may be invariably critical for function and underlie specificity differences. Most of these residues are also highlighted as active-site relevant in models built on the recently released X-ray structure of the uracil permease homolog UraA (Protein Data Bank ID: 3QE7) (Lu et al., 2011).

3.2.1 The research question: distinction between xanthine and uric acid (8-oxy-xanthine)

Most of the experimentally known members of the NAT/NCS2 family have been characterized as purine nucleobase transporters, which are specific for the proton gradient-driven uptake of xanthine, uric acid (8-oxy-xanthine) or both. This group of related transporters include 11 bacterial, fungal or plant homologs, namely the xanthine transporters XanQ (UniProtKB accession number P67444) and XanP (P0AGM9) from *Escherichia coli* and PbuX (P42086) from *Bacillus subtilis*, the uric-acid transporters UacT (or YgfU) (Q46821) from *E. coli* and PucK (O32140) and PucJ (O32139) from *B. subtilis*, and the dual-selectivity uric-acid/xanthine transporters UapA (Q07307) and UapC (P487777) from the filamentous fungus *Aspergillus nidulans*, AfUapA (XP748919) from its pathogenic relative *A. fumigatus*, Xut1 (AAX2221) from the yeast *Candida albicans*, and Lpe1 (AAB17501) from maize (*Zea mays*) (see Karena & Frillingos, 2011). Based on the spectrum of their known

specificities, a major research challenge is to understand the mechanism of differential recognition between xanthine and uric acid (8-oxy-xanthine) and between different binding-site preferences for xanthine analogs with variations at the imidazole moiety (8-methylxanthine, 8-azaxanthine, oxypurinol) (Goudela et al., 2005; Karatza & Frillingos, 2005).

Inspection of conserved sequence motifs and sequence alignment analysis of the different xanthine and/or uric acid-transporting homologs indicated interesting patterns of correlation with changes between xanthine-selective and xanthine/uric acid dual-selectivity NAT transporters, especially at a characteristic sequence region of transmembrane segment TM10 known as the NAT-signature motif (Georgopoulou et al., 2010). However, such sequence differences did not correlate with clear-cut changes in substrate selectivity of corresponding mutants that were made in either XanQ (Georgopoulou et al., 2010; Karatza et al., 2006) or UapA (Papageorgiou et al., 2008; Koukaki et al., 2005). In particular, the most pronounced change in XanQ was accomplished with replacement of Gly-333 to Arg (at the carboxyl-terminal end of the motif sequence) yielding aberrant recognition of 8-methylxanthine (which is not a wild-type ligand), but without affecting the selectivity preference for xanthine (Georgopoulou et al., 2010). Recognition of 8-methylxanthine has also been observed with a number of other single-replacement XanQ mutants and even with UapA/XanQ chimeras (Papakostas et al., 2008), implying that several changes at different sites in this xanthine-specific transporter can confer a degree of promiscuity for the recognition of analogs at the imidazole moiety of xanthine. However, since none of these changes resulted in a clear selectivity change (most notably, none allowed recognition or uptake of uric acid), it is evident that the strict preference of XanQ for xanthine is not easily modifiable and a more systematic approach is needed to address the basis of xanthine/8-oxy-xanthine selectivity differences. Such an approach is offered through the exploitation of evidence from a systematic Cys-scanning analysis of XanQ and the elucidation of the function of a new, uric-acid selective homolog (UacT), as described in the next section.

3.2.2 Selection of the homolog to study and the mutagenesis targets

The xanthine-specific permease XanQ has been subjected to a systematic Cys-scanning and site-directed mutagenesis study to address the role of each amino acid residue (Georgopoulou et al., 2010; Karatza et al., 2006; Karena & Frillingos, 2009, 2011; Mermelekas et al., 2010; Papakostas et al., 2008). Of more than 180 residues analyzed to date, a small set emerges as crucial for the mechanism at positions at which a native residue is functionally irreplaceable, replaceable with a limited number of side chains or sensitive to alkylation of a substituted Cys with *N*-ethylmaleimide leading to inactivation (Figure 4). Homology modeling showed that these functionally important residues could be implicated in substrate binding (Glu-272, Gln-324, Asp-276, Ala-323) or involved in crucial hydrogen bonding (Asn-325, His-31) or disposed to the cytoplasmic halves of TM10 and TM8, which contain key binding residues (Karena & Frillingos, 2011). Site-directed alkylation analysis of XanQ has suggested that Gln-324 and Asn-325 may participate directly in the XanQ binding site (Georgopoulou et al., 2010), while His-31 and Asn-93 are essential for the proper binding affinity and selectivity, as evidenced from ligand inhibition assays (Karena & Frillingos, 2009). In the light of the homologous UraA structure (Lu et al., 2011), it appears that the effect of His-31 (TM1) might be indirect through its interaction with Asn-325

(TM10), while Asn-93 (TM3) is at the binding pocket and may be involved in more direct interactions with substrate or substrate-binding residues (Karena & Frillingos, 2011).

The information derived from the Cys-scanning analysis of XanQ provides a basis to study structure-function relationships in other related members of the NAT/NCS2 family, which are not yet characterized or are poorly studied. In this respect, of particular interest are new homologs with distinct selectivity profiles relative to XanQ. One such homolog is UacT (more commonly known as YgfU), a low-affinity uric acid transporter from *E. coli* characterized recently (Papakostas & Frillingos, 2012). UacT is a proton-gradient-dependent, low-affinity (K_m 0.5 mM) and high-capacity transporter for uric acid that also transports xanthine, but with disproportionately low capacity. Although UacT shares low sequence homology with XanQ (28% identity of residues), it retains most of the residues of the important set identified in XanQ with Cys-scanning analysis (Figure 4). It thus offers a good substrate to apply the Cys-scanning-based approach for elucidation of changes involved in the switch of substrate preference from xanthine (XanQ) to uric acid (UacT).

To delineate targets of mutagenesis in UacT, we have taken into account residues of the important set of XanQ that are conserved or not conserved in the different-selectivity homolog, as depicted in Figure 4. The initial round of mutagenesis in UacT included (i) conservative replacements of residues that are invariant and functionally irreplaceable in XanQ (Glu-270, Asp-298, Gln-318, Asn-319); (ii) rationally designed replacements of side chains that correspond to affinity- or specificity-related residues in XanQ (His-37, Thr-100); (iii) replacements of non-conserved residues of the important set with the corresponding amino acid found in XanQ (T259V, M274D, L278T, V282S, S317A, V320N, R327G, S426N).

3.2.3 Cross-homolog chimeras and mutants

In their majority, the set of important residues identified for XanQ cluster at contiguous regions of transmembrane segments TM8 and TM10, as well as at specific sites in TM1, TM3 and TM14 (Figure 4). In a previous attempt to replace extended sequence portions containing multiple important residues of XanQ with the corresponding regions of the dual-selectivity UapA transporter from *A. nidulans* and search for deviations in substrate preference, it was striking that most of the engineered chimeric constructs were unstable and failed to express in the membrane (see Figure 4). Only one chimera of this set was expressible, but without displaying any transport activity. It was the one that replaced TM14 with the corresponding segment of UapA in the background of XanQ (Papakostas et al., 2008). Interestingly, this chimera could be rescued for active xanthine uptake with reintroduction of two residues from the important set (Asn-430, Ile-432) in the UapA-derived graft (Papakostas et al., 2008). In addition, further combinatorial replacements in this region progressively lead to restoration of full activity and the wild-type profile for xanthine selectivity and ligand specificity (Georgopoulou, K., Botou, M. & Frillingos, S., in preparation).

The difficulty in obtaining structurally stable and active chimeric constructs between the two different NAT transporters (XanQ, UapA) cannot be accounted for by the heterologous origin of the fungal UapA sequence. The engineered chimeras are transferred, induced for expression and tested in an *E. coli* K-12 host, yet similar difficulty is observed with cross-

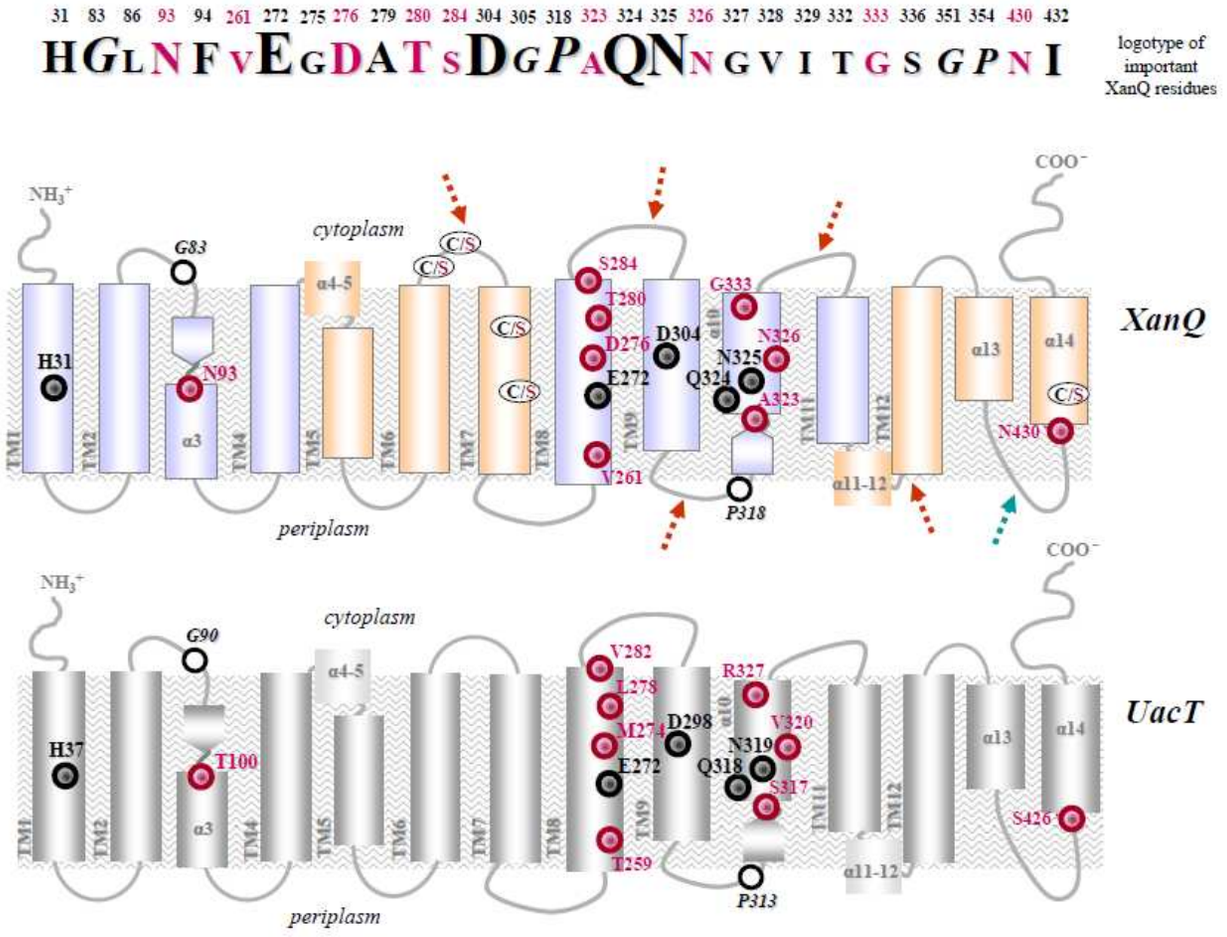

Fig. 4. **Topology models of XanQ and UacT and the important set of residues in XanQ**. A logotype of the important residues deduced from Cys-scanning analysis of XanQ is shown on top, with larger-size letters indicating irreplaceable residues, medium size indicating residues that are replaceable with few alternative side chains, smaller size indicating residues at which a Cys replacement is sensitive to inactivation by *N*-ethylmaleimide (IC_{50} < 0.1 mM) and *italics* denoting residues that are crucial (smaller size) or irreplaceable (larger size) for expression in the membrane (Karena & Frillingos, 2009, 2011; Georgopoulou et al., 2010; Mermelekas et al., 2010). Topology models are derived from the X-ray structure of UraA (PDB 3QE7) in combination with prediction algorithms and experimental data on the accessibility of loops to hydrophilic reagents (Georgopoulou et al., 2010) and homology threading of XanQ and UacT (Karena & Frillingos, 2011; Lu et al., 2011). The α-helical segments are indicated in rectangles (*blue* and *orange* in XanQ denote helices of the core and the gate domain, respectively). *Broken arrows* in the XanQ model denote junctions of XanQ/UapA chimeras (Papakostas et al., 2008). [UapA is a fungal homolog with dual selectivity, *i.e.*, for both uric acid and xanthine.] The activities of the chimeras are denoted with *teal* (expressed in the membrane and activated upon reintroduction of particular residues; see text) and *red* (not expressed in the membrane). The positions of the important XanQ residues are shown in both models, with *circles* and mutagenesis targets *bolded* and shown in *black* (conserved residues) or in *red* (residues that differ in UacT). The five native Cys residues of XanQ and the amino acid replacing each Cys in the Cys-less permease version are shown in ellipses.

homolog chimeras between XanQ and its *E. coli* paralog UacT, although the phylogenetic distance between XanQ and UacT (28% sequence identity) is equivalent to the one between XanQ and UapA (30% sequence identity) (Georgopoulou, K. & Frillingos, S., in preparation). A more plausible interpretation stems from the intertwined-domain organization of the NAT transporters that was revealed recently from the crystal structure of the UraA homolog (Lu et al., 2011). These transporters are organized in a core and a gate domain, which are composed of two separate contiguous regions each (Figure 4). Although discontinuous in sequence, each domain represents a pair of internal repeats and forms a distinct fold in the structure (Figure 5). The core domain is thought to be pivotal for substrate binding and proton symport, and the gate domain is thought to be crucial for the conformational changes that allow access and release of substrate from the binding site (Lu et al., 2011). However, the relative arrangement of the helices of each domain, which interlace between the repeats to form the dynamic binding site, is highly sensitive to deregulation by changes at key sites. Deregulations leading to instability and loss of the protein expression can be introduced by discontinuities between TM8, TM9, TM10 and TM11 in the chimeric constructs or even by single amino acid changes at the beginning of the crucial TM10 (Pro-318) (Karatza et al., 2006) or TM3 (Gly-83) (Karena & Frillingos, 2011). Thus, sequence rearrangements within the gate domain, which is intimately associated with the binding site architecture, can be grossly deregulating or detrimental for the structural fold. The situation is different with the chimeras involving homologs of LacY (section 3.1) because each domain in the LacY fold is contiguous in sequence and the dynamic binding site is formed at the interface between the two bundles of helices, allowing more flexibility (Kaback et al., 2001).

3.2.4 Site-directed mutagenesis of UacT and mirror-image replacements in XanQ

The most significant conclusions from the analysis of individual site-directed replacements of UacT at the positions of putatively important residues (Figure 4) are that (a) functionally irreplaceable residues of XanQ (such as the substrate binding-relevant Glu-272 and Gln-324) are also irreplaceable in UacT, highlighting the functional conservation of the purine binding site in different-selectivity homologs, and (b) replacements lowering the bulk and polarity of the side chain at one position (Thr-100; TM3) allow conversion of the uric acid-selective UacT to a dual-selectivity variant (mutant with Ala in lieu of Thr-100) that transports both uric acid and xanthine (Papakostas & Frillingos, 2012). Thus, the side chain of Thr-100 at the middle of TM3 is associated directly with defining the purine substrate selectivity with respect to position 8 of the imidazole moiety. This conclusion is strengthened by mirror-image replacements made in XanQ, including an extensive site-directed mutagenesis at the corresponding amino acid found in TM3 (Asn-93) (Karena & Frillingos, 2009, 2011). Mutagenesis at Asn-93 revealed replacements that allow conversion of the xanthine-selective XanQ to dual-selectivity variants (mutants with Ala or Ser in lieu of Asn-93), even though these variants transported the non-wild-type substrate (uric acid) with very low capacity (Karena & Frillingos, 2011). The above considerations are reinforced by the fact that no other single-replacement mutants of either XanQ or UacT has been shown to convert the native transporter to a dual-selectivity one (Karena & Frillingos, 2011). In further support, a similar specificity-related effect is observed with mutants replacing the corresponding TM3 residue (Ser-154) in the fungal homolog UapA, in which introduction of an Ala in lieu of Ser-154 leads to higher affinity for xanthine relative to uric acid, thus shifting the dual-selectivity profile to the xanthine-selective direction (Amillis et al., 2011).

In summary, a major conclusion is that a polar side chain at positions Thr-100/Asn-93/Ser-154 at the middle of TM3 (Figure 4) is associated with the xanthine/uric acid selectivity. However, the selectivity changes observed with the relevant mutants are not sufficiently dramatic to emulate the properties of the other, different-selectivity homologs (Karena & Frillingos, 2011). In this respect, combinatorial replacements are needed to lead to more clear-cut shifts, involving, for example, other sites at which or sequence regions in which mutations have been shown to modify the specificity profile with respect to the imidazole moiety of the substrate (for example, recognition of 8-methylxanthine by XanQ mutants) to a lesser extent (Georgopoulou et al., 2010; Karatza et al., 2006; Karena & Frillingos, 2009).

3.2.5 The refined structure-function-selectivity models

The apparently unique selectivity-related role of Thr-100/Asn-93 in UacT (and XanQ) can be explained by taking into account the distinct conservation pattern of this residue in NAT transporters and homology modeling on the template of the UraA structure (Karena & Frillingos, 2011). In general, Asn-93 is poorly conserved as an amidic side chain even in close XanQ relatives (Karena & Frillingos, 2011); and the same is true of Thr-100 with respect to UacT relatives. However, the polar character of Thr-100/Asn-93 is conserved invariably in the known nucleobase-transporting NAT members (Asn, Thr or Ser), while the ascorbate-transporting SVCTs have an Ala at this position. Furthermore, all the dual-selectivity uric-acid/xanthine transporters (Xut1, UapA, UapC, AfUapA, Lpe1) have a Ser at the corresponding position. To understand the structural relevance of this difference, we have built structural models for the uric acid-selective UacT and the dual-selectivity fungal homologs and compared them with the one of the xanthine-selective XanQ (Figure 5 and data not shown). First of all, the models indicate that this position of TM3 is vicinal to the presumed substrate binding site formed between residues of the middle parts of TM3, TM8 and TM10 in NAT transporters (Figure 5). Strikingly, however, in UacT and all dual-selectivity NATs, the Thr or Ser replacing Asn-93 is distal from the conserved, substrate-relevant glutamate of TM8 (minimal distance between oxygen atoms, 6.0 Å), while Asn-93 in XanQ is significantly closer (distance between oxygen atoms, 4.5 Å). This difference is most prominent in the models of UacT (Figure 5) or UapA (Karena & Frillingos, 2011), which conserve nearly all the other side chains of functionally important residues of TM1, TM3, TM8 or TM10, except Asn-93.

In the dual-selectivity UapA, Ser-154 (corresponding to Asn-93 of TM3) is oriented away from the carboxyl group of Glu-356 (corresponding to Glu-272 of TM8) and leaves more space between TM3 and TM8 in the substrate binding pocket (Amillis et al., 2011; Karena & Frillingos, 2011). Thus, occupation of the Asn-93 position by Ser may relax a constraint for the recognition of analogs modified at position 8 of the imidazole moiety of xanthine and allow binding and transport of uric acid (8-oxy-xanthine), which modifies the NAT selectivity towards a less stringent, dual-substrate profile. Accordingly, the XanQ mutants replacing Asn-93 with Ser (or Ala) yield efficient recognition of 8-methylxanthine and low, but significant, uptake of uric acid, mimicking in part the fungal, dual-selectivity NATs (Karena & Frillingos, 2011).

In the uric acid transporter UacT, Thr-100 (corresponding to Asn-93) is oriented away from the carboxyl group of Glu-270 (corresponding to Glu-272 of TM8) leaving more space in the substrate binding pocket; but, at the same time, the pKa of Glu-270 may be distorted

significantly relative to the corresponding carboxylic acid in XanQ due to its proximity to hydrophobic groups from Thr-100 and Met-274 (Figure 5E). These changes on the substrate binding glutamate Glu-272/Glu-270 (Lu et al., 2011) might account for the selectivity difference between UacT (uric acid) and XanQ (xanthine). Interestingly, however, replacement of Asn-93 with Thr in XanQ cannot imitate the UacT profile, but leads to low affinity for all xanthine analogs, possibly due to interference of the methyl group of Thr-93 in the vicinity of the essential Glu-272 that is not counterbalanced by other permissive mutations (Karena & Frillingos, 2009, 2011). Based on this observation, it is evident that further combinatorial replacements are needed to promptly convert XanQ to the UacT selectivity profile or vice versa; and targets for such replacements have to be selected from the residues of the important set (Figure 4), which correspond to binding site-relevant positions (Karena, E., Papakostas, K. & Frillingos, S., in preparation).

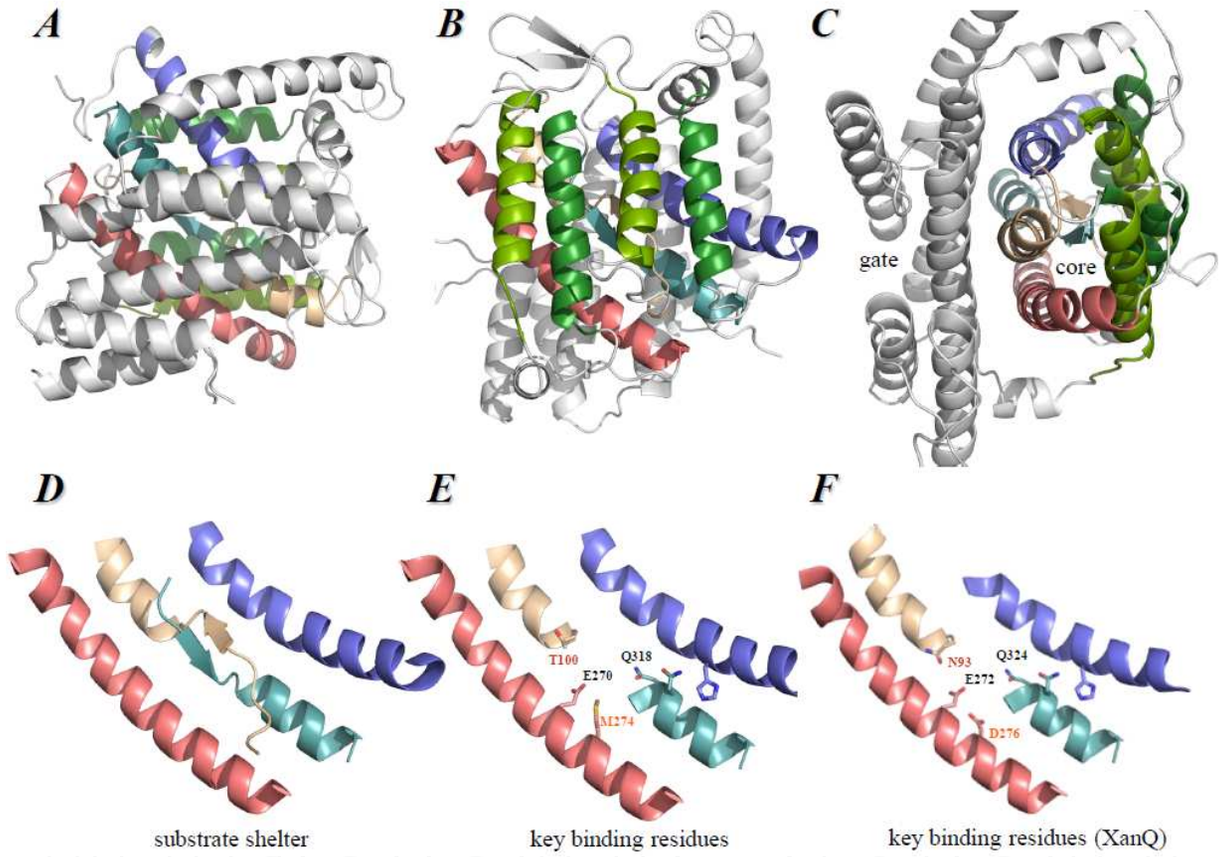

Fig. 5. **Structural models of UacT and comparison with the prototypic homolog (XanQ)**. The sequence of UacT (***A-E***) or XanQ (***F***) was threaded on the known X-ray structure of UraA (PDB 3QE7) (Lu et al., 2011) using the SWISSPROT modeling server, and the structural models were displayed with PyMOL v1.4. The overall helix packing model of UacT is shown in three different views (***A-C***). View ***C*** (from the periplasm) highlights the two domains (core and gate), which are interplexed and not readily discerned in the side views (***A*** and ***B***). Transmembrane segments of the core domain (associated with substrate binding) are shown in *blue* (TM1), *wheat* (TM3), *teal* (TM10), *salmon* (TM8), *pea green* (TM2, TM4) and *forest green* (TM9, TM11), while the gate domain (associated with the conformational changes allowing access and release of substrate from the binding site) is shown in *grey*. The arrangement of the four substrate-coordinating segments (TM1, TM3,

TM8 and TM10) is shown more clearly in ***D-F***. Panel ***D*** highlights the central position of the two short antiparallel β-strands of TM3 and TM10, which provide a shelter for the nucleobase substrate (Lu et al., 2011). Panel ***E*** (and ***F*** showing XanQ) highlights key binding-site residues, with residues differing between UacT and XanQ indicated with a *red label* (Thr-100/Asn-93, implicated in the purine selectivity preference) and an *orange label* (Met-274/Asp-276). For clarity, only the helical segments of TM3 and TM10 are shown in ***E*** and ***F***.

4. Conclusion and perspectives

The two paradigms described above highlight the applicability of approaches that employ Cys-scanning analysis data from a reference molecule (the study prototype) to guide the *ab initio* analysis of structure-function relationships of new transporters in evolutionarily conserved families of structurally related homologs. In particular, they provide a strategy for effective site-directed mutagenesis designs to dissect the molecular determinants underlying substrate selectivity shifts between closely related homologs. In parallel, they explore the mechanism of change to novel selectivity profiles through a combination of site-specific replacements in native and chimeric transporter backgrounds. Apart from the obvious contributions to the research of groups of transporters with high potential for translation to biomedical and other applications, a more systematic and generalized application of this strategy would certainly have a major methodological impact in the field. It should allow rapid and cost-effective mutagenesis designs on newly identified membrane transport proteins, even in cases in which a high-resolution model is unavailable.

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