What lies between
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Summary and Concluding Remarks

1. Introduction

The major facilitator superfamily constitutes the largest family of secondary transporters and contains members from all three kingdoms of life (101). Mechanistic details obtained for these transporters are therefore highly relevant. Although a wealth of functional data on MFS proteins is available, thus far, for only three proteins medium or high resolution structural data have been produced. The structures of OxIT (49), the oxalate:formate antiporter from *Oxalobacter formigenes*, GlpT (54), the glycerol-3-phosphate:phosphate antiporter, and LacY (3), the lactose:proton symporter from *E. coli*, reveal a strikingly similar architecture and helix arrangement and suggest structural conservation within the MFS. Their helix packing differs significantly from other non-MFS secondary transporters as NhaA (131), the Na⁺:proton antiporter and AcrB (72), the drug:proton antiporter from *E. coli*, and GltPh, the glutamate:Na⁺:proton symporter from *Pyrococcus horikoshii* (135), indicating the absence of a common structure for all secondary transporters. The structures of the MFS proteins have greatly contributed to our understanding of the mechanism of transport, but several questions remain to be answered. For example, both LacY and GlpT were crystallized in the inward-facing conformation and the structure of the outward-facing conformation is unknown, although educated guesses have been made (3,52). Also, the translocation mechanism is still highly elusive as the highest resolution of a MFS transporter is only 3.3 Å (3,54). Thus, the study of other MFS proteins is still highly appropriate to increase our insights, if not, to verify the general validity of observations. Additionally, both LacY and GlpT are proposed to function as monomers (54,99), whereas higher oligomers are formed by other MFS proteins, like TetL and TetA (97,98), the tetracycline-divalent cation:proton exchangers from *Bacillus subtilis* and *E. coli*, respectively, GLUT1 (140), the glucose unipporter from human erythrocytes, XylP (48), the xyloside:proton symporter from *Lactobacillus pentosus*, and LacS (32), the lactose:proton symporter from *Streptococcus thermophilus*.

2. Functional overexpression of membrane proteins

The overexpression of membrane proteins, while preserving the correct structure, is one of the key steps in the path to structural information. The functional assembly of membrane proteins is preceded by targeting to and insertion of the polypeptides into the membrane. Overloading the machinery involved in these steps leads to the production of membrane proteins in insoluble form or their rapid degradation. Due to the difficulties involved in refolding of membrane proteins from inclusion bodies, overexpression in the membrane is highly preferred. The AraC/P_{BAD}-expression system allows tuning of the expression level over a wide range (approximately three orders of magnitude) by varying the concentration of the inducer L-arabinose (44). The system has been proposed to be highly suited for the overexpression of membrane proteins, because careful adjusting of the rate of synthesis to the rate of downstream processing would prevent overloading of the machinery involved in targeting, membrane insertion and assembly and thereby maximize amplification in the membrane. However, it has been shown that, at non-saturating induction levels, the population of cells was heterogenous and consisted of uninduced and fully induced cells (104), making this expression...
system less favourable than others that do show homogenous populations after induction, like the IPTG-inducible tac and trc promoter-based systems (64). However, the tightness and high reproducibility, amongst other reasons, is still an incentive to use the AraC/P\textsubscript{BAD} system.

The expression of LacS, governed by the AraC/P\textsubscript{BAD}-system, could indeed be modulated over a wide range and up to high levels (Chapter 2). However, functional analysis revealed a clear optimum in lactose transport at intermediate inducer concentrations (Fig. 1). High induction levels led to decreased lactose transport rates, suggesting that most of the protein produced was not functional. To verify whether the protein was produced in inclusion bodies, a C-terminal fusion with the green fluorescent protein (GFP) of \textit{Aequorea victoria} was constructed. Since GFP needs to be properly folded in order to become fluorescent, it can be used as a folding indicator (126). Overexpression of membrane proteins in inclusion bodies prevents correct folding of the GFP moiety and thus formation of the fluorophore (26). Confocal imaging of cells expressing the LacS-GFP fusion showed membrane-associated fluorescence, indicating correct localization. The GFP-fluorescence of cells induced with increasing concentrations of L-arabinose showed a profile identical to that of the transport activity, indicating that the decreased transport rate results from incorrect processing and misfolding of a large fraction of the protein produced. To avoid expression in a misfolded state, it is thus recommended to determine the optimal inducer concentration based on functional or structural parameters (e.g. activity or GFP-fluorescence) rather than protein levels.

Figure 1. Initial rates of lactose counterflow transport as a function of L-arabinose concentration. Lactose counterflow transport was determined in whole \textit{E. coli} MC1061 cells expressing LacS\textsubscript{ΔIIA} and the initial rates were estimated from the amount of lactose taken up after 10 seconds. The inducer concentration was varied from 0 to 2.0 x 10\textsuperscript{-1}\% (wt/vol) (13 mM) L-arabinose.
3. Regulation of lactose transport by LacS-IIA

The lactose transport capacity of *S. thermophilus* is highly regulated both at the level of transcription and at the level of LacS activity. This regulatory network assures a prolonged high lactose uptake rate when the external lactose and galactose concentrations decrease and increase, respectively (42). Phosphorylation of the LacS-IIA domain results in an increase in the rate of lactose counterflow (41). Prior to this study, it was unclear whether this was caused by a relief of inhibition by dephosphorylated LacS-IIA or stimulation by phosphorylated LacS-IIA. To settle this point, the characteristics of transport of a LacS truncation mutant lacking the IIA domain were compared to phosphorylated and dephosphorylated full-length LacS (Chapter 3). This mutant, designated LacSΔIIA, catalyzed Δp-driven lactose transport and lactose counterflow with characteristics similar to dephosphorylated LacS, suggesting that the phosphorylated LacS-IIA domain stimulates the activity of the carrier (Fig. 2). This mode of regulation contrasts from that known as inducer exclusion, where the dephosphorylated IIA^Glc^ domain inhibits sugar permeases. Additionally, analysis of tandem fusions of two LacS carrier domains, harbouring only one LacS-IIA domain per dimer, demonstrated that regulation by LacS-IIA takes place intramolecularly (Chapter 5).

![Figure 2](image_url)

Figure 2. A model of the interactions between the carrier and IIA domain of LacS. The membrane-embedded carrier domain of LacS is represented by a white box to which the soluble IIA domain (represented by a white oval) is coupled via a flexible linker. The phosphorylation state of LacS-IIA is represented by X and P, reflecting the unphosphorylated and the phosphorylated state, respectively. The two-way arrows indicate lactose counterflow transport and their size reflects the rate of transport. A, upon phosphorylation of the IIA domain by HPr(His~P), the IIA domain interacts with the carrier domain; B, this interaction accelerates the counterflow transport. The other subunit of the dimer (depicted in grey) is not affected by the opposing IIA domain. For clarity only one phosphorylated subunit per dimer is shown.

4. Structural and functional aspects of the LacS dimer

Within the MFS, LacS forms a representative of the members that have a higher order oligomeric structure. In the detergent-solubilized state, LacS undergoes reversible self-association with a monomer to dimer mode of association, whereas membrane-reconstituted LacS forms dimers only (32,109). Functional interactions between the subunits take place within the LacS dimer (124). As a first step in studying the mechanism between these...
interactions, regions near the subunit interface were determined (Chapter 4). Residues at or near the membrane interface of TMs were substituted by cysteine residues and submitted to cysteine cross-linking. Four regions, two at each side of the membrane, were susceptible to cross-linking, indicating their proximity to the dimer interface. Extreme care was taken to exclude that these cross-links were formed via randomly colliding particles. The identified positions reside on the cytoplasmic side of TMs VI and VII and on the extracellular side of TMs V and VIII. This result complies to the helix packing model of LacS, that is, the identified residues are located on the periphery of the protein (Fig. 3). However, in the helix arrangement observed for GlpT (54), OxlT (49), and LacY (3), these regions are located on opposing sides of the protein, which is not in agreement with a specific interface. It remains to be established whether this common architecture is applicable to LacS as well.

Previously, a partially defective mutant was shown to interact functionally with an opposing wildtype subunit during $\Delta p$-driven lactose transport, while both subunits functioned independently during lactose counterflow (124). By fusing two carrier units with distinct properties, it became possible to study subunit cooperativity in vivo (Chapter 5). Functional interactions between a mutant completely inactive and a wildtype unit were detected for counterflow and $\Delta p$-driven transport. Furthermore, the inactive subunit was almost fully complemented by the wildtype subunit, which contrasts the dominant negative phenotype observed previously (124). These results indicate that functional interactions between the subunits take place in the diverse modes of transport.

Figure 3. The relative orientation of TMs in the subunits and the dimer of LacS. A, The helix packing model of LacS. This model is based on second-site suppressor studies on LacS and MelB, the amphipathicity of TMs II, IV, XI, and residues important for binding of the sugar or the coupling ion (adapted from (122)). B, A possible organization of the TMs near the center of the dimer (marked by the cross) on the extracellular and the cytoplasmic face of the membrane based on the data presented here. TMs of different subunits are in different shades, the dotted line separates the different subunits.
5. Functional consequences of the presence of LacZ on lactose transport

Analysis of the S. thermophilus genome and the lactose transporter LacS revealed that this organism is highly adapted to living in milk (8,125). Internalized lactose is hydrolyzed by β-galactosidase (LacZ) to glucose and galactose, and, subsequently, the galactose moiety is excreted by LacS. Clustering the site of lactose import and lactose hydrolysis, by associating the respective enzymes LacS and LacZ, could contribute to the high efficiency of the overall process by preventing the diffusion of the galactose moiety into the cytoplasm. This would lead to an elevated galactose concentration near the LacS transporter, resulting in an increased rate of galactose export. Indications for an association of LacS and LacZ came from co-purification of LacZ with LacS and co-migration on Blue Native gels. Furthermore, LacZ from S. thermophilus contains an insert remarkably similar to a sequence on the surface of the LacS-IIA domain. These sequences, TTGLVDSLSSVDKHFASG and TTGYLVDSLSSVNDEHFASG, in LacZ and LacS, respectively, could contribute to the interaction site.

A physical association of LacS and LacZ could not be confirmed using Blue Native gel-electrophoresis. Due to the anomalous migration of a subpopulation of LacS molecules, it could not be determined whether LacS specifically comigrated with LacZ. In addition, functional interactions between both proteins could not be demonstrated. However, it was shown that the mere presence of LacZ accelerated the lactose transport rate of LacS by the production of the high-affinity countersubstrate galactose.

6. Outlook

Research on the lactose transporter from Streptococcus thermophilus, LacS, was initiated in the second half of the 1980s, and, since then, attracted the devotion of many scientists resulting in nearly 30 papers and 4 Phd. theses. Their efforts led to an extensive characterization of functional aspects of the protein and its role in the physiology of S. thermophilus, and the development of methodologies applicable to other membrane transporters as well. To interpret the wealth of functional information on the transport mechanism obtained thusfar, the research on LacS would greatly benefit from a high-resolution 3D structure.

Nevertheless, such detailed structural information is not available for LacS or homologues, and one could wonder “[w]hat to do while awaiting crystals of a membrane transport protein […]” (61)? Although alternative methodologies could be explored to obtain structural information, e.g., cysteine-crosslinking and Förster Resonance Energy Transfer (FRET), these will only yield low resolution information, insufficient to relate structure and function. Instead, alternative aspects should be explored. LacS is thusfar one of the best described oligomeric transporters and structural information will not suffice to answer several questions on the dimeric state of LacS. For example, it is not known whether the quaternary state of LacS is dynamic in the membrane, allowing exchange of subunits between dimers, or inert. Fluorescence Cross-Correlation Spectroscopy (FCCS) of LacS dimers, labeled with different fluorophores and reconstituted in Giant Unilamellar Vesicles (GUVs), could lead to answers on this question. Furthermore, transport characteristics of the covalently linked dimer suggest that lactose counterflow and Δp-driven lactose transport are affected differently by the covalent linkage (chapter 5). By co-expressing the Tobacco Etch Virus protease and the covalent LacS dimer, the effect of the forced close association can be studied in vivo. Since the study of functional
aspects of oligomeric membrane proteins only starts to emerge, the characterization of the LacS dimer would greatly contribute to the transporter field.

Next to this, attempts to crystallize LacS should be continued. The protein can be expressed to high levels in S. thermophilus and is stable in a large range of detergents. The soluble LacS-IIA domain provides the protein with a relatively large hydrophilic surface that could be important for the formation of crystal contacts. Thus far, all crystallization trials were done using the full-length protein in the dephosphorylated state. However, based on the kinetic data presented in chapter 3, most probably, the LacS-IIA domain does not interact with the carrier domain in this state, and could introduce disorder, unfavourable for crystallization. The phosphorylation of LacS-IIA, prior to crystallization, could result in more order, provided that the modification is complete. In this respect, it is important to note that the phosphorylated state of LacS-IIA is orders of magnitude more stable than IIAGlc~P.

An alternative approach, would be to use LacS-IIA His-552 substitution mutants. During this study, it was noted that upon substitution of the residue in LacS-IIA that receives the phosphate group from HPr(His~P), His-552 (85), for either arginine or glutamine, the mutant protein still exhibited a phenotype similar to the phosphorylated wildtype protein (Fig. 4). As these mutants could no longer be phosphorylated, this suggests that these substitutions of His-552 result in a mimick of the structure of phosphorylated LacS-IIA. This observation is not

Figure 4. Initial rates of lactose transport by LacS derivatives. E. coli MC1061 cells harbouring different plasmids were cultivated, induced with 1 x 10^{-3}\% (wt/vol) of L-arabinose, prepared and assayed as described under Material and Methods of chapter 3. LacS is the parent protein and corresponds to LacS(C320A) with a C-terminal His-tag; LacS\( \triangle \)IIA is the 160 amino acid truncation mutant of LacS; LacS\( \triangle \)IIA-GFP consists of a C-terminal fusion of GFP to LacS\( \triangle \)IIA. LacS(H552Q) and LacS(H552R) are substitution mutants of the phosphorylated His residue. Open and hatched bars represent \( \Delta p \)-driven lactose transport and counterflow transport, respectively.
unprecedented. For the *E. coli* IIAGlc mutant H90Q (corresponding to H552Q in LacS-IIA), it was shown that the capacity to inhibit some non-PTS transporters was lost, while dephosphorylated IIAGlc was able to do so (92). This was not caused by a decreased expression level or an aberrant protein structure, as the crystal structure of IIAGlc(H90Q) is virtually identical to the wildtype protein (80). In addition, the separately expressed dephosphorylated IIA domain of LacS was able to inhibit glycerol kinase, but no effect of IIALacS(H552R) was observed (43). Taken together, these studies, combined with the data presented here, suggest that the phosphorylation site histidine substitution mutants of IIAGlc or IIA-like proteins do not mimic the dephosphorylated state and may resemble the phosphorylated state of the IIA proteins. Additional structural verification of a physical interaction between the carrier domain and the IIA domain of a LacS(His-552) mutant might come from cysteine cross-linking. Ultimately, by integrating structural, functional, and dynamic data on LacS, the mechanism of the transport reaction catalyzed by LacS will be revealed.