Site-directed alkylation and the alternating access model for LacY

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In a functional lactose permease mutant from *Escherichia coli* (LacY) devoid of native Cys residues, almost every residue was replaced individually with Cys and tested for reactivity with the permeant alkylating agent *N*-ethylmaleimide in right-side-out membrane vesicles. Here we present the results in the context of the crystal structure of LacY. Engineered Cys replacements located near or within the inward-facing hydrophilic cavity or at other solvent-accessible positions in LacY react well with this alkylating agent. Cys residues facing the low dielectric of the membrane or located in tightly packed regions of the structure react poorly. Remarkably, in the presence of ligand, increased reactivity is observed with Cys replacements located predominantly on the periplasmic side of the sugar-binding site. In contrast, other Cys replacements largely on the cytoplasmic side of the binding site exhibit decreased reactivity. Furthermore, both sets of Cys replacements in the putative cavities are located at the periplasmic (increased reactivity) and cytoplasmic (decreased reactivity) ends of the same helices and distributed in a pseudosymmetrical manner. The results are consistent with a model in which the single sugar-binding site in the approximate middle of the molecule is alternately exposed to either side of the membrane due to opening and closing of cytoplasmic and periplasmic hydrophilic cavities.

membrane proteins | membranes | permease | symport | transport

The lactose permease of *Escherichia coli* (LacY) is encoded by the *lacY* gene and catalyzes the coupled stoichiometric translocation of a galactopyranoside and an H^+. As such, LacY is a paradigm for membrane proteins that transduce free energy stored in an electrochemical ion gradient into a solute concentration gradient or vice versa. LacY has been solubilized from the membrane, purified to homogeneity in a completely functional state (reviewed in ref. 1), and acts functionally and structurally as a monomer (reviewed in ref. 2).

A functional LacY molecule devoid of eight native Cys residues (C-less LacY) has been constructed (3), and placement of single-Cys residues at almost every position of the molecule has provided a library of mutants that is particularly useful for structure/function studies (see ref. 4). By using Cys, which is average in bulk, relatively hydrophobic, and amenable to highly specific modification, site-directed mutagenesis can be used in conjunction with biochemical and biophysical techniques *in situ* to study accessibility of intramembrane residues to the aqueous or lipid phase of the membrane (5–10), as well as the proximity between transmembrane domains (reviewed in refs. 11 and 12). Notably, x-ray structures of LacY solved at nominal resolutions of 3.5 Å (13) and 2.9 Å (14) confirm many of the conclusions derived from biochemical and biophysical observations (reviewed in ref. 2).

LacY is composed of pseudosymmetrical N- and C-terminal domains, each with six transmembrane helices, many of which are highly irregular in shape (13). Perpendicular to the plane of the membrane, the molecule is heart shaped with a surprisingly large interior hydrophilic cavity open on the cytoplasmic side, which represents an inward-facing conformation. Within the cavity, there is a single bound sugar molecule located in the approximate middle of the membrane. Site-directed biochemical, spectroscopic, and immunological techniques, in conjunction with functional studies and the crystal structure, have led to a working model for lactose/H^+ symport in which hydrophilic cavities allowing access to the sugar-binding site are alternately open to either side of the membrane (the alternating access model).

Site-directed alkylation of single-Cys LacY mutants in right-side-out (RSO) membrane vesicles with the permeant alkylating agent *N*-ethylmaleimide (NEM) (15) is an invaluable tool for obtaining clues regarding static and dynamic features of LacY (6, 15–22). Alkylation reflects the reactivity and/or accessibility of a Cys replacement at any given position, which depends on the environment in the vicinity of the thiol side chain and is limited by close contacts between transmembrane helices and/or the low dielectric of the environment and/or accessibility. Any change in reactivity of a Cys side chain upon substrate binding is indicative of an alteration in the local environment.

Distances approximated from cross-linking of certain paired Cys residues across the inward-facing cavity were underestimated compared with the x-ray structure, and the structure shows no pathway to the sugar-binding site from the external surface (13). Therefore, it was suggested that during transport, the inward-facing cavity closes with opening of an outward-facing cavity so that the sugar-binding site is alternately accessible to either face of the membrane, and a similar model was proposed for the glycerol phosphate/phosphate antiporter GlpT (23), another member of the Major Facilitator Superfamily. In this communication, observations from site-directed alkylation of Cys residues at almost every position in LacY accumulated over more than a decade are presented in the context of the structure of the molecule. The results are consistent with the alternating access model.

Results

The results summarized here are derived from a series of publications describing the reactivity of single-Cys mutants at almost every position in LacY in RSO membrane vesicles (6, 15–22). In all instances, reaction with radioactive NEM was carried out for 10 min. Because the time course of labeling of Cys-148 (helix V), a highly reactive side chain, is essentially linear for at least 10 min (24), it is a reasonable assumption that the single time points are proportional to the rates of labeling. It is also noteworthy that the transport activity of each single-Cys

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Abbreviations: LacY, lactose permease of *Escherichia coli*; RSO, right-side-out; NEM, *N*-ethylmaleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside.

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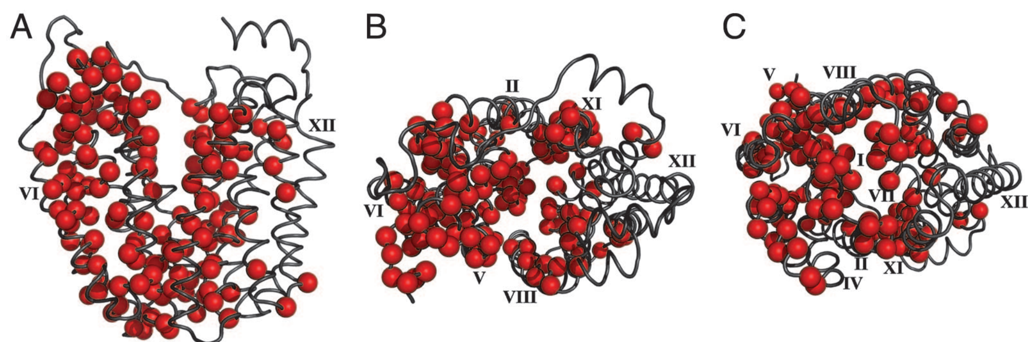

Fig. 1. Alkylation of single-Cys LacY mutants with NEM. Positions of Cys replacements (red spheres) are superimposed on the backbone of LacY (Protein Data Bank ID code 1PV7; www.pdb.org). LacY viewed perpendicular to the membrane with the N-terminal helix bundle on the left and the C-terminal bundle on the right (*A*); cytoplasmic view (*B*); and periplasmic view (*C*). In *A*, the cytoplasmic surface is at the top. A movie showing a rotating model of the perpendicular view (*A*) is shown in SI Movie 1. Positions that do not react in the absence of TDG are not shown (see Fig. 2*A*). [Note that none of the single-Cys replacements in helix XII react with NEM (P. Gao and H.R.K., unpublished observations).]

mutant, as well as the effect of NEM on activity, has been measured (reviewed in ref. 4). The vesicles are almost quantitatively RSO (25, 26); in any case, NEM is permeant and has access to both sides of the membrane (15).

Site-Directed Alkylation of Single-Cys Mutants. The red spheres shown in Fig. 1 [see supporting information (SI) Movie 1] depict the α-carbon at each position in the main chain of LacY (13), where a single-Cys replacement reacts significantly with NEM. Cys replacements in helices VI and XII label poorly or not at all, respectively, which is consistent with exposure of the cysteines to the low dielectric of the bilayer. Furthermore, Cys replacements on the faces of other transmembrane helices at the periphery of the molecule are also unreactive, and the average length of these unreactive faces is $\approx$30 Å, which corresponds to the approximate thickness of the hydrophobic interior of the bilayer. This finding agrees well with the results of intermolecular cross-linking studies (27, 28). As expected, almost all Cys replacements lining the inward-facing hydrophilic cavity (Fig. 1*A*), as well as those tested on the cytoplasmic (Fig. 1*B*) and periplasmic (Fig. 1*C*) surfaces, label well.

Effect of Ligand Binding on Reactivity of Cys Replacements. The lactose homologue β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), which binds to LacY with relatively high affinity, either increases or decreases the reactivity of the Cys replacements in a relatively specific fashion (Fig. 2). The gold spheres represent positions where TDG significantly increases reactivity, and it is apparent that the majority of these positions fall on the periplasmic side of the sugar-binding site (Fig. 2*A* and SI Movie 2) in a pattern generally consistent with the notion that a periplasmic cavity opens in the presence of ligand. Furthermore, some of these single-Cys mutants label only in the presence of TDG within the time course of the experiments (positions 18, 24, 29, 30, 45, 53, 295, 298, 315, 362). There are also a relatively small number of replacements on the cytoplasmic side of the sugar-binding site that exhibit enhanced labeling in the presence of ligand.

Although there is overlap with the positions where ligand binding leads to increased reactivity, there is another group of Cys replacements that exhibits decreased reactivity in the presence of TDG (Fig. 2*B* and SI Movie 3, blue spheres). These Cys replacements are disposed mainly on the cytoplasmic side of the sugar-binding site and in helices that form the inward-facing cavity.

The changes in reactivity are consistent with the formation of a new water-accessible cavity on the periplasmic side and closing on the cytoplasmic cavity. However, the distribution of these

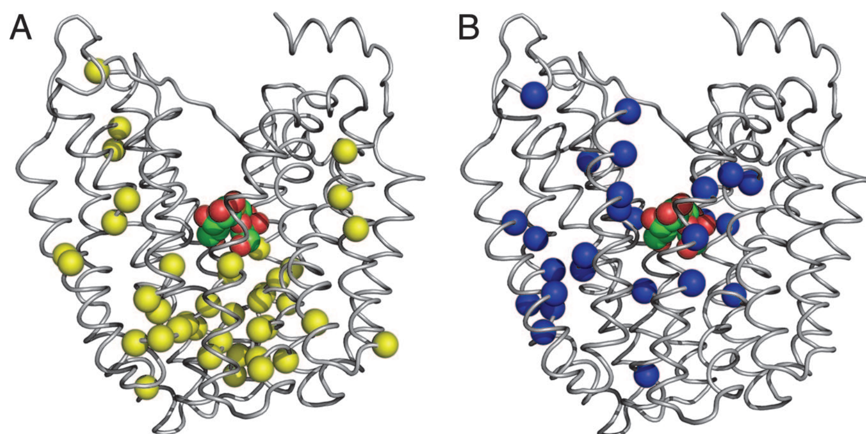

Fig. 2. Distribution of Cys replacements that exhibit changes in reactivity with NEM in the presence of TDG. (*A*) Positions of Cys replacements that exhibit a significant increase in reactivity with NEM (gold spheres) superimposed on the backbone of LacY viewed perpendicular to the plane of the membrane. TDG is shown as a Corey–Pauling–Koltun model at the apex of the inward-facing cavity. (*B*) Positions of Cys replacements that exhibit a significant decrease in reactivity with NEM (blue spheres) superimposed on the backbone of LacY viewed perpendicular to the plane of the membrane. The cytoplasmic surface is at the top, and TDG is shown as a Corey–Pauling–Koltun model at the apex of the inward-facing cavity. Movies of *A* and *B* are shown in SI Movies 2 and 3.

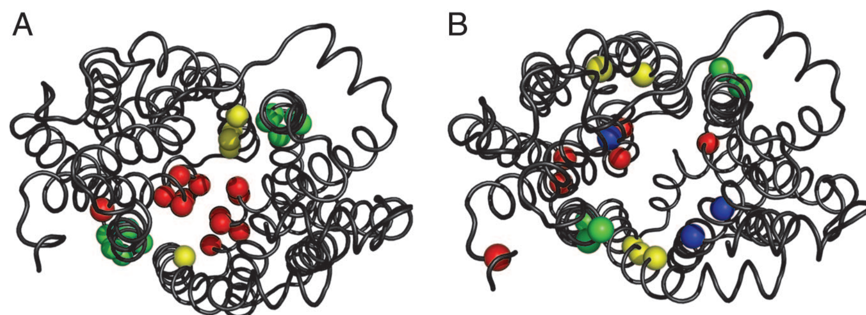

Fig. 3. Pseudosymmetrical distribution of Cys replacements in putative translocation pathway that exhibit increased or decreased reactivity with NEM. (*A*) Cytoplasmic view showing positions of Cys replacements exhibiting increased reactivity with NEM. Helices I and VII (red spheres), II and VIII (yellow spheres), and V and XI (green spheres) are shown. (*B*) Cytoplasmic view showing positions of Cys replacements exhibiting decreased reactivity with NEM. Helices I and VII (red spheres), II and VIII (yellow spheres), IV and X (blue spheres), and V and XI (green spheres) are shown. In both *A* and *B*, the positions of the Cys replacements that show altered activity are superimposed on the backbone of LacY in the inward-facing conformation.

changes suggests a more general change in the structure of the protein as well.

Symmetry. Focusing on the Cys replacements that exhibit ligand-induced reactivity changes in the middle portion of the LacY molecule, both types of replacements appear to be disposed in a pseudosymmetrical manner (Fig. 3). Therefore, the periplasmically disposed Cys replacements that exhibit increased reactivity in the presence of TDG are paired at the periplasmic ends of the following helices: I and VII (red spheres), II and VIII (yellow spheres), and V and XI (green spheres). Furthermore, the cytoplasmically disposed Cys replacements that exhibit decreased reactivity in the presence of ligand are also paired in a similar manner at the cytoplasmic ends of the following helices: I and VII (red spheres), II and VIII (yellow spheres), IV and X (blue spheres), and V and XI (green spheres). The findings suggest that the global conformational change occurring during turnover involves symmetrical movements of the N6 and C6 helix bundles.

Discussion

Findings derived from site-directed alkylation of Cys-replacement mutants at almost every position of LacY collected over more than 10 years (6, 15–22) are presented here in the context of the x-ray structure of LacY. In large measure, the observations are consistent with the alternating access model (13). According to this notion, a single sugar-binding site located in the approximate middle of the molecule at the apex of a deep hydrophilic cavity has alternating access to either side of the membrane as a result of reciprocal opening and closing of hydrophilic cavities on either side of the membrane. Although this idea is consistent with a number of observations (2, 13, 29), there is no direct evidence for either opening of a cavity on the periplasmic side of LacY or closure of the cavity on the cytoplasmic side.

The overall results of site-directed alkylation are consistent with the alternating accessibility hypothesis in that they provide a clear indication that binding of TDG increases the reactivity of Cys replacements located predominantly on the periplasmic side of the sugar-binding site. Furthermore, there is another group of Cys replacements located mainly on the cytoplasmic side of the sugar-binding site that exhibits decreased activity in the presence of ligand. The results are consistent with opening of a pathway for sugar on the periplasmic side of LacY that appears to be correlated with closure of the cytoplasmic cavity. Although opening and closing of these pathways are not necessarily coupled, it is notable that certain Cys replacements exhibiting ligand-induced increased or decreased reactivity are pseudosymmetrically disposed across the N- and C-terminal helix bundles of LacY. In addition, the Cys replacements at the periplasmic and cytoplasmic ends of the same helices exhibit increased or decreased ligand-induced reactivity, respectively.

In summary, the changes in reactivity observed here are consistent with opening of a new periplasmic, water-accessible cavity and closure of the cytoplasmic cavity (i.e., the alternating access model). However, the relatively broad distribution of these changes is indicative of a more global change in addition, which is consistent with many lines of evidence showing that ligand binding induces widespread structural changes in LacY (reviewed in ref. 2).

Materials and Methods

Materials. *N*-[*ethyl*-1-^{14}C]maleimide (40 mCi/mmol; 1 Ci = 37 GBq) was purchased from DuPont NEN (Boston, MA). ^{125}I-Protein A was from Amersham (Arlington Heights, IL). Immobilized monomeric avidin and avidin-conjugated horseradish peroxidase were from Pierce (Rockford, IL). Site-directed rabbit polyclonal antibody against a dodecapeptide corresponding to the C terminus of lac permease was prepared as described in ref. 30. All other materials were reagent grade and obtained from commercial sources.

Plasmid Construction. Construction of the single-Cys mutants and insertion of a DNA fragment encoding the biotin acceptor domain from a *Klebsiella pneumoniae* oxaloacetate decarboxylase either at the C terminus or in the middle cytoplasmic loop of the mutants by restriction fragment replacement has been described (7, 31).

Growth of Bacteria. *E. coli* T184 ($lacY^-Z^-$) transformed with plasmid pT7–5 encoding a given mutant was grown aerobically at 37°C in Luria–Bertani broth containing streptomycin (10 μg/ml) and ampicillin (100 μg/ml). Fully grown cultures were diluted 10-fold and grown for 2 h before induction with 0.5 mM isopropyl TDG. After additional growth for 2 h, cells were harvested and used for the preparation of RSO membrane vesicles.

Preparation of RSO Membrane Vesicles. RSO membrane vesicles were prepared from 1- to 4-liter cultures of *E. coli* T184 expressing a specified mutant by lysozyme-EDTA treatment and osmotic lysis (32, 33). The vesicles were resuspended to a protein concentration of 16–22 mg/ml in 100 mM potassium phosphate (KP_i; pH 7.3)/10 mM $MgSO_4$, frozen in liquid N_2, and stored at −80°C until use.

NEM Labeling. Alkylation with [^{14}C]NEM was performed essentially as described (17). RSO membrane vesicles [0.4–1.1 mg of protein in 50 μl of 100 mM KP_i (pH 7.3)/10 mM $MgSO_4$] harboring a single-Cys LacY mutant were incubated with [^{14}C]NEM (40 mCi/mmol; 0.4 mM final concentration) in the absence or presence of 10 mM TDG at 0 or 25°C. Labeling was terminated after 10 min by addition of 15 mM DTT, and the membranes were solubilized with

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2.0% (wt/vol; final concentration) *n*-dodecyl-β-D-maltopyranoside (DDM) for 5 min at 25°C. The DDM extract containing solubilized membranes was incubated with immobilized monomeric avidin-sepharose previously equilibrated in 50 mM KP_i (pH 7.3)/100 mM NaCl/0.02% DDM (wt/vol; equilibration buffer) for 30 min at 0°C. The resin was then extensively washed with equilibration buffer, and biotinylated LacY was eluted with 5 mM *d*-biotin in equilibration buffer. SDS/12% PAGE ($NaDodSO_4$/PAGE) followed by autoradiography was used to analyze NEM labeling. The relative amounts of autoradiographic bands were quantified with a STORM 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis. Fractions containing affinity-purified biotinylated LacY were analyzed by $NaDodSO_4$/PAGE (34). Protein was electroblotted onto poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore, Bedford, MA) and probed with either avidin-conjugated horseradish peroxidase or a site-directed polyclonal antibody against the C terminus of LacY (30). For detection of antibody binding, the PVDF membrane was subsequently incubated with ^{125}I-protein A (30 mCi/mg, 100 mCi/ml) and autoradiographed. Quantification of the relative amounts of permease was carried out with a STORM 860 PhosphorImager (Molecular Dynamics).

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