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Identification of the Dimer Interface of the Lactose Transport Protein from *Streptococcus thermophilus*

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The lactose transporter from *Streptococcus thermophilus* catalyses the symport of galactosides and protons. The carrier domain of the protein harbours the contact sites for dimerization, and the individual subunits in the dimer interact functionally during the transport reaction. As a first step towards the elucidation of the mechanism behind the cooperation between the subunits, regions involved in the dimer interface were determined by oxidative and chemical cross-linking of 12 cysteine substitution mutants. Four positions in the protein were found to be susceptible to intermolecular cross-linking. To ensure that the observed cross-links were not the result of randomly colliding particles, the cross-linking was studied in samples in which either the concentration of LacS in the membrane was varied or the oligomeric state was manipulated. These experiments showed that the cross-links were formed specifically within the dimer. The four regions of the protein located at the dimer interface are close to the extracellular ends of transmembrane segments V and VIII and the intracellular ends of transmembrane segments VI and VII.

Keywords: membrane transport protein; functional dimer; dimer interface; cysteine cross-linking; quaternary structure

Introduction

Lactose transport in *Streptococcus thermophilus* is facilitated by the membrane transporter LacS.[1] This protein is composed of 12 α-helical transmembrane segments (TMS)[2] and can use the electrochemical energy stored in a proton gradient to drive the accumulation of galactosides.[3] LacS is a member of a family of secondary sugar transporters, designated the galactoside-pentoside-hexuronide (GPH) family,[4] and, together with the melibiose transporter from *Escherichia coli* MelB, LacS is the best studied member of this family. The LacS subfamily differs from the other members of the GPH family by the presence of a carboxyl-terminal domain, which is homologous to IIA proteins of the phospoenolpyruvate-phosphotransferase system.[1] Phosphorylation of this domain by HPr results in the regulation of the transport activity of the membrane-embedded carrier domain.[5]

On the basis of second-site suppressor studies, cysteine accessibility, and intra-molecular cross-linking in LacS and MelB, a helix packing model has been proposed for the carrier domain. Basically, this model consists of a so-called catalytic core of transmembrane segments I, II, IV, VII, X and XI, and a secondary ring of the remaining transmembrane segments surrounding this core.[6,7]

The quaternary structure of LacS has been determined for the protein in the detergent-solubilized and the membrane-reconstituted state, using a variety of biophysical techniques. Both analytical ultracentrifugation[8] and blue native gel electrophoresis (BN-GE)[9] showed that detergent-solubilized LacS undergoes reversible self-association with a monomer to dimer mode of association. Furthermore, both methods demonstrated that the oligomeric state in solution can be manipulated by varying the concentration of the detergent. The quaternary structure of membrane-embedded LacS was determined by freeze-fracture electron microscopy[8] and saturation-transfer

Abbreviations used: TMS, transmembrane segment; GPH, galactoside-pentoside-hexuronide; BN-GE, blue native gel electrophoresis; ST-ESR, saturation-transfer electron spin resonance; BMH, bis-maleimidohexane; MalNEt, *N*-ethyl-maleimide; oPDM, *N*,*N*′-1,2-phenylenedimaleimide; pPDM, *N*,*N*′-1,4-phenylenedimaleimide; DDM, *n*-dodecyl-β-D-maltoside; CuPhe, Cu(II)-(1,10-phenanthroline)$_3$.

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electron spin resonance (ST-ESR),[10] which showed that the protein is most likely present in the membrane as a dimer, but the results of the latter technique were also consistent with a trimeric state. The xyloside transporter from *Lactobacillus pentosus*, XylP, a member of the GPH family lacking the IIA domain, has been observed as a dimeric species, suggesting that this quaternary structure is a property of the membrane-embedded carrier domain only.[9] The projection map of MelB, however, does not comply with a dimeric state, since the relatively small subunit interaction surfaces are between adjacent protein molecules in up and down orientations.[11] It remains to be established which state reflects the *in vivo* relevant quaternary structure of MelB.

The structural unit of a membrane protein does not necessarily have to be the functional unit, since self-association can be promoted by effects such as excluded volume and a high concentration of protein in the membrane.[12] Independent functioning of the subunits within the LacS dimer was observed for the counterflow mode of transport, indicating that each subunit is equipped with a substrate-binding site, alternately exposed to the outer and inner face of the membrane, and a functional translocation path. Conversely, for the electrochemical proton gradient (Δp)-driven lactose uptake mode of transport, which comprises an additional kinetic step, cooperativity between the subunits in the dimer could be observed.[13] This functional cooperativity between the subunits is most likely associated with this additional step, the reorientation of the unliganded-binding site from the inside to the outside, which is known to be the slowest step in the translocation cycle.[14]

The quaternary state of most membrane transporters is not known. In most cases where structural interactions between identical subunits were observed, one or more functions proved to be associated with the oligomer. A first step in studying the mechanism behind the functional interaction involves the determination of the site of interaction; that is, the identification of the subunit interface. Attempts in this direction have been made for a number of transport systems, of which the datasets for the *E. coli* mannitol permease EII^{mtl},[15] the Na^+/H^+ antiporter NhaA[16] and the multidrug transporter EmrE,[17] and the human erythrocyte anion-exchanger AE1[18] are most complete. Using a cysteine cross-linking approach, close proximity of one to five regions in or near TMS was demonstrated. Here, we report on the identification of four zones in the vicinity of transmembrane segments that comprise parts of the dimer interface of LacS.

Results

Transport capacity of the single cysteine mutants

Residues at or near the membrane interface were substituted for cysteine residues (Figure 1), because cysteine residues at these positions are expected to be more susceptible for labelling than residues in the hydrophobic milieu of the membrane and may have fewer degrees of freedom than positions in the middle of interhelical loops. T83C, located in the middle of loop 2-3, forms a notable exception. The effect of the single cysteine substitutions on the transport activity of the LacS protein was determined for downhill, Δp-driven, and counterflow mode of translocation. Table 1 shows the transport capacity of the mutants compared to the Cys-less (C320A) protein. Most mutants displayed significant transport activities (30–130% of C320A). Mutants S145C, I229C and S384C were severely hampered in Δp-driven transport, while the other transport modes were less affected, a phenotype observed before in many single Cys

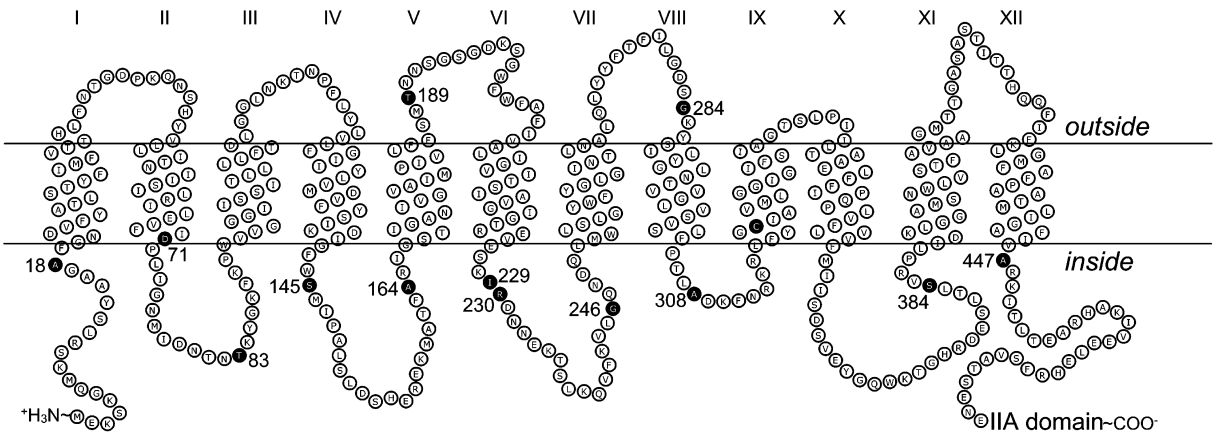

Figure 1. Topology model of LacS. This model is based on the topology model of MelB.[2] For simplicity, the remaining 169 C-terminal amino acid residues, constituting the IIA-domain, a factor Xa cleavage site and the hexa-His-tag, are not shown. The gray horizontal lines indicate the membrane interfaces. The residues in the cytoplasmic and extracellular loops that were the target of cysteine substitutions are depicted in black. In these mutants, the endogenous Cys320 in TMS IX (also shown in black) was replaced by an alanine residue.

Table 1. Transport capacity of the single cysteine mutants

Mutant	Codon change	Downhill	Δp-driven	Counterflow
C320A	[a]	++	++	++
Empty vector	[a]	−	−	−
A18C	GCT → TGT	+	+	+
D71C	[a]	−	−	−
T83C	ACT → TGT	+	+++	++
S145C	TCA → TGC	++	−	++
A164C	GCC → TGC	+++	+++	++
T189C	ACG → TGC	+	++	+
I229C	ATT → TGT	++	+	++
G246C	GGT → TGT	++	+++	+++
G284C	GGT → TGT	++	++	++
A308C	GCT → TGT	++	++	+
S384C	TCA → TGC	+++	+	++
A447C	GCT → TGT	+	++	++

Symbols −, +, ++, and +++, refer to no transport, 30–60%, 60–100%, and 100–130% of the C320A initial uptake rates, respectively. Codon changes resulting in cysteines were made in the previously constructed vector pSKE8His C320A, bearing the gene coding for cysteine-free LacS.[6] Initial downhill, Δp-driven and counterflow transport rates are shown as percentage of C320A.

[a] See Veenhoff *et al.*[6]

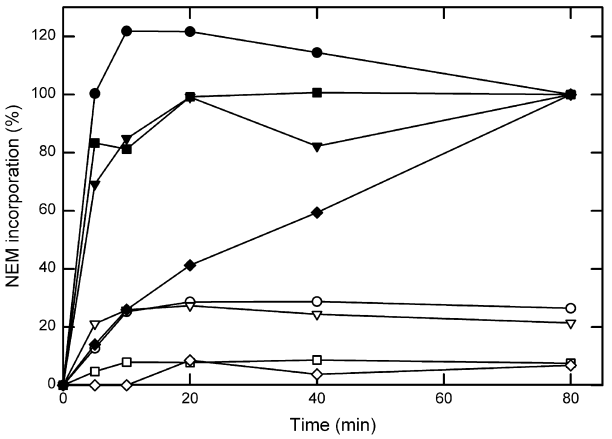

Figure 2. Accessibility of the LacS single cysteine mutants for MalNEt and BMH. Untreated and BMH-prelabelled LacS was purified from membrane vesicles and exposed to [^{14}C]MalNEt for different time-intervals. The reaction was quenched by the addition of β-mercaptoethanol, and samples were analysed by SDS-PAGE and subsequent phosphor-imaging. For each timepoint, the amount of radioactivity incorporated into LacS was normalized to the radioactivity associated with the not-prelabelled protein after 80 minutes exposure to [^{14}C]MalNEt. The kinetics of MalNEt labelling for the not-prelabelled (filled symbols) and BMH-prelabelled proteins (open symbols) are shown only for the extreme cases (A18C (circles), S384C (diamonds)) and two mutants with intermediate kinetics (S145C (triangles) and A308C (squares)). Radioactivity was not associated with the Cys-less protein (results not shown).

mutants in TMS II.[6] D71C was previously shown to be inactive in transport, but could be protected from biotin-maleimide labelling in a substrate-dependent fashion, indicating a functional substrate-binding site.[6] Taken together, the functional analyses indicate that all the mutants will have a membrane topology and overall conformation very similar to wild-type LacS.

Accessibility and reactivity of cysteine residues for maleimides

One of the prerequisites for the formation of a dimaleimide-mediated intermolecular cross-link is the ability of a cysteine residue to react with the cross-linking reagent. Therefore, the susceptibility of the single cysteine mutants towards labelling with bis-maleimidohexane (BMH), the reagent that is most flexible and has the longest span width,[19] was determined. Labelling was done in membrane vesicles, and, after purification of the LacS protein, the degree of labelling was estimated by comparing the susceptibility of the BMH-prelabelled and the not-prelabelled protein for alkylation by the small ^{14}C-labelled probe *N*-ethylmaleimide ([^{14}C]MalNEt). The Cys-less protein did not show any label incorporation. All mutants showed at most 25% MalNEt incorporation when pre-incubated with BMH as compared to the unlabelled sample (Figure 2). This result indicates that all the engineered cysteine residues are well accessible for alkylation by maleimides. The rates of MalNEt labelling differed per mutant (the most extreme cases are shown in Figure 2), indicating a difference in susceptibility of the cysteines towards MalNEt labelling. The accessibility and reactivity of D71C for alkylation has been demonstrated.[6]

Intermolecular cross-linking at the cytoplasmic face of LacS

To identify contact points between the subunits at the cytoplasmic face of LacS, membrane vesicles harbouring the mutant LacS proteins were subjected to oxidative cross-linking catalysed by Cu(II)-(1,10-phenanthroline)$_3$ (CuPhe) and to cross-linking with three dimaleimide cross-linkers differing in flexibility, span-width[19] and reactivity.[20] BMH, *N*,*N*′-1,2-phenylenedimaleimide (oPDM) and *N*,*N*′-1,4-phenylenedimaleimide (pPDM), the dimaleimide reagents used in this study, can cross-link neighboring cysteine residues at distances of 10.2(±2.4) Å, 11.1(±0.5) Å, and 9.4(±0.5) Å,[19] respectively, whereas the β-carbon atoms of the cysteine residues need to be within 4.6 Å of each other to allow the formation of a disulfide bridge.[21] Taken together, these four agents span a wide range of characteristics and they were applied to minimize the number of false negatives. Due to the flexibility of the agents and/or the target cysteine residues, cross-links were not used to exactly quantify distances between neighboring cysteine residues. The cross-linking was done mostly on membrane vesicles to avoid possible artefacts due to structure perturbation upon

solubilization, e.g. dissociation of the dimer (see below). After the cross-linking, proteins were separated by SDS-PAGE and LacS was detected by Western blotting using an antibody directed against its hexa-His-tag.

No effect of the reagents on the electrophoretic mobility of LacS(C320A) on SDS-PAGE was observed (Figure 3), indicating that non-specific cross-linking does not take place. As expected, after exposure to the agents most mutants (A18C, D71C, T83C, S145C, A164C, A308C, S384C, and A447C) showed only a band corresponding to monomeric LacS, migrating at approximately 55 kDa as a typical split band (results not shown). However, incubation of both I229C and G246C with the appropriate concentrations of dimaleimide linker, resulted in the formation of a second species in the gel, migrating at approximately 115 kDa. This size corresponds to the expected size of a LacS dimer. I229C and G246C are located at the cytoplasmic face near TMS VI and VII, respectively. The extent of dimer formation was higher for I229C than for G246C, as can be seen best from the decreased signal of the monomer band. Both mutants showed optimal dimer formation at a dimaleimide concentration of 50 μM, and only a low-intensity dimer band was observed when the dimaleimide concentration was tenfold higher. Upon oxidative cross-linking, the monomer band of I229C decreased, suggesting the formation of cross-linked product. Indeed, a faint dimer band was formed (not well visible in Figure 3). G246C did not show any reactivity upon incubation with CuPhe. Lactose (5 mM), the substrate of the LacS protein, had no effect on the cross-linking efficiency. The formation of a dimaleimide cross-linked dimer suggests that both the regions around I229C and G246C are in close proximity of the center of the homodimer. However, both positions are not susceptible to extensive oxidative cross-linking, indicating that their average distance from the β-carbon atom of the opposing cysteine residue of the other subunit is more than 4.6 Å. The inability of the majority of mutants to cross-link suggests that the regions near those residues do not reside in the vicinity of the dimer interface.

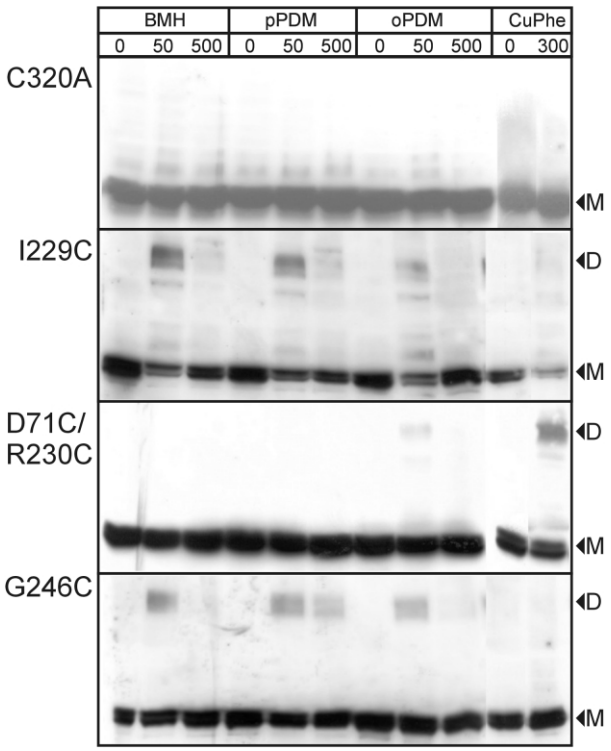

Figure 3. Immunoblots of Cys-less LacS and LacS mutants containing cysteine(s) facing the cytoplasm that were exposed to cross-linking reagents. Membrane vesicles were incubated for 20 minutes at 30 °C with dimaleimide linkers, at 50 μM and 500 μM, final concentration, or CuPhe at 300 μM, final concentration, or with the solvents (0 samples) used to dissolve the reagents. LacS was detected using an antibody directed against the C-terminal His-tag. M and D indicate the LacS-monomer (55 kDa) and dimer (115 kDa). The top panel, C320A, is illustrative for all the single Cys mutants that did not form a cross-link with each of the reagents, they are A18C, D71C, T83C, S145C, A164C, A308C, S384C, and A447C.

Intermolecular cross-linking at the extracellular face of LacS

Two cysteine substitutions were constructed at the extracellular face of LacS near TMS V and VIII (Figure 1). Analysis of these mutants showed that exposure to the cross-linking agents resulted for both T189C and G284C in the formation of LacS dimers (Figure 4). Dimers were formed upon exposure to each of the reagents. The extent of cross-linking was highest for T189C, whose monomer band almost disappeared. Dimers of this mutant were even formed with CuPhe, indicating that the sulfhydryl groups of the two Cys residues are within a few Ångström units from each other. Only a small amount of T189C dimer was formed upon exposure to oPDM. Albeit less intense,

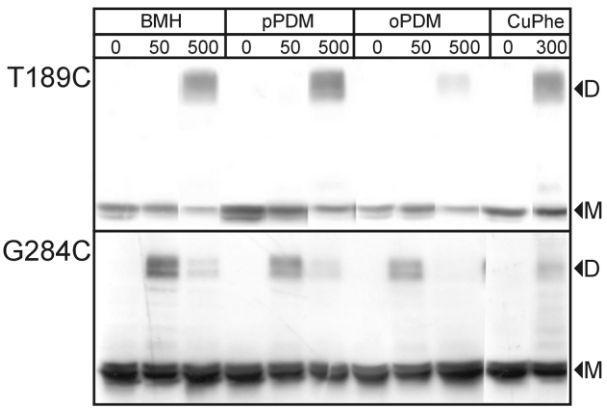

Figure 4. Immunoblots of LacS mutants with their cysteine on the extracellular face of the protein that were exposed to cross-linking agents. Samples were prepared and analysed as described in the legend to Figure 3.

dimer formation upon exposure to CuPhe was observed for G284C, indicating that both regions around T189C and G284C can access the center of the dimer. The presence of 5 mM lactose had no effect on the cross-linking efficiency.

Intermolecular cross-linking of D71C/R230C double cysteine mutant

The ability of I229C to form an intermolecular cross-link, and the inability of D71C to do so, prompted us to investigate the double cysteine mutant D71C/R230C, which was derived from a second-site suppressor study.[6] Indeed, a clear dimer signal was observed upon incubation with CuPhe, and a low intensity dimer band was formed upon incubation with oPDM (Figure 3). The dimer band, resulting from oxidative cross-linking, could be removed upon incubation with a reducing agent (results not shown). Incubation with the other agents did not result in intermolecular cross-linking. There are no indications for intramolecular cross-linking (e.g. gel-shift) in the D71C/R230C double cysteine mutant. Detailed analysis of the peptides obtained from the monomer bands upon in-gel digestion with trypsin and CNBr,[22] and subsequent analysis of the extracted peptides by mass spectrometry did not reveal the presence of an intramolecularly linked fragment (M. K. Doeven, unpublished results).

Mechanism of the intermolecular cross-linking

To exclude that the observed cross-links were the result of randomly colliding particles, as was suggested for intermolecularly formed cross-links in the lactose transport protein of *E. coli* LacY,[23] a series of control experiments was performed to determine the mechanism *via* which the cross-linking reaction proceeds. We assume that intermolecular cross-linking of randomly colliding particles does not depend on the species (monomers or dimers) involved in the collision but merely on the presence of surface-exposed cysteine residues. The formation of intradimeric cross-linked products, however, would depend highly on the quaternary structure. Therefore, we set out to determine how the quaternary state of LacS influences the cross-linking efficiency. In order to exclude the possibility that the cross-linking proceeded *via* a different mechanism on the different faces of the protein, two mutants were analysed that faced either the cytoplasmic (G246C) or the periplasmic (T189C) side of the membrane. As mentioned before, the oligomeric state of LacS can be manipulated in the detergent-solubilized state.[9] By varying the detergent concentration and detergent type after purification, samples were prepared in which mainly dimers (~95%), dimers and monomers (approx. 40% dimers), or mainly monomers (~95%) were present as determined by BN-GE analysis (Figure 5A and C). Upon monomerisation, as shown in Figure 5B and D, the

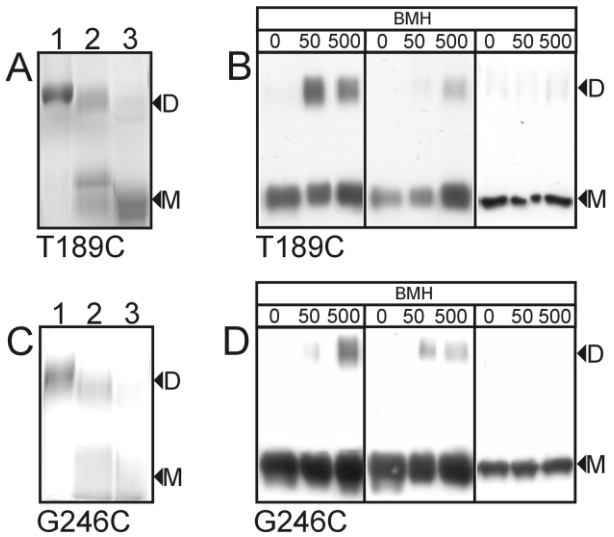

Figure 5. Effect of the quaternary structure of LacS on the cross-linking efficiency. Two mutants with their cysteine residues located on opposite sides of the membrane were purified as dimers in 0.025% (w/v) Triton X-100 as shown by BN-GE analysis in lane 1 of A and C. Subsequently, the percentage of monomers was increased by the addition of 0.5% (w/v) Triton X-100 (lane 2 in A and C) or 0.3% (w/v) DDM (lane 3 in A and C). Samples containing mostly dimers (B and D, left panel), dimers and monomers (B and D, middle panel), or mostly monomers (B and D, right panel) were incubated without (0 samples), or with 50 μM or 500 μM BMH for 20 minutes at 30 °C and analysed by immunoblotting. D and M depict the position at which the LacS dimer and monomer migrate, respectively.

extent of BMH-mediated cross-link formation decreased for both T189C and G246C. This complies with a mechanism of intermolecular cross-linking in which the cross-link is formed within the dimer. Similar results were obtained for oxidative cross-linking of solubilized membrane vesicles in which T189C was present as mixed species (dimers and monomers) or mainly monomers (results not shown), indicating that the effect is independent of the cross-linking agent.

Another way to approach the specificity problem is to study the effect of particle dilution, which for membrane proteins can be established by fusion of the membrane vesicles with empty liposomes. By increasing the membrane surface, the amount of particles, and thereby the amount of collisions, will decrease and this will result in a decreased cross-link formation when these are caused by randomly colliding particles. We could demonstrate membrane fusion upon three cycles of freezing–thawing of *E. coli* membrane vesicles and liposomes consisting purified *E. coli* total lipids (75%) plus of L-α-phosphatidyl choline (25%) from egg yolk (results not shown). Fusion, resulting in two to six times the initial amount of phospholipids in the membrane surface, and thus a decrease in LacS concentration, did not decrease the dimer formation upon oxidative cross-linking

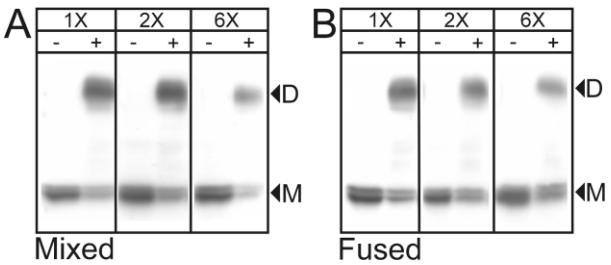

Figure 6. Effect of the LacS concentration in the membrane on the cross-linking efficiency. Membrane vesicles containing T189C and liposomes were fused by three freezing-thawing cycles (B), or merely mixed after freezing-thawing the seperate components (A). Fusions were done in the absence of liposomes (1 ×) or in the presence of a quantity of liposomes that yielded a total membrane surface consisting of two (2 ×) or six times (6 ×) the original amount of phospholipids. The material was incubated for five minutes at 30 °C in the absence (−) or in the presence of 300 μM CuPhe. Equal amounts of T189C were analysed by immunoblotting. D and M depict the LacS dimer and monomer, respectively.

of T189C. As is evident by comparing fused and mixed membranes (Figure 6A and B), the blotting and/or detection efficiency of LacS was less efficient when an excess of lipids (fused with membrane vesicles or present as liposomes) was present. Taken together, these results demonstrate that the observed cross-links are formed between the subunits within the LacS dimer and not *via* the cross-linking of surface-exposed cysteine residues upon dynamic collisions.

Discussion

Assessment of the quaternary structure of LacS showed that the solubilized protein exists as monomers and dimers, and that the membrane-embedded protein complex is probably present as dimers only. Although the rotational correlation times in previous ST-ESR studies were consistent with dimers and trimers,[10] other types of analyses never yielded indications for an oligomeric state other than dimeric. Independent functioning of the subunits within the LacS dimer was observed for the counterflow mode of transport. However, for substrate accumulation driven by the Δp, functional interaction of the subunits within the dimer was observed.[13] On the basis of these previous findings, it was anticipated that the contact points between the subunits of the LacS dimer would be present on a specific side and not scattered over the full periphery of the protein. It is very likely that the functional interactions between the subunits take place at this so-called dimer interface. As a first step in determining the mechanism of the intradimeric communication, regions at the dimer interface and in the vicinity of TMS were identified.

Although cysteine replacements within the TMS would have yielded more precise positional constraints, the low dielectric milieu of the bilayer prevents efficient labelling of membrane-embedded cysteine residues.[17,23,24] Therefore, based on the membrane topology model of MelB,[2] residues predicted to be near the ends of the α-helices of the carrier domain of LacS were replaced by cysteine, except for Thr83, which is located in the middle of the second cytoplasmic loop. On the basis of transport and substrate-binding assays, the conservation of the native structure was confirmed for each of the mutants. Furthermore, the suitability of the mutants for the cross-linking approach was established by determining the accessibility of the cysteine residues for the longest, most flexible cross-linker BMH. All the single cysteine mutants were exposed to a dimaleimide concentration range, because it was anticipated that a potential dimaleimide-mediated cross-link could be overlooked when the concentration of the reagent was too high. This would cause separate labelling of both cysteine residues rather than cross-linking *via* the dimaleimide. A strong dimaleimide concentration-dependence of cross-linking was indeed observed, suggesting that a concentration range should be applied in the initial screening for cross-links.

On the cytoplasmic side, only I229C and G246C yielded cross-linked products, indicating their close proximity to the center of the dimer. Of these residues, I229C even formed a disulfide bridge, albeit at a very low rate. Since the formation of a disulfide bridge can occur only when the C^{β} atoms of both cysteine residues are within 4.6 Å of each other,[21] I229C must have (limited) access to the dimer center. Since D71C did not show any intermolecular linking, it is likely that Cys230 in the double cysteine mutant D71C/R230C is responsible for the dimer formation under oxidizing conditions. This dimer band was more intense than that for Cys229, which could be explained by a better positioning of the residues for disulfide formation or a greater flexibility. It is well possible that not all interface regions on the cytoplasmic side of the protein have been identified in this study, because only residues close to the center of the dimer can be cross-linked with the strategy used.

On the extracellular face of the protein, two positions were analysed. T189C near TMS V and G284C near TMS VIII were able to form cross-links. Since oxidative cross-links were formed with great efficiency, both residues must have access to the very center of the dimer. Analysis on this side of the protein is not complete, and participation of other zones on the extracellular face of the dimer center cannot be excluded.

The validity of the cross-linking approach was assessed to exclude the possibility that collisions of monomeric or dimeric species with surface-exposed cysteine residues were responsible for the observed cross-links, even though there is extensive evidence that LacS is present in the membrane as a structural and functional dimer. Other lines of *a priori* facts that make it likely that the cross-links are formed specifically within the dimer relate to

our experimental setup, which was chosen to minimize the amount of cross-linked protein formed by random collisions. Namely, all the cross-linking experiments were done in membrane vesicles in which LacS constitutes only a small percentage (0.5–2%) of total membrane protein. Furthermore, the outcome of the experiment shows that all the positions in the protein susceptible to cross-linking are close to each other in the primary structure and located on one side, rather than scattered over the full periphery of the helix packing model of the protein. The latter would be expected for randomly colliding particles.

To determine the mechanism *via* which the cross-linking reactions proceeded, the dimaleimide-mediated and the oxidative cross-linking of G246C and T189C was studied in more detail. These mutants have their cysteine residues located on opposite sides of the membrane and were used to exclude the possibility that cross-link formation on these sides proceeded *via* different mechanisms. As shown previously,[9] the oligomeric state of detergent-solubilized LacS can be manipulated by varying the detergent concentration and/or detergent type. We found that the transition from a dimeric to a monomeric structure coincides with a large decrease in cross-link efficiency, demonstrating that the intermolecular cross-links observed are formed within the LacS dimer and do not take place between randomly colliding particles with surface-exposed cysteine residues. In addition, the mechanism of the reaction was assessed by manipulating the particle density in the membrane by fusing membrane vesicles with liposomes. Even at lower concentrations of LacS, the cross-links were formed and the rate of the reaction was not severely affected (results not shown). We thus conclude that our data have truly revealed parts of the dimer interface of LacS.

The functions of the zones identified at the interface in the intradimeric communication remain to be elucidated. For T189C and G284C, and the nearby TMS V, VI, and VIII, there are no data to support a direct role of these regions in either the binding of substrate and/or cation, or translocation of the solutes. Based on this and the known functions of TMS I, II, IV, VII, X and XI in substrate binding and/or translocation,[6,7] the putative α-helices V, VI and VIII, and accompanying extracellular loop regions are thought to be positioned on the periphery of the protein. These latter regions might be involved in the communication between the subunits by coupling the functional regions of both subunits. However, none of these zones has been shown to interact directly with a catalytically important region. The available data on TMS VI suggest that none of the residues in this putative α-helix faces an aqueous pore.[25] In TMS V, a suppressor mutation of L391C in TMS XI was isolated,[26] but it is not clear whether the corresponding residues interact directly. Data on the loops containing T189C and G284C, and TMS VIII are not available.

Contrary to the extracellular regions, several lines of evidence suggest functional roles for the cytoplasmic loop containing both I229C and G246C, and the nearby located TMS VII. Firstly, a second-site suppressor study on LacS demonstrated a possible interaction between Arg230 in the cytoplasmic loop near TMS VI, and Asp71 in TMS II. The latter residue has been implicated in cation binding.[27,28] In this study, I229C, located directly above Arg230, has been shown to have access to the center of the dimer. Presumably, R230C, in the double mutant D71C/R230C, is the residue responsible for the formation of the cross-linked species. Future studies need to reveal if the proposed location of this residue near the dimer centre is important for the suppression of the translocation defect in D71C. Secondly, in MelB, several mutations in TMS VII were found to result in an altered cation[29] and sugar specificity,[30] and second-site suppressor mutations suggested an interaction between TMS VII and TMS II.[31] The mutations identified in these three studies all reside on one face of TMS VII. This side of the helix is likely to face the aqueous pore, which could leave the other face free to interact with the opposing subunit and provide a rationale for the G246C cross-link.

Overall, the results presented here are in agreement with the previously proposed helix packing model of LacS;[6] that is, most regions that were found to be in the vicinity of the dimer interface are near helices that were thought to be located on the periphery of the protein (Figure 7A). One possible organisation of TMS at the dimer interface involves close proximity of the extracellular ends of the putative α-helices V and VIII as well as close proximity of the cytoplasmic ends of the α-helices VI and VII (Figure 7B). The fact that most

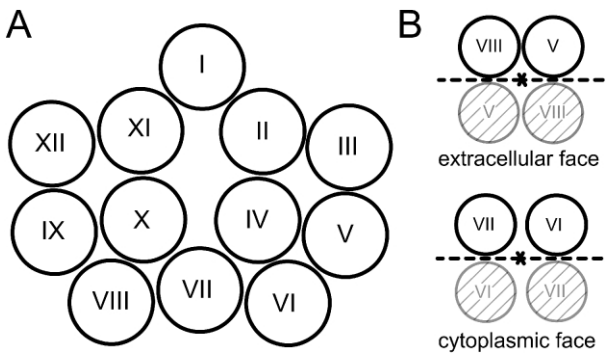

Figure 7. A, The helix packing model of LacS. This model is based on second-site suppressor studies on LacS and MelB, the amphipathicity of TMS II, IV, XI, and residues important for binding of the sugar or the coupling ion (adapted from Veenhoff *et al.*[6]). B, A possible organization of the transmembrane segments 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. Transmembrane segments of different subunits are in different shades, the line separates the different subunits.

of the identified regions at the interface and nearby TMS lack a prominent catalytic role is in agreement with earlier observations, which indicated that each individual subunit is equipped with a substrate and proton-binding site and a translocation path. We speculate that the cooperativity between the subunits might be mediated by transmembrane segments or regions that are of structural rather than catalytic importance.

Materials and Methods

Chemicals

Bis-maleimidohexane (BMH) was purchased from Pierce, *N*,*N*′-1,2-phenylenedimaleimide (oPDM) and *N*,*N*′-1,4-phenylenedimaleimide (pPDM) from Fluka. D-[Glucose-1-^{14}C]lactose was obtained from the Radiochemical Center, Amersham Pharmacia Biotech. Bacteriological media were purchased from Difco. Other chemicals were reagent grade and obtained from other commercial sources.

Bacterial strains and plasmids

E. coli strains HB101 (relevant genotype: *lacY1*)[32] and DW2 (relevant genotype: *Δlac*(*ZY*))[30] were grown at 37 °C in Luria Broth (LB) under vigorous aeration. When appropriate, the medium was supplemented with 50 μg/ml of ampicillin and/or 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Mutant alleles of *lacS* yielding single cysteine mutants of LacS were obtained using the Quikchange mutagenesis kit (Stratagene) with a template plasmid coding for His-tagged cysteine-free LacS (pSKE8His C320A).[6] The mutations introduced are listed in Table 1. Mutations were verified by DNA sequencing (Bio-Medical Technology Center, Groningen, The Netherlands).

Transport assays

E. coli cells used for transport assays were grown overnight in LB, washed twice in KPM (50 mM KPi (pH 7.2), 2 mM $MgSO_4$), resuspended to 30 mg protein/ml, and stored on ice. Transport of [^{14}C]lactose in dilute cell suspensions was assayed at 30 °C. The transport reactions were stopped at different timepoints by dilution into 2 ml of 0.1 M LiCl, followed by rapid filtration on 0.45 μm nitrocellulose filters (Schleicher & Schuell Inc.). After washing the filters with another 2 ml of 0.1 M LiCl, the radioactivity associated with the filter was determined by liquid scintillation counting.

Downhill uptake

Lactose transport down the concentration gradient was measured in *E. coli* HB101 cells that were grown in the presence of 0.5 mM IPTG to induce the expression of β-galactosidase. Downhill lactose uptake was started by the dilution of 6 μl of cell suspension into 200 μl of KPM containing 50 μM [^{14}C]lactose.

Proton motive force (Δp)-driven uptake

Lactose transport driven by the Δp was measured in *E. coli* DW2 cells. Aliquots of 6 μl of cell suspension were diluted into 200 μl of KPM containing 10 mM D-Li-lactate as electron donor, and aerated for two minutes. After this pre-energization, the transport reaction was started by the addition of 100 μM [^{14}C]lactose.

Counterflow transport

Lactose counterflow was measured in *E. coli* DW2 cells. Concentrated cell suspensions were supplemented with 10 mM lactose and allowed to equilibrate overnight at 4 °C. The next day, the lactose-loaded cells were de-energized by incubation with 50 μM SF6847 plus 30 mM NaN_3 for two hours at 4 °C. The suspension was concentrated to 56 mg/ml of protein. Counterflow transport was started by the dilution of 2 μl of cell suspension into 200 μl of KPM, bringing the final outside [^{14}C]lactose concentration to 130 μM.

Membrane vesicle isolation

E. coli HB101 cells were ruptured by a twofold pass through the French pressure cell at 10,000 psi (1 psi ≈ 6.9 kPa), and the inside-out membrane vesicles were collected as described.[33] Membrane vesicles were resuspended in 50 mM KPi (pH 7), and 3 mM dithiothreitol (DTT), frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined using the DC Protein Assay (Bio-Rad).

[^{14}C]*N*-Ethylmaleimide (MalNEt) accessibility

Membrane vesicles were deprived of DTT by three subsequent washing steps or using a Bio Micro-spin 6 column (Bio-Rad) pre-equilibrated with 50 mM KPi (pH 7.2), resuspended to 5.6 mg/ml of protein and split into two batches. Both batches were incubated for 20 minutes at 30 °C in the absence or in the presence of 500 μM BMH at a protein concentration of 5 mg/ml. After the addition of 50 mM β-mercaptoethanol, the membrane vesicles (approx. 30 mg total protein in a total volume of 3 ml) were solubilized in solubilization buffer (15 mM imidazole (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol) supplemented with 0.5% (w/v) *n*-dodecyl-β-D-maltoside (DDM) and incubated on ice for 20 minutes. The solubilized material was mixed with 0.4 ml Ni-NTA resin that was washed with ten volumes MilliQ and two volumes of elution buffer (200 mM imidazole (pH 7.0), 10% glycerol) and pre-equilibrated with four volumes of solubilization buffer supplemented with 0.05% (w/v) DDM. The mixture was incubated for one hour at 4 °C with continuous mixing. For all subsequent steps, the solutions were supplemented with 0.05% (w/v) DDM. After one hour, the column was drained, washed with ten volumes of solubilization buffer, ten volumes of wash buffer (25 mM imidazole (pH 8.0), 100 mM NaCl, 10% glycerol), and eluted with elution buffer. Purified protein (0.5–1 μM) was incubated with 60 μM [^{14}C]MalNEt for different time intervals at room temperature. Labelling was quenched by the addition of 8 mM β-mercaptoethanol. Samples were analysed by SDS/7.5% PAGE and radioactivity was determined with a Phosphorimager 425 (Molecular Dynamics).

Cross-linking in membrane vesicles

The dimaleimide cross-linkers were dissolved in DMF as tenfold concentrated stocks. Cu(II)-(1,10-phenanthroline)$_3$ (CuPhe) was prepared fresh as a

tenfold concentrated stock (3 mM $CuSO_4$, 9 mM 1,10-phenanthroline). Inside-out membrane vesicles were deprived of DTT as described above, resuspended to 5.6 mg/ml of protein in 50 mM KPi (pH 7.2). The reaction was started by the addition of cross-linking agent (or solvent) from a tenfold concentrated stock. Samples were incubated for 20 minutes at 30 °C and quenched by the addition of 50 mM β-mercaptoethanol (for maleimide cross-linking) or 20 mM K-EDTA (pH 7.0), plus 5 mM MalNEt (for oxidative cross-linking) and ten minutes incubation at 4 °C. Reducing or non-reducing sample buffer was added afterwards. Samples were analysed on SDS/7.5% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech) by semi-dry electroblotting according to standard techniques. A home-built automated Western blot processor, designated Blot-o-Matic, was used for the subsequent washing and incubation steps. LacS was immunodetected with a primary antibody directed against a hexa-His-tag (Amersham Pharmacia Biotech) and the secondary anti-mouse IgG alkaline phosphatase conjugate (Sigma). Chemiluminescence detection was done using the Western-Light™ kit with CSPD™ (Tropix Inc.) as substrate, according to the manufacturer's instructions.

Cross-linking in the detergent-solubilized state

The initial purification steps of untreated LacS mutants from *E. coli* membrane vesicles were done as described above. After draining the Ni-NTA resin column, all subsequent wash and elution buffers were supplemented with 0.025% (w/v) Triton X-100 instead of 0.05% (w/v) DDM. In order to change the oligomeric state of the purified protein samples, 0.5% (w/v) Triton X-100 or 0.3% (w/v) DDM were added, and the samples were allowed to equilibrate for 30 minutes at room temperature. The quaternary state of the samples was determined by BN-GE analysis as described.[9] Purified LacS (2–3 μM) was incubated without or with 50 μM or 500 μM BMH for 20 minutes at 30 °C. The reaction was quenched by the addition of 11 mM β-mercaptoethanol and ten minutes incubation at 4 °C. Samples were analysed as described above.

Cross-linking in fused membrane vesicles

Membrane vesicles were deprived of DTT as described above. The average lipid to protein ratio in the cytoplasmic membrane of *E. coli* was estimated at about 0.4 (w/w).[34] Membrane vesicles (0.36 mg of lipids) were fused with 0.36 mg or 1.8 mg of liposomes consisting of L-α-phosphatidyl choline from egg yolk and purified *E. coli* total lipid extract[35] (in a ratio of 1:3) by three rapid freezing (−196 °C)/slow thawing (room temperature) cycles. Fusion between membrane vesicles and liposomes was monitored using the octadecyl rhodamine B chloride (R18) dequenching assay.[36] R18 was incorporated in the membrane vesicles to a non-saturating concentration range ($\sim 1 \times 10^{-2}$ mol% with respect to total lipid). Membrane vesicles and liposomes that seperately underwent three freezing/thawing cycles, and mixed prior to oxidative cross-linking, were used as control samples. Oxidative cross-linking and subsequent analysis was done as described above.

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