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Helix dynamics in LacY: helices II/IV

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Abstract

Biochemical and biophysical studies based upon crystal structures of both a mutant and wild-type lactose permease from *Escherichia coli* (LacY) in an inward-facing conformation have led to a model for the symport mechanism in which both sugar- and H⁺-binding sites are alternatively accessible to either side of the membrane. Previous findings indicate that the face of helix II with Asp68 is important for the conformational changes that occur during turnover. As shown here, replacement of Asp68 at the cytoplasmic end of helix II, particularly with Glu, abolishes active transport, but the mutants retain the ability to bind galactopyranoside. In the x-ray structure, Asp68 and Lys131 (helix IV) lie within ~4.2 Å of each other. Although a double mutant with Cys replacements at both position 68 and 131 cross-links efficiently, single replacements for Lys131 exhibit very significant transport activity. Site-directed alkylation studies show that sugar binding by the Asp68 mutants causes closure of the cytoplasmic cavity, like wild-type LacY; but strikingly, the probability of opening the periplasmic pathway upon sugar binding is markedly reduced. Taken together with previous mutagenesis and cross-linking studies, the findings lead to a model in which replacement of Asp68 blocks a conformational transition involving helices II and IV that is important for opening the periplasmic cavity. Evidence is also presented suggesting that movements of helices II and IV are coupled functionally with movements in the pseudo-symmetrically paired helices VIII and X.

Keywords

permease; transport; membranes; membrane proteins; transport mechanisms

Introduction

Members of the Major Facilitator Superfamily (MFS)¹ catalyze transport of a variety of solutes including amino acids, Krebs cycle intermediates, sugars and antibiotics. This large group of evolutionarily related transport proteins is found in membranes from archaea to the mammalian central nervous system^{2, 3} and contains the lactose permease of *E. coli* (LacY), arguably the most intensively studied ion gradient-coupled symport protein. LacY catalyzes

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the coupled stoichiometric symport of a galactopyranoside and an H^+ across the cytoplasmic membrane by transducing the energy stored in the H^+ electrochemical ion gradient ($\Delta\mu_{H^+}$, interior negative and/or alkaline) into a concentration gradient (reviewed in ^{4; 5}). In the absence of $\Delta\mu_{H^+}$, LacY also catalyzes the opposite reaction, utilizing free energy released from downhill translocation of sugar to drive uphill translocation of H^+ with generation of $\Delta\mu_{H^+}$, the polarity of which depends upon the direction of the substrate concentration gradient. Importantly, LacY catalyzes exchange or counterflow of sugar without translocation of H^+ , and these reactions are unaffected by $\Delta\mu_{H^+}$ ^{6; 7; 8}. Therefore, the primary driving force for the global conformational change is concurrent occupancy of co-substrate binding sites.

Crystal structures of a conformationally restricted mutant ^{9; 10}, as well as wild-type LacY ¹¹, have been solved to a resolution of 2.9 Å and 3.5 Å, respectively. In all LacY structures determined thus far, the N- and C-terminal six-helix bundles form a large internal cavity open to the cytoplasm only, representing the inward-facing conformation. There is a single sugar-binding site at the apex of the cavity near the approximate middle of the molecule, and the periplasmic side is tightly closed, thereby preventing access of sugar on the periplasmic side of the membrane from the binding site.

A combination of x-ray crystallographic structures with extensive biochemical and biophysical studies ^{12; 13; 14; 15; 16; 17; 18; 19} provides strong support for an alternating access mechanism in which the sugar and H^+ binding sites are alternatively accessible to either side of the membrane. However, despite an abundance of evidence that LacY opens on the periplasmic side, an x-ray structure in this conformation has not been obtained yet. Although the global conformational change proposed in the alternating access model explains how LacY catalyzes translocation of sugar across the membrane, the movement of transmembrane helices within the N- and C-terminal bundles that leads to opening and closing of the cavities remains a puzzle. In this regard, Cys-scanning mutagenesis and site-directed alkylation studies show that one face of helix II is lined with a set of residues that are inactivated by replacement with Cys (Fig. 1A) ²⁰. Moreover, the activity of an adjoining set of active Cys-replacement mutants is compromised by alkylation. Therefore, it seems likely that this surface of helix II is important for the conformational changes associated with turnover.

Asp68 is a conserved residue located in helix II (Fig. 1 & Fig. 7D) in the oligosaccharide/ H^+ symport subfamily of the MFS ¹. The residue was thought originally to be a key residue in the conserved motif, GXXXD(R/K)XGR(R/K) in loop II/III ^{21; 22}. However, the x-ray structures of LacY ^{9; 10} show that Asp68 is at the cytoplasmic end of helix II and within H-bonding distance of Lys131 at the end of helix IV (Fig. 1B and C). In any event, the motif has been shown to play an important role in transport by mutational analysis ^{6; 20; 21; 22; 23; 24; 25; 26}. It is also notable that most second-site suppressor mutations for Asp68 mutants are in the C-terminal half of LacY on the other side of the membrane ²⁵, suggesting that Asp68 mutations have a global effect on the conformational dynamics of LacY.

In this study, site-directed mutagenesis, transport assays, thiol cross-linking and site-directed alkylation are used in conjunction to assess the role of Asp68 and the Asp68/Lys131 pair in the dynamics of LacY. The results show that Asp68 is exquisitely sensitive to replacement, and even the most conservative replacement with Glu inactivates transport. In contrast, replacement of Lys131 with Arg leads to high activity, and replacement with neutral residues inhibits only partially. Furthermore, as shown by thiol cross-linking, the two positions cross-link readily in the presence of Cu(II) *o*-phenanthroline [Cu-Ph] or *bis*-methanethiosulfonate-based cross-linking agents. In addition, the Asp68 mutants bind substrate normally, and loss of transport activity in the mutants is likely caused by

decreasing the probability of opening the hydrophilic pathway on the periplasmic side of LacY upon sugar binding. A mechanism for the phenotype that is consistent with many experimental observations is proposed.

Results

Cross-linking

A general approach to thiol cross-linking involves expression of LacY in two contiguous, non-overlapping fragments comprised of the N- and C-terminal transmembrane helices each with a single Cys residue at a defined position on either side of the discontinuity^{27; 28; 29; 30; 31; 32}. Disulfide formation or chemical cross-linking of the two fragments in the absence or presence of sugar then approximates the distance between the paired Cys residues and ligand-induced changes. The x-ray crystal structures shows that Asp68 and Lys131 are located at the cytoplasmic ends of helices II and IV, respectively (Fig. 1), and although lactose accumulation is nil in N₂C₁₀³³, the construct was utilized to test the possibility of a dynamic interaction between the two positions.

RSO membrane vesicles containing D68C/K131C in the LacY N₂C₁₀ split were incubated with the oxidizing agent Cu-Ph or the homobifunctional cross-linking reagents 1,1-methanediyl *bis*-methanethiosulfonate (MTS-1-MTS) or 1,3-propanediyl *bis*-methanethiosulfonate (MTS-3-MTS) in the absence or presence of β -D-galactopyranosyl 1-thio- β -D-galactopyranoside (TDG) (Fig. 2). Cross-linked and non-cross-linked products were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and probed with anti-C-terminal antibody. While the C₁₀ fragment migrates at about 25 kDa in the control lanes, N₂C₁₀ cross-linked with Cu-Ph, MTS-1-MTS, or MTS-3-MTS migrates at about 33 kDa, and cross-linking is complete by 10 min with all three reagents (Fig. 2A). In order to study the time course of cross-linking, RSO membrane vesicles containing the same LacY construct were incubated with MTS-1-MTS for given times at 25 °C (Fig. 2B) or 4 °C (Fig. 2C) in the absence or presence of 10 mM TDG. At 25 °C, complete cross-linking is achieved as early as 1 min and no obvious effect of TDG is observed. At 4 °C, cross-linking with MTS-1-MTS is also essentially complete except at 1 min in the presence of TDG, where it appears that there may be slight inhibition of cross-linking.

Transport activity

Consistent with earlier observations^{20; 22}, Asp68 mutations (Asp68→Ala, Cys, Asn or Glu) do not catalyze lactose accumulation to any significant extent (Fig. 3 and 4). Although mutation of Asp68 abrogates activity, several second-site revertants exhibit high activity²⁵ indicating that Asp68 is not irreplaceable for the transport mechanism.

In contrast, mutations in Lys131 do not abolish transport, but have varied effects depending on the mutation (Fig. 4). Mutant K131R catalyzes lactose accumulation at least as well or even better than the wild type, while mutants K131A and K131C accumulate lactose at a low rate to about 50-60% of the steady-state level of accumulation observed with wild-type LacY.

Two pairs of double neutral mutants, D68A/K131A and D68C/K131C were constructed to test whether neutralization of both residues rescues loss of activity due to generation of an unpaired charge in the single mutants^{34; 35}. Both double mutants accumulate lactose slowly to a steady state of ~20% of wild type (Fig. 4). Although this represents a low level of activity, the finding suggests that removal of positive charge at Lys131 rescues at least some activity in Asp68 single mutants. Furthermore, all attempts to rescue the transport activity of the D68A mutant by neutral replacements for Lys residues on the cytoplasmic side of LacY are inactive as is mutant D68K/K131D (Fig. 4).

Ligand binding

In order to test whether loss of activity in the Asp68 mutants is due to a defect in sugar binding, RSO membrane vesicles containing wild-type LacY, mutant D68E or mutant D68N were assayed for binding of *p*-nitrophenyl- α -D-galactopyranside (NPG) by flow dialysis (Fig. 5). At the inception of the experiments, [³H]NPG is added to the upper chamber containing membrane vesicles. Radioactivity in the dialysate increases rapidly to a maximum and then decreases at a slow rate. When excess TDG is added to the upper chamber, bound [³H]NPG is displaced, and radioactivity in the dialysate increases. As shown clearly, a marked increase in the dialyzable NPG concentration with both D68E and D68N mutants is observed upon addition of TDG, which is comparable to that observed with wild-type LacY. The observation is consistent with the conclusion that ligand binding is intact in the D68 mutants.

Asp68 mutations restrict opening of the periplasmic cavity

Site-directed alkylation experiments indicate that sugar binding induces closing of the hydrophilic cytoplasmic cavity with opening of a wide hydrophilic pathway on the periplasmic side of LacY^{12; 15; 16}, and a variety of additional biophysical and biochemical experiments provide strong support for this conclusion^{13; 14; 19; 36}. With Cys-less LacY containing the biotin acceptor domain (BAD), as well as mutants D68E and D68N in the same background, binding of TDG causes decreased reactivity of Cys replacements at positions 60 and 64 (Fig. 6B), which are located in the cytoplasmic cavity (Fig. 6A). In dramatic contrast, although increased reactivity is observed with Cys replacements at positions 245 or 248 with Asp at position 68 (Fig. 6B), no increase in reactivity whatsoever is observed when Asp68 is replaced with either Glu or Asn (Fig. 6B). Taken as a whole, the findings indicate that in the Asp68 mutants, the probability of opening the periplasmic pathway remains low upon ligand binding, while the cytoplasmic cavity is able to close. Thus, in the presence of ligand, these LacY mutants may favor a novel locked conformation.

Discussion

In this study, site-directed mutagenesis in combination with cross-linking, active transport, binding assays and site-directed alkylation was employed to study the role of Asp68 and its interaction with Lys131. N₂C₁₀ split LacY with mutations D68C/K131C does not cross-link spontaneously, but does so completely at a rapid rate when catalyzed by Cu-Ph induced oxidation or treatment with homobifunctional MTS reagents (Fig. 2). Thus, although the two positions are relatively close physically, as shown in the crystal structure^{9; 10} where the distance between the functional groups is ~4.2 Å (Fig. 1C), and mutant the distance should be somewhat greater in the double-Cys, which may explain the lack of spontaneous cross-linking. In any event, the double-Cys mutant cross-links in the presence of Cu-Ph or methanethiosulfonate reagents, and binding of sugar probably does not change the rate of cross-linking.

All replacements for Asp68 tested inactivate, particularly Glu, while Lys131 is more permissive; K131R LacY transports at least as well as wild-type, and K131A or K131C single mutants have low but significant activity. Despite the sensitivity of Asp68 to replacement, high transport activity is observed with second-site suppressors²⁵, indicating that this carboxyl side chain is not absolutely required for symport. Double neutral replacements for Asp68 and Lys131, as described in this study, also rescue low but significant transport activity (Fig. 4). Importantly, flow dialysis studies show clearly that loss of function in Asp68 mutants is not due to a defect in sugar binding (Fig. 5). Rather, site-directed alkylation of single-Cys replacements in the cytoplasmic or potential periplasmic opening in LacY (Fig. 6) indicates that loss of function in the Asp68 mutants is

due to inability of sugar binding to increase the open probability of the periplasmic pathway, which severely impairs a key step in the transport cycle.

In addition to the Asp68/Lys131 charge pair in LacY, two other important pairs have been described, Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X) ^{34; 35; 37; 38; 39; 40}. The Asp237/Lys358 charge pair, which is important for membrane insertion and stability of LacY, is promiscuous. Thus, Asp237 can be replaced with Glu, carboxymethyl-Cys or sulfonylethylthio-Cys with Lys or Arg at position 358 with retention of good activity. Similarly, LacY tolerates replacement of Lys358 with Arg or ammoniummethylthio-Cys with Asp or Glu at position 237. Remarkably, moreover, LacY with Lys, Arg, or ammoniummethylthio-Cys in place of Asp237 is highly active when Lys358 is replaced with Asp or Glu, thereby demonstrating that the polarity of the charge interaction can be reversed without loss of activity ^{35; 37}. In contrast, the Asp240/Lys319 charge pair, which comprises part of the H-bond/charge pair network involved in H⁺ translocation and coupling, behaves similarly to the Asp68/Lys131 charge pair except that single neutral replacement of Lys131 retains significant activity (Fig. 4). But as with Asp240, replacement with Glu abolishes lactose transport, and activity is not rescued by replacing Lys319 with Arg nor will either pair tolerate interchange.

Unlike Asp237/Lys358, the interaction between Asp68 and Lys131, like that of the Asp240/Lys319 pair, exhibits marked sensitivity to changes in the nature of the side chains. Even short extension of either Asp residue by replacement with Glu inactivates transport. Since Asp68 appears to interact relatively weakly with Lys131, replacement of the former with the longer Glu side chain may result in a stronger charge-pair interaction, which inhibits the dynamics of LacY. If the interaction between helices II and IV is dynamic and Asp68 and Lys131 undergo association-dissociation during turnover of LacY, the strength of the interaction would be important for optimal activity. Thus, alterations in the length of the side chains at these positions may favor or disfavor interaction depending upon steric constraints.

All crystallographic structures of LacY to date are in the same inward-facing conformation ^{9; 10; 11}. However, many independent biochemical and biophysical studies show that LacY exists in multiple conformations, which are essential for the overall transport cycle ⁴¹. Site-directed thiol cross-linking ²⁸, shows that helices II and VII cross-link in a manner suggesting that ligand binding induces a translational or scissors-like movement between the two helices. Since helix II is kinked at Phe59, there is probably also clockwise rotation of the C-terminal portion of helix II with Asp68 (Fig. 7C, stereo view). Consequently, replacement of Asp68 with Glu may result in a stronger interaction with Lys131, which interferes with rotation of helices II and IV.

Mapping the cross-linked pairs V125C/F354C and I129C/T350C on helices IV and XI ³¹ reveals that positions 125 and 129 are on the side of helix IV facing away from helix XI (Fig. 7A and C). In order for these pairs to cross-link, helix IV likely rotates counterclockwise towards helix XI (Fig. 7C, stereo view). This idea is supported by experimental results obtained from double electron-electron resonance distance measurements with Cys pairs labeled with nitroxide probes ¹⁴. Ligand-induced distance changes between neighboring positions in helix IV (136 and 137) exhibit behavior that is inconsistent with rigid body movements. The major population in the absence of sugar exhibits the same interspin distance between nitroxide probes at positions 136 and 137 on helix IV paired with nitroxides at either position 340 in helix X (41 Å) or position 401 in helix XII (44 Å), but shifts differently upon ligand binding (41 Å to 30 Å for S136C/Q340C and to 23 Å for N137C/Q340C; 44 Å to 40 Å for S136C/S401C and to 31 Å for N137C/S401C). This behavior may be due to a combination of partial unwinding and counterclockwise rotation of C-terminal end of helix IV. The rotation of helices II and IV

against each other may also move Glu130 and Lys69 into closer proximity with formation of a weak charge pair or H-bond that acts to stabilize the outward-facing conformer (Fig. 7C, stereo view). Of the 417 amino acid residues in LacY, only six side chains are absolutely irreplaceable with respect to active transport: Glu126 (helix IV) and Arg144 (helix V), which are critical for substrate binding, and Glu269 (helix VIII), Arg302 (helix IX), His322 and Glu325 (helix X), which are essential for H⁺ translocation/coupling^{4; 5}. Asp68 is required for transport, but not absolutely irreplaceable, and the residue is not involved in substrate binding and probably not directly involved in H⁺ translocation or coupling between sugar and H⁺ translocation. Even the most conservative replacement with Glu blocks sugar-induced opening of the periplasmic cavity. Second-site mutations that restore transport activity are located at the periplasmic end of helices II, VII and XI where the helices are tightly packed (Fig. 7A and C). Moreover, the second-site suppressors of Asp68 mutations are generally replacements with relatively bulky side-chains on opposing faces of helices II and XI, suggesting that the mutations restore transport activity by loosening the tight packing between helices II, VII and XI in the periplasmic region. Packing of helices II and XI in the crystal structures^{9; 10; 11} also shows that these helices are tightly packed at the location of the conserved Gly46 and Gly370 pair (Fig. 7A, C and D). Upon sugar-induced rotation of helices II and IV, interaction between Gly46 and Gly370 would be weakened, and tight contact between the two helices would be abolished.

Finally, LacY exhibits pseudo-symmetry between the N- and C-terminal helix bundles. Therefore, it is highly noteworthy that helices VIII and X in the C-terminal bundle exhibit the same relationship to each other as helices II and IV in the N-terminal bundle (Fig. 8). Clearly, if sugar binding induces movement of helices VIII and X in the same manner as suggested for helices II and IV, their concerted movement plays an important role in opening the periplasmic pathway.

In any event, the Asp68 mutants probably exist in a novel locked conformation in the presence of sugar, and these mutants are candidates for crystallization trials to trap another conformational state(s) of LacY.

Materials and Methods

Plasmid construction

Single mutants were constructed by Quik-Change Site-Directed Mutagenesis (Stratagene) using template plasmid pT7-5/WT-LacY/10xHis. Construction of Cys-less N₂C₁₀ split LacY was described³³. Generation of double Cys mutants in Cys-less N₂C₁₀ split LacY was carried out by replacing the *Pst*I-*Hind*III fragment of Cys-less N₂C₁₀ split LacY containing one mutation with the corresponding fragment containing the second point mutation. Single-Cys mutants for site-directed alkylation studies were constructed in Cys-less LacY with a biotin acceptor domain (BAD) at the C-terminus, as described previously^{15; 42}. Introduction of D68E and D68N mutations into single-Cys mutants was carried out by Quik-Change Site-Directed Mutagenesis (Stratagene). All mutations were confirmed by a standard sequencing protocol.

Growth of cells and preparation of right-side-out (RSO) membrane vesicles

E. coli T184 transformed with given LacY mutants was grown in Luria-Bertani medium with ampicillin (100 µg/ml). Overnight cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2-3 h at 37 °C, cells were harvested by centrifugation and washed with 100 mM KP_i (pH 7.5). RSO membrane vesicles were prepared from 1.2 L cultures

expressing given mutants by lysozyme/ethylenediaminetetraacetic acid treatment and osmotic lysis as described^{43; 44}.

Cross-linking

RSO membrane vesicles containing LacY with the D68C/K131C double-Cys mutations in the N₂C₁₀ construct were cross-linked at a protein concentration of 1 mg/ml at 4 °C or 25 °C using either 0.5 mM Cu-Ph or 0.05 mM given methanethiosulfonate (MTS) reagents in the absence or presence of 10 mM TDG. Oxidation with Cu-Ph or cross-linking with MTS reagents was terminated at given times by addition of 10 mM methylmethane thiosulfonate or *N*-ethylmaleimide (NEM) (final concentrations). Samples were then subjected to sodium dodecyl sulfate SDS-(12%)PAGE, transferred onto poly vinylidene fluoride membranes (Millipore) and immunoblotted with a site-directed polyclonal antibody against the C terminus of LacY (residues 402-417) followed by horseradish peroxidase-coupled anti-rabbit antibody (Amersham Biosciences, NA-934)⁴⁵.

Transport assays— $\Delta\mu_{H^+}$ -driven lactose transport was measured in *E. coli* T184 (*Z⁻Y⁻*) transformed with a given plasmid. Briefly, overnight cell cultures were diluted 10-fold in Luria-Bertani broth, grown for ~2-3 h at 37 °C until the OD₄₂₀ reached 0.8-1.0 and then induced with 1 mM IPTG. After 2 h induction, cells were harvested, washed with ice-cold 100 mM KP_i (pH 7.5) and with the same buffer containing 10 mM EDTA. Cells were then resuspended in 100 mM KP_i (pH 7.5) to an OD₄₂₀ of 10. Transport of [¹⁴C]lactose (5.0 mCi/mmol; 37 MBq) at a final concentration of 0.4 mM was assayed by rapid filtration⁴⁶.

Flow dialysis

Binding of [³H]NPG was measured by flow dialysis as described⁴⁷. The upper chamber contained 200 μ L of RSO membrane vesicles in 100 mM KP_i (pH 7.5) with constant stirring at a protein concentration of 22-25 mg/mL. To ensure complete de-energization, 20 μ M valinomycin and 0.4 μ M nigericin (final concentrations) were added to the vesicles in the upper chamber. Buffer [100 mM KP_i (pH 7.5)] was pumped through the lower chamber at a flow rate of 0.5 mL/min, and 1-mL fractions were collected. Aliquots (0.9 mL) were assayed for radioactivity by addition of 5 mL of ScintiSafe Econo 2 (Fisher Scientific) scintillation mixture and liquid scintillation spectrometry.

Site-directed alkylation

RSO membrane vesicles (0.1 mg of total protein) in 50 μ L of 100 mM KP_i (pH 7.5)/10 mM MgSO₄ containing a given single-Cys mutant with the BAD at the C terminus were incubated with 40 μ M tetramethylrhodamine-5-maleimide (TMRM) in the absence or presence of 10 mM TDG at 0 °C for 30 min. Dithiothreitol was added to a final concentration of 10 mM to stop the reaction. The membranes were then solubilized in 2% *n*-dodecyl- β -D-maltopyranoside (DDM), and biotinylated LacY was purified with immobilized monomeric avidin Sepharose chromatography as described¹⁵. Purified proteins were subjected to SDS-(12%)PAGE. The wet gels were imaged directly on an Amersham TyphoonTM 9410 Workstation (λ_{ex} = 532 nm and λ_{em} = 580 nm). The gels were then silver-stained to reveal the proteins.

Protein assays and immunoblotting

Total protein was determined with the Micro BCA protein determination kit (Pierce). Samples from cross-linking experiments were electrophoresed in SDS-(12%)PAGE gel and transferred to PVDF membrane. Membranes were blocked for 1 h at room temperature with 5% BSA/1 $\times$ TBST (0.2% Triton X-100/10 mM Tris-HCl (pH 7.4)/150 mM NaCl) and then incubated with 1:10,000 dilution of primary antibodies (anti-C-terminal antibodies) for 1 h

at room temperature. Membranes were washed 3×10 min with $1 \times$ TBST and then incubated with 1:10,000 dilution of secondary antibodies (Protein A-horse radish peroxidase) for 1 h at room temperature in 5% BSA/ $1 \times$ TBST. Membranes were washed 2×10 min with $1 \times$ TBST and then incubated with Pierce's Super Signal West Pico Chemiluminescent substrate, and exposed to Hyperfilm ECL (Amersham Pharmacia).

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Abbreviations. The abbreviations used are

LacY	lactose permease
RSO	right-side-out
TDG	β -D-galactopyranosyl 1-thio- β -D-galactopyranoside
NPG	<i>p</i> -nitrophenyl- α ,D-galactopyranoside
Cu-Ph	copper(II) <i>o</i> -phenanthroline
MTS	methanethiosulfonate
MTS-1-MTS	1,1-methanediyl bismethanethiosulfonate
MTS-3-MTS	1,3-propanediyl bismethanethiosulfonate
DDM	<i>n</i> -dodecyl β -D-maltopyranoside
TMRM	tetramethylrhodamine-5-maleimide
SDS-(12%)PAGE	sodium dodecyl sulfate (12%) polyacrylamide gel electrophoresis
N₂C₁₀ split	LacY expressed in two fragments as indicated
OD	optical density

Figure 1.

Locations of Asp68 (Helix II, pink) and Lys131 (Helix IV, light blue) in the x-ray structure of LacY (PDB ID 1PV7). A) Helical wheel plots of residues in helix II viewed from the periplasmic side of the membrane. Residues from Thr45 to Leu62 are plotted in the upper panel, and residues from Ala51 to Asp68 are plotted in the lower panel. Positions sensitive to NEM treatment (>60% inhibition of initial rate) are shown as enlarged boldface letters. Positions where a single-Cys replacement by itself results in low activity ($\leq 25\text{-}30\%$ of C-less) are outlined. From citation ²⁰. B) Side view of the overall structure. D68 (red) and K131 (blue) are showed as spheres. C) Zoom into the Asp68/Lys131 vicinity. Images were generated using Chimera software (UCSF).

Figure 2.

Chemical cross-linking with RSO membrane vesicles containing D68C/K131C N₂C₁₀ split permease. A) RSO membrane vesicles were incubated with 0.5 mM Cu-Ph, 50 μ M MTS-1-MTS or MTS-3-MTS for 10 min at 25 °C in the presence or absence of 10 mM TDG. B) and C) Time course of cross-linking was studied by incubation of RSO membrane vesicles with 50 μ M MTS-1-MTS at indicated times at both room temperature (B) or 4 °C (C). Reactions were terminated by addition of methylmethane thiosulfonate to a final concentration of 5 mM. Samples were then subjected to SDS-(12%)PAGE and Western blot analysis with anti-C-terminal antibody. Control samples were treated with 2.5% DMSO, the solvent in which the cross-linking agents were dissolved.

Figure 3.

Lactose transport by *E. coli* T184 expressing given mutants. Lactose transport was assayed at 10 min as described in *Materials and Methods*. Results are expressed as a percentage of activity measured with cells expressing wild-type LacY. The data were correlated with nonspecific uptake as determined with T184 cells harboring empty vector (pT7-5 with no *lacy* insert).

Figure 4.

Time course of active lactose transport by *E. coli* T184 ($Z^{-}Y^{+}$) expressing wild-type LacY or mutants D68A, D68C, D68E, D68N, K131A, K131C, K131R, D68A/K131A, D68C/K131C and no permease (pT7-5 vector only). Cells were grown at 37 °C, as described in *Materials and Methods*. Aliquots of cell suspensions (50 μ L) in 100 mM KPi (pH 7.2)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [^{14}C]lactose (5 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at the times given by addition of 3.0 mL of 100 mM KPi (pH 5.5)/100 mM LiCl and rapid filtration through Whatman GF/F filters.

Figure 5.

Binding of NPG to RSO membrane vesicles containing wild-type LacY, mutant D68E or mutant D68N. Binding of [^3H]NPG to nonenergized RSO membrane vesicles was assayed by flow dialysis as described in *Experimental Procedures*. An aliquot of RSO membrane vesicles (200 μL) in 100 mM KPi (pH 7.5) at a concentration of 20-25 mg of protein/ml was placed in the upper chamber. An aliquot (3 μL) of 1 mM [^3H]NPG [950 mCi/mmol, approximately $4\text{--}4.5 \times 10^6$ cpm] was added to a final concentration of 20 μM at fraction 1. Where indicated by the arrow, 2 μL of 1 M TDG was added to the upper chamber to a final concentration of 10 mM.

Figure 6.

Site-directed alkylation of LacY mutants. A) The ribbon structure of LacY containing single-Cys replacements at positions 60, 64, 245 or 248 is shown as black spheres. B) LacY, mutant D68E or mutant D68N, each in a Cys-less background with a C-terminal BAD, were incubated with 40 μ M TMRM in the absence or in the presence of 10 mM TDG at 0 $^{\circ}$ C for 30 min. Dithiothreitol was added to 10 mM to terminate the reactions. The membranes were then solubilized in 2% *n*-dodecyl- β , β -maltopyranoside, and biotinylated LacY was purified with immobilized monomeric avidin Sepharose chromatography and analyzed by SDS-PAGE. The wet gels were imaged directly on an Amersham TyphoonTM 9410 Workstation (λ_{ex} = 532 nm and λ_{em} = 580 nm).

Figure 7. Model for opening of LacY elements involving helices II and IV on the periplasmic side. A) Ribbon representation of LacY. Positions 68 and 131 are shown as red segments and positions 69 and 130 as pink segments on the ribbon model; side-chains are shown as sticks. The positions of Gly46 and Gly370 are shown as red-orange segments. Yellow spheres represent the positions of cross-links between helices IV and XI³¹ (helix II, green; helix IV, blue; helix VII, grey; helix XI, cyan; remaining helices, white). B) Amplification of helices II (green) and IV (blue). Cys-scanning results are indicated as follows: yellow, positions where Cys replacements inactivate transport by >75%; pink, positions where alkylation of active Cys replacements are inactivated by >60%²⁰. C) Stereo view of helices II, IV, VII and XI, viewed from the cytoplasmic side (color-coding is same as in panel A). The

postulated rotations of helices II and IV are indicated as blue and magenta arrows on top of the respective helices. D) Sequence alignment of the regions corresponding to helices II, IV and XI of given sugar transport proteins from various organisms. Color coding: Asp68 and Glu130, red; Lys69 and Lys 131, magenta; Gly46 and Gly370, red-orange;

Figure 8.

Congruence of helix-pairs II/IV and VIII/X in LacY. A) Cytoplasmic view of LacY (helix II, red; helix IV, blue; helix VIII, orange; helix X, green; remaining helices, gray). B) Side-view of helix pair II/IV. C) Side-view of helix pair VIII/X. D) Superimposition of helix pair VIII/X on II/IV. Helices are shown as a minimal backbone trace, color-coding the same as in A).