Probing the Conformation of the Lactose Permease of *Escherichia coli* by *in Situ* Site-Directed Sulfhydryl Modification†

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ABSTRACT: By using site-directed chemical labeling of lactose permease, conformational changes induced by ligand binding are observed in the native membrane of *Escherichia coli*. Membranes containing permease mutants with a single-Cys residue and a biotin-acceptor domain were labeled with radioactive *N*-ethylmaleimide (NEM) in the presence or absence of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) or a proton electrochemical gradient, followed by solubilization in *n*-dodecyl β-D-maltopyranoside and adsorption to avidin. TDG-induced enhancement of the reactivity of membrane-embedded Val315→Cys (helix X) permease is observed, while the reactivity of Val331→Cys (helix X) permease is inhibited by ligand binding or imposition of a proton electrochemical gradient. In contrast, the reactivity of permease with a single native Cys residue at position 148 (helix V) is blocked by TDG, but unaffected by the proton electrochemical gradient. Furthermore, as shown with right-side-out and inside-out membrane vesicles, the accessibility of Cys148 to either NEM or impermeant methanethiosulfonate derivatives is comparable from both sides of the membrane. On the other hand, TDG protects Cys148 from alkylation more effectively in right-side-out vesicles (apparent $K_D = 20-50$ μM) than inside-out vesicles (apparent K_D ca. 1.0 mM). The findings provide strong support for the conclusion that the permease retains close to native conformation in *n*-dodecyl-β-D-maltopyranoside. In addition, the results are consistent with the idea that lactose permease has two binding sites: one with higher affinity on the periplasmic surface of the membrane and another with lower affinity on the cytoplasmic surface.

The lactose (lac)[1] permease of *Escherichia coli* is a membrane transport protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ [i.e., symport or cotransport; reviewed in Kaback (1983, 1989, 1992) and Poolman and Konings (1993)]. Encoded by the *lacY* gene, this polytopic cytoplasmic membrane protein has been purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport as a monomer [see Sahin-Tóth et al. (1994)]. Based on circular dichroism and hydropathy analysis, a secondary structure was proposed in which the permease is composed of 12 hydrophobic α-helical domains that traverse the membrane in a zig-zag fashion connected by hydrophilic loops. A variety of experimental approaches [see Kaback (1983, 1989, 1992)], including an extensive series of lac permease−alkaline phosphatase (*lacY*−*phoA*) fusions (Calamia & Manoil, 1990), has provided unequivocal support for the 12-helix motif and demonstrated that the N and C termini are on the cytoplasmic face of the membrane. Furthermore, second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence have led to a model describing the packing of helices VII−XI in the C-terminal half of the permease [Jung et al., 1993; reviewed in Kaback et al. (1993, 1994)]. The model has been confirmed and extended recently by engineering divalent metal-binding sites (bis-His residues) within the permease (Jung et al., 1995; He et al., 1995a,b) and by site-directed chemical cleavage (Wu et al., 1995b).

Remarkably few amino acid residues in lac permease are essential for active transport, although widespread conformational changes involving movement of transmembrane helices are important in the mechanism [reviewed in Kaback et al. (1994) and Kaback (1996)]. Clearly, however, delineating the mechanism at the molecular level requires structure/function information at high resolution. In order to facilitate such studies on membrane proteins like lac permease which are inherently difficult to crystallize, molecular biological approaches are being used to engineer the permease for chemical and biophysical studies.

In particular, introduction of Cys at specified positions has provided a useful and versatile means of manipulation by allowing site-specific labeling with radioactive, fluorescent, or spin-labeled probes [see Kaback et al. (1993, 1994), Kaback (1996), Hubbell and Altenbach (1994), and Akabas et al. (1992)]. In this regard, a functional lac permease mutant devoid of Cys residues has been described (C-less permease), and almost all of the 417 residues in the permease have been mutagenized by Cys-scanning and/or site-directed mutagenesis [reviewed in Kaback et al. (1994) and Kaback

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; DM, *n*-dodecyl β-D-maltopyranoside; $\Delta\bar{\mu}_H^+$, the H^+ electrochemical gradient across the membrane; NEM, *N*-ethylmaleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $MTSEA^+$, methanethiosulfonate ethylammonium; $MTSES^-$, methanethiosulfonate ethylsulfonate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RSO, right-side-out; ISO, inside-out; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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(1996)]. In addition to providing evidence that very few residues are mandatory, the Cys-replacement mutants represent unique experimental tools for structure/function studies.

Both static and dynamic aspects of lac permease have been obtained from site-directed fluorescence with purified Cys-replacement mutants either in *n*-dodecyl β-D-maltopyranoside (DM) (Wu & Kaback, 1994; Wu et al., 1994, 1995a) or after reconstitution into proteoliposomes (Jung et al., 1994a,b). It has been documented that the permease undergoes widespread conformational changes upon interaction with ligand, enhancing the reactivity of molecules with single-Cys replacements for Pro28, Pro31, Gly147, Glu269, Val315, His322, or Val331. Moreover, the properties of V315C[2] permease suggest that ligand binding or a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) may cause the permease to assume a similar conformation (Sahin-Tóth & Kaback, 1993; Jung et al., 1994b). On the other hand, the reactivity of Cys148 permease is completely blocked by ligand, and the reactivity of M145C which is presumed to be on the same face of helix V is partially blocked (Wu & Kaback, 1994). These and other findings [see Jung et al. (1994c)] demonstrate that Cys148 is component of a binding site, interacting hydrophobically with the galactosyl moiety of the substrate, with Met145 on the periphery. Finally, studies with V331C permease provide further evidence for the argument that lac permease has at least two binding sites which differ in affinity by a factor of 20−30 [see Wu et al. (1994)].

Evidence confirming the spectroscopic findings, many of which were obtained with purified permease in *n*-dodecyl β-D-maltopyranoside (DM), is provided here by using site-directed sulfhydryl modification of single-Cys permease mutants in the native membrane. The reactivity of V315C or V331C permease is shown to be altered by the high-affinity ligand β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), while the reactivity of C148 permease is blocked by TDG. It is also demonstrated with impermeant sulfhydryl reagents that Cys148 is accessible from both sides of the membrane. However, TDG protection of Cys148 occurs with higher affinity from the periplasmic side of the membrane (apparent K_D 20−50 μM) than from the cytoplasmic side (apparent K_D ca. 1.0 mM). The results provide confirmation for the argument that lac permease maintains close to native structure in DM and are consistent with previous findings [see Wu et al. (1994)] suggesting that lac permease has at least two binding sites: a relatively high-affinity site on the periplasmic face and a low-affinity site on the cytoplasmic face.

MATERIALS AND METHODS

Materials. *N*-[*ethyl*-1-^{14}C]Ethylmaleimide (40 mCi/mmol) and N-[*ethyl*-2-^{3}H]-ethylmaleimide (56 Ci/mmol) were purchased from DuPont NEN (Boston, MA). [^{125}I]Protein A was from Amersham (Arlington Heights, IL). Immobilized monomeric avidin and Ultralink-immobilized neutrAvidin were from Pierce (Rockford, IL). Methanethiosulfonate ethylammonium ($MTSEA^+$) and methanethiosulfonate ethylsulfonate ($MTSES^-$) were the generous gifts of Arthur Karlin. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984) was prepared by BabCo (Richmond, CA). All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmid Construction. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*) *rspL Met*$^-$ *Thr*$^-$ *recA hsdM hsdR/F′ lacI*^{q}*O*$^+$*Z*^{D118} (*Y*$^+$*A*$^+$)] (Teather at al., 1980) harboring plasmid pKR35/*lacY*-L6XB (Consler et al., 1993) encoding wild-type lac permease, C-less lac permease (van Iwaarden et al., 1991), or C-less permease with given Cys substitutions with a biotin acceptor domain in the middle cytoplasmic loop was used for expression from the *lacZ* promoter/operator by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG). A cassette *lacY* gene (EMBL-X56095) encoding C-less permease in plasmid pT7-5 was used as a template for site-directed mutagenesis to construct single-Cys mutants C148 (Weitzman & Kaback, 1995), V315C (Sahin-Tóth & Kaback, 1993), or V331C (Sahin-Tóth & Kaback, 1993), and the mutants were transferred into plasmid pKR35/*lacY*-L6XB by restriction fragment replacement. Mutations were verified by sequencing the length of the inserted DNA fragment through the ligation junctions by using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulsen, 1978) after alkaline denaturation (Hattori & Sakaki, 1986).

Growth of Bacteria. *E. coli* T184 (*Z*$^-$*Y*$^-$) transformed with each plasmid described was grown aerobically at 37 °C in LB broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and allowed to grow for another 2 h before induction with 0.3 mM IPTG. After additional growth for 2 h at 37 °C, cells were harvested and used for preparation of membranes.

Membrane Preparations. Crude membrane fractions were prepared from 50 mL cultures of *E. coli* T184 by osmotic lysis and sonication as described (Frillingos et al., 1994). Right-side-out (RSO) membrane vesicles were prepared from 4 L cultures by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis (Kaback, 1971; Short et al., 1975). Inside-out (ISO) membrane vesicles were prepared from 10 L cultures by a single passage through a French Pressure cell (Aminco) at 8000 psi (Reenstra et al., 1980). Membrane vesicles were resuspended at a protein concentration of 12 mg/mL in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and stored at −80 °C until use.

Labeling with [^{14}C]NEM. Membranes (1.5 mg of protein in 65 μL) were incubated in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ containing 0.4 mM [1-^{14}C]NEM (40 mCi/mmol) at 25 °C. Reactions were quenched by addition of 5 mM dithiothreitol (DTT), and the membranes were solubilized with 2.0% DM (w/v) for 5 min. The DM−extract was then mixed with immobilized monomeric avidin−Sepharose that had been pretreated with 2 mM *d*-biotin (Consler et al., 1993) and equilibrated in 50 mM KP_i (pH 7.5)/150 mM NaCl/0.02% DM (w/v) (Wu & Kaback, 1994). After a 20-min incubation at 0−4 °C, the resin was washed sequentially with 10, 20, and 40 volumes of equilibration buffer, and biotinylated permease was eluted with 5 mM *d*-biotin in equilibration buffer, followed by electrophoresis and autoradiography. Quantitation of the relative amounts of the autoradiographic

[2] Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type lac permease, and then a second letter denoting the amino acid replacement at this position. The permease mutant containing a single native Cys at position 148 is designated Cys148.

bands was performed with a Model 425F PhosphorImager (Molecular Dynamics).

Labeling with [^{3}H]NEM. Membrane vesicles (0.9 mg of protein in 100 μL) were incubated with 0.4 mM [2-^{3}H]NEM (560 mCi/mmol) at 28 °C. At given times, the reaction was terminated by addition of 5 mM DTT, and the membranes were solubilized with DM as described above. The DM extract was mixed with preequilibrated neutrAvidin−Sepharose, and the mixture was incubated at 0−4 °C, for 1 h. The resin was washed 4 times with 10 volumes of equilibration buffer [i.e., 50 mM KP_i (pH 7.5)/150 mM NaCl/0.02% DM (w/v)] followed by 6 times with 10 volumes of 2.5 M guanidine hydrochloride (pH 4.5). Finally, the resin was suspended in 100 μL of 8 M guanidine hydrochloride (pH 1.5), transferred into 10 mL of scintillation fluid (CytoScint; ICN Biomedicals), and assayed by liquid scintillation spectrometry.

Western Blot Analysis. Fractions containing biotinylated lac permease were analyzed electrophoretically on a 12% sodium dodecyl sulfate ($NaDodSO_4$)−polyacrylamide gel (Newman et al., 1981). Protein was electroblotted to poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984). The PVDF membrane was subsequently incubated with horseradish peroxidase-conjugated protein A (Amersham) and finally developed with fluorescent substrate (Renaissance; DuPont NEN) before exposure to film. Alternatively, after treatment with the anti-C-terminal antibody, the blot was incubated with [^{125}I]protein A (30 mCi/mg, 100 μCi/mL) and autoradiographed, and the amount of permease was quantitated with a Model 425F PhosphorImager (Molecular Dynamics) using purified biotinylated lac permease as standard (Sun et al., 1996).

Protein Determinations. Protein was assayed in the presence of $NaDodSO_4$ (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Specificity of N-Ethylmaleimide. Initially, the membrane permeant thiol reagent *N*-ethylmaleimide (NEM) was used to alkyate Cys in crude membranes containing wild-type lac permease, C-less permease, or given single-Cys mutants. Under the conditions employed, C-less lac permease is not labeled significantly even after exposure to NEM for 1 h in the absence or presence of ligand (Figure 1, lanes 3, 4), while wild-type lac permease with eight native Cys residues (lanes 1, 2) or the single-Cys mutants V315C (lane 6) and Cys148 (lane 7) are readily labeled after 10-min incubation with [^{14}C]-NEM under the conditions described. Therefore, NEM is highly specific for Cys, and no significant reactivity with amino or imidazole groups is observed (Smyth et al., 1964).

Effect of Ligand on the Reactivity of Wild-Type Permease or Single-Cys Mutants. Crude membranes with wild-type or single-Cys permease mutants were incubated with [^{14}C]-NEM in the absence or presence of the high-affinity ligand TDG. Although it is not readily apparent from the data shown (Figure 1, lanes 1−2), in the presence of TDG, the reactivity of wild-type lac permease is slightly attenuated, since only Cys148 of the eight native Cys residues is protected by ligand (Beyreuther et al., 1981). On the other hand, the reactivity of single-Cys148 permease is totally

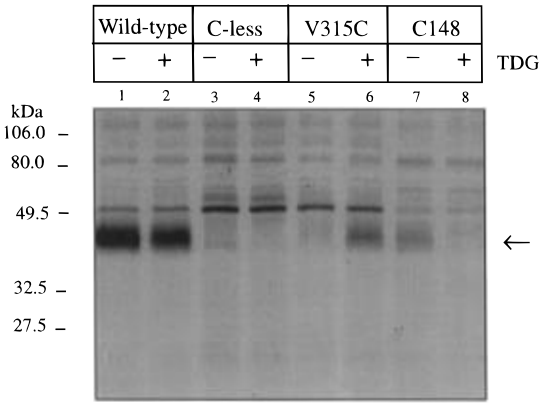

FIGURE 1: Effect of TDG on the reactivity of wild-type, C-less, V315C, and C148 permeases with [^{14}C]NEM in crude membranes. Membranes (1.5 mg of protein in 250 μL) prepared from *E. coli* T184 transformed with pKR35/cassette *lacY* L6XB (lanes 1, 2), pKR35/C-less cassette *lacY* L6XB (lanes 3, 4), pKR35/V315C *lacY* L6XB (lanes 5, 6), or pKR35/C148 *lacY* L6XB (lanes 7, 8) were labeled with 0.4 mM [1-^{14}C]NEM in the absence or presence of 10 mM TDG. The reactions were quenched at 60 min (lanes 3, 4) or 10 min (all other lanes), and biotinylated permease was solubilized and purified as described under Materials and Methods. Aliquots containing 8 μg of protein were separated by 12% $NaDodSO_4$−polyacrylamide gel electrophoresis, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography. The positions of molecular mass markers phosphorylase B (106.0 kDa), bovine serum albumin (80.0 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), and soybean trypsin inhibitor (27.5 kDa) are shown at the left. The arrow at the right denotes the position of purified C148-L6XB permease.

blocked by TDG (lanes 7−8). In contradistinction, the reactivity of V315C permease is markedly enhanced by ligand (Figure 1, lanes 5−6; see Figure 2 in addition), in agreement with previous studies (Sahin-Tóth & Kaback, 1993; Jung et al., 1994b). Finally, the reactivity of V331C permease (helix X) is inhibited by TDG both in crude membranes (not shown) and in RSO vesicles (Figure 2), which is consistent with the finding that TDG protects V331C permease from NEM inactivation [see Figure 2B in Wu et al. (1994)].

Distinction between Steric and Conformational Effects. As discussed previously (Wu et al., 1994), the effect of TDG on the reactivity of V331C permease with NEM may be interpreted in either of the following ways: (i) Val331 is a binding site residue that interacts directly with ligand; or (ii) the reactivity of Val331 is altered by a ligand-induced conformational change. Wu et al. (1994) demonstrated by site-directed fluorescence with purified V331C permease in DM that changes in the reactivity of V331C permease on addition of ligand are conformationally induced. Nonetheless, in order to differentiate between the two possibilities *in situ*, the reactivity of V331C permease was compared with that of Cys148 permease, since it has been shown clearly (Jung et al., 1994c; Wu & Kaback, 1994) that TDG protection of Cys148 is due to a direct steric effect. The reactivity of V331C permease is inhibited by either TDG or $\Delta\bar{\mu}_H^+$ (Figure 3B,D). In contrast, the reactivity of Cys148 permease is completely blocked in the presence of TDG, but unaffected by generation of $\Delta\bar{\mu}_H^+$ (Figure 3A,C). The data extend previous observations (Wu et al., 1994) indicating that the Cys at position 331 in V331C permease does not interact with ligand directly but is altered by a ligand-induced conformational change. Furthermore, the observations are

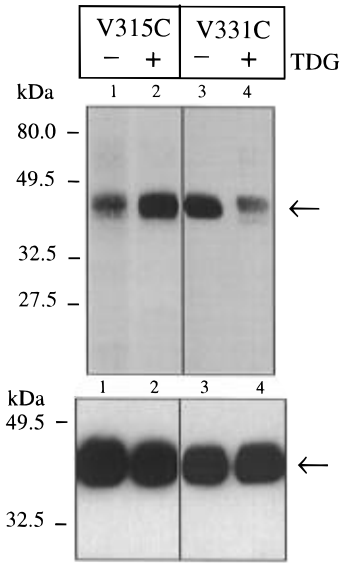

FIGURE 2: Effect of TDG on the reactivity of V331C and V315C permeases with [^{14}C]NEM in RSO membrane vesicles. RSO membrane vesicles (1.5 mg of protein in 65 μL) prepared from *E. coli* T184 transformed with pKR35/V315C *lacY* L6XB (lanes 1, 2) or pKR35/V331C *lacY* L6XB (lanes 3, 4) were incubated with 0.4 mM [1-^{14}C]NEM in the absence or presence of 10 mM TDG. The reactions were quenched with DTT at 10 min, and biotinylated permease was solubilized and purified as described under Materials and Methods. Aliquots containing 15 μg of protein were separated by 12% NaDodSO$_4$-polyacrylamide gel electrophoresis, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography (upper panel). A fraction of the eluted molecules (2 μg of protein) was analyzed on Western blot with anti-C-terminal antibody (lower panel). The positions of molecular mass markers are shown at the left. The arrow at the right denotes the migration position of pure C148-L6XB permease.

consistent with the contention that the permease retains close to native conformation in DM.

Cys148 Is Accessible from Both Sides of the Membrane. The low specific activity of [^{14}C]NEM and the multiple contaminating bands obtained with crude membrane fractions (Figure 1) make rate measurements difficult without electrophoresis and autoradiography. On the other hand, with purified membrane vesicles, background contamination is minimal (compare Figures 1 and 2). Therefore, the original protocol was modified. Briefly, the specific activity of the probe was increased by using [^{3}H]NEM, and neutrAvidin which is multivalent was used in place of monomeric avidin so that nonspecific binding could be minimized by extensive washing with guanidine prior to liquid scintillation spectrometry (see Materials and Methods).

As shown in Figure 4, RSO and ISO vesicles containing biotinylated Cys148 permease react with membrane-permeant [^{3}H]NEM at comparable rates, labeling rapidly for about the first 2 min and achieving completion in about 10 min. The low stoichiometry observed (0.10−0.12 mol of NEM/mol of permease) is apparent, as only about 20% of the permease is biotinylated *in vivo* (Consler et al., 1993; Privé et al., 1994; Privé & Kaback, 1995), and small losses occur during washing of the neutrAvidin. In any case, addition of 10 mM TDG almost completely blocks Cys148 reactivity with vesicles of either orientation.

The effect of the charged, impermeant thiol reagents MTSEA$^+$ or MTSES$^-$ (Akabas et al., 1992; Stauffer & Karlin, 1994) was tested in order to investigate the accessibility of Cys148 from either surface of the membrane. Thus,

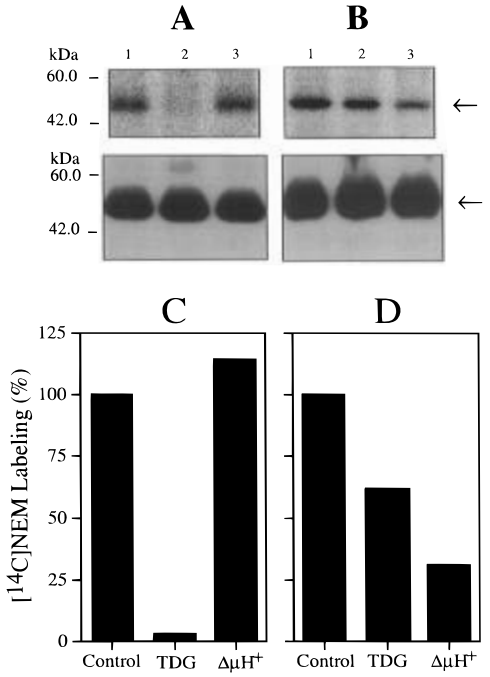

FIGURE 3: Comparison of the effect of $\Delta\bar{\mu}_{H^+}$ or TDG on the reactivity of single-Cys148 and V331C permeases. RSO membrane vesicles (1.5 mg of protein in 65 μL) prepared from *E. coli* T184 transformed with pKR35/C148 *lacY* L6XB (A) or pKR35/V331C *lacY* L6XB (B) were labeled with 0.4 mM [1-^{14}C]NEM alone (lane 1), in the presence of 10 mM TDG (lane 2), or in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate under oxygen (Konings et al., 1971) to generate a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline; lane 3). The reactions were quenched at 10 min, and biotinylated permease was solubilized, purified, and analyzed by autoradiography (upper panels) and Western blotting (lower panels) exactly as in Figure 2. The amount of permease labeled with [^{14}C]NEM as shown in the upper panels was quantitated with a PhosphorImager and normalized for the total amount of permease determined by quantitative Western blots as shown in the lower panels. The quantitative data are presented as percentages of the amount measured in the absence of TDG or $\Delta\bar{\mu}_{H^+}$ (lane 1) for either C148-L6XB (C) or V331C-L6XB permease (D).

RSO or ISO membrane vesicles were pretreated with a given methanethiosulfonate derivative for the times indicated and then exposed to [^{3}H]NEM for 20 min in order to determine the amount of unreacted Cys148 remaining (Figure 5). With both reagents, the rate of reaction of Cys148 in RSO or ISO vesicles approximates linearity with time in semilog plots. MTSEA$^+$ at 20 μM exhibits a rate constant of about 0.022 min^{-1}, while MTSES$^-$ at 200 μM inactivates with a rate constant of about 0.038 min^{-1}. The difference is consistent with the finding that MTSEA$^+$ is inherently about 4 times more reactive than MTSES$^-$ (Stauffer & Karlin, 1994). In any event, it is remarkable that the reactivity of Cys148 with either impermeant thiol reagent is similar in RSO and ISO vesicles, indicating that the residue is accessible from both surfaces of the membrane.

TDG Protection of Cys148 Is More Effective from the Outside Surface of the Membrane. Several lines of evidence (Kaczorowski et al., 1979; Lolkema & Walz, 1990; Lolkema et al., 1991; van Iwaarden et al., 1993; Wu et al., 1994) support the postulate that lac permease contains at least two binding sites which differ in affinity by a factor of 30−50, and it has been suggested that the high- and low-affinity sites are on the periplasmic and cytoplasmic surfaces of the membrane, respectively. Therefore, the ability of TDG to

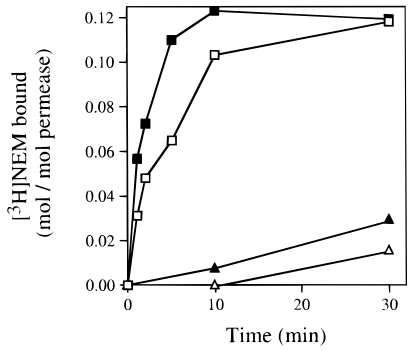

FIGURE 4: Time course of labeling of single-Cys148 permease with [^{3}H]NEM in oriented membrane vesicles. RSO (open symbols) or ISO (closed symbols) membrane vesicles (0.9 mg of protein in 100 μL) prepared from *E. coli* T184 transformed with pKR35/C148 *lacY* L6XB were labeled with 0.4 mM [2-^{3}H]NEM in the absence (squares) or presence (triangles) of 10 mM TDG. The reactions were quenched at given times, and biotinylated permease was solubilized and bound to neutrAvidin. After removal of nonspecifically bound material by extensive washing, the neutrAvidin−permease complexes were solubilized, and radioactivity was assayed as described under Materials and Methods. Results were corrected for binding measured at each time point with the corresponding membrane vesicles prepared from *E. coli* T184 transformed with pKR35/C-less *lacY* L6XB, and the amount of specific binding was normalized for the amount of C148-L6XB permease present in each vesicle preparation as determined by quantitative Western blotting (data not shown). The lac permease content per milligram of membrane protein was 10.2 μg (RSO C148-L6XB vesicles), 9.0 μg (ISO C148-L6XB vesicles), 6.5 μg (RSO C-less−L6XB vesicles), or 11.6 μg (ISO C-less−L6XB vesicles). The maximal binding measured for single-Cys148 permease at 30 min in the absence of ligand was 30 fmol of [^{3}H]NEM/mg of protein (RSO vesicles) or 35 fmol of [^{3}H]NEM/mg of protein (ISO vesicles), while nonspecific binding measured at 30 min with C-less permease was 6 fmol of [^{3}H]NEM/mg of protein or 10 fmol of [^{3}H]NEM/mg of protein, respectively.

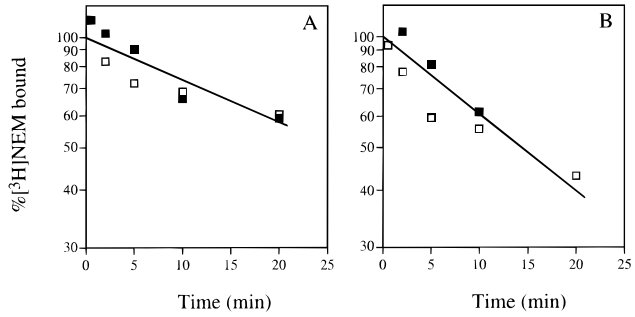

FIGURE 5: Accessibility of single-Cys148 permease to MTSEA$^+$ (A) or MTSES$^-$ (B). RSO (open symbols) or ISO (closed symbols) membrane vesicles (0.9 mg of protein in 100 μL) prepared from *E. coli* T184 transformed with pKR35/C148*lacY* L6XB were incubated with 20 μM MTSEA$^+$ (A) or 200 μM MTSES$^-$ (B) for given times and labeled with 0.4 mM [2-^{3}H]NEM. The reactions were quenched at 20 min, and radiolabeled biotinylated permease was assayed with neutrAvidin as described in Figure 4. Results were corrected for binding measured with the corresponding membrane vesicles prepared from *E. coli* T184 transformed with pKR35/C-less *lacY* L6XB. In both cases, maximal binding measured for single-Cys148 untreated with methanethiosulfonate derivatives was approximately 25 fmol of [^{3}H]NEM/mg of protein, while nonspecific binding measured with C-less permease was 7 fmol of [^{3}H]NEM/mg of protein.

block the NEM reactivity of Cys148 was studied as a function of ligand concentration in RSO and ISO membrane vesicles (Figure 6). In RSO vesicles, NEM reactivity decreases markedly from 0.001 mM to about 1.0 mM TDG, and half-maximal inhibition is observed at 0.02−0.05 mM.

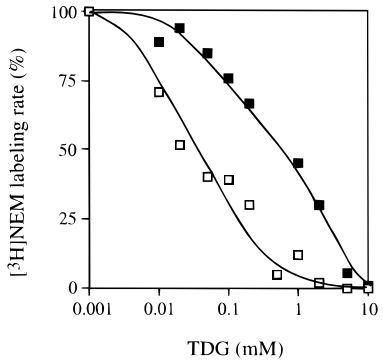

FIGURE 6: Titration of the TDG effect on the reactivity of single-Cys148 permease in RSO or ISO vesicles. RSO (open symbols) or ISO (closed symbols) membrane vesicles (0.9 mg of protein in 100 μL) prepared from *E. coli* T184 transformed with pKR35/C148 *lacY* L6XB were labeled with 0.4 mM [2-^{3}H]NEM in the presence of TDG at given concentrations. The reactions were quenched at 2 min, and radiolabeled biotinylated permease was assayed with neutrAvidin as described in Figure 4. Results were corrected for binding measured at 2 min with the corresponding membrane vesicle population prepared from *E. coli* T184 transformed with pKR35/C-less *lacY* L6XB. The binding measured for single-Cys148 permease in the absence of ligand was 14 fmol of [^{3}H]NEM/mg of protein (RSO vesicles) or 17 fmol of [^{3}H]NEM/mg of protein (ISO vesicles), while nonspecific binding measured with C-less permease was 3 fmol of [^{3}H]NEM/mg of protein or 4 fmol of [^{3}H]NEM/mg of protein, respectively. Values are the average of three independent measurements.

In ISO vesicles, little or no inhibition is observed up to about 0.05 mM TDG, and reactivity is essentially completely blocked at 7−10 mM TDG, with half-maximal inhibition observed at close to 1.0 mM. Thus, the apparent K_Ds for TDG are 20−50 μM or ca. 1.0 mM in RSO or ISO vesicles, respectively, a difference that is in good agreement with the previous studies.

DISCUSSION

Site-directed spectroscopy with Cys-replacement mutants has been developed recently as a means of studying structure/function relationships in purified lac permease solubilized in DM (Wu & Kaback, 1994; Wu et al., 1994, 1995a; Voss et al., 1995a,b) or after reconstitution into proteoliposomes (Jung et al., 1993, 1994a,b). A novel site-directed chemical modification assay is described here that allows the reactivity and/or accessibility of Cys residues in lac permease to be probed *in situ*, and a number of the observations obtained with purified single-Cys permease mutants have now been confirmed. Thus, TDG-induced enhancement of the reactivity of membrane-embedded V315C permease is observed, while the reactivity of V331C permease is inhibited by ligand binding or imposition of $\Delta\bar{\mu}_H^+$. In contrast, the reactivity of permease with a single native Cys residue at position 148 is blocked by TDG, but unaffected by $\Delta\bar{\mu}_H^+$. On one level, therefore, the data presented here argue strongly for the physiological significance of the site-directed spectroscopic approach with purified permease mutants and also provide additional support for the contention that the permease maintains close to native conformation when solubilized in DM.

NEM, one of the sulfhydryl reagents used here, irreversibly inactivates lac permease, and protection against inactivation is afforded by ligands such as TDG (Fox & Kennedy, 1965) which led originally to the hypothesis that a Cys residue in

lac permease is in or near a binding site. The substrate-protectable residue was later identified as Cys148 (Beyreuther et al., 1981), and subsequently, site-directed mutagenesis demonstrated that Cys148 is not essential for lactose/H^+ symport (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986). These observations and more recent findings (Wu & Kaback, 1994; Wu et al., 1994, 1995a; Jung et al., 1994a,b) demonstrating that many single-Cys permease mutants exhibit marked changes in reactivity in the presence of ligand cast doubt on the idea that Cys148 interacts directly with substrate. However, kinetic studies with a variety of amino acid replacements for Cys148 (Jung et al., 1994c) coupled with site-directed fluorescence spectroscopy (Wu & Kaback, 1994) provide sound support for the original postulate of Fox and Kennedy (1965). As shown here, the reactivity of membrane-embedded single-Cys148 permease is completely abrogated by TDG. Although the protective effect of ligand is also observed with other alkylating agents [e.g., 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS; Wu & Kaback, 1994) or *N*-iodoacetyltyrosine and *N*-bromoacetyltyramine (S. Frillingos, D. Sigman, and H. R. Kaback, unpublished results)], the reactivity of Cys148 is completely unaffected by $\Delta\bar{\mu}_{H^+}$ [see Cohn et al. (1981) and Viitanen et al. (1985) in addition].

It is particularly noteworthy that Cys148 in lac permease appears to be accessible from the aqueous phase on both sides of the membrane. Thus, $MTSEA^+$ or $MTSES^-$, each of which is highly water-soluble (Akabas et al., 1992; Stauffer & Karlin, 1994), reacts with Cys148 in both RSO and ISO vesicles at comparable rates, although $MTSEA^+$ is approximately 5-fold more effective. Since Cys148 interacts hydrophobically with the galactosyl moiety of the substrate (Jung et al., 1994c; Wu & Kaback, 1994), the findings clearly imply that at least part of the substrate-binding site in the permease is accessible to solvent from both sides of the membrane. This conclusion is also consistent with the effect of ambient pH on the transport activity of C148D permease (Jung et al., 1994c) and with the observation that iodide effectively quenches the fluorescence of MIANS-labeled single-Cys148 permease (Wu & Kaback, 1994).

Although Cys148 appears to be accessible to thiol reagents from both surfaces of the membrane, TDG protects against alkylation by NEM more effectively from the periplasmic surface than from the cytoplasmic surface, exhibiting an apparent K_D of 20−50 μM in RSO vesicles and an apparent K_D of ca. 1.0 mM in ISO vesicles. Since Cys148 is accessible to NEM which is permeant, as well as $MTSEA^+$ and $MTSES^-$ which are impermeant, the difference in apparent affinities for TDG cannot be attributed to differences in accessibility. Rather, the data indicate that lac permease has at least two binding sites for substrate: a relatively high-affinity site on the periplasmic surface and a low-affinity site on the cytoplasmic surface. Initial evidence that the permease may contain two binding sites was provided by studies in which the kinetics of lactose influx and efflux were studied (Kaczorowski et al., 1979; Robertson et al., 1980), and it was observed that the apparent affinity for influx increases about 50-fold from about 20 mM to 0.4 mM in the presence of $\Delta\bar{\mu}_{H^+}$, while the apparent affinity for efflux remains constant at about 2−3 mM. Subsequently, Lolkema and Walz (1990) demonstrated that *p*-nitrophenyl α-D-galactopyranoside binding exhibits both a high- and a low-affinity K_D, and the idea received additional support from kinetic studies of equilibrium exchange in proteoliposomes reconstituted with purified permease (Lolkema et al., 1991; van Iwaarden et al., 1993). More recently, Wu et al. (1994) utilized site-directed fluorescence spectroscopy of V331C permease to obtain more direct evidence for the notion of two binding sites. With MIANS-labeled V331C permease in DM, fluorescence quenching studies suggest a high-affinity binding site for TDG with an apparent K_D of about 0.12 mM. In contrast, a blue-shift in the emission maximum of the MIANS-labeled protein is observed with an apparent K_D of 3−5 mM that corresponds to the apparent K_D obtained for TDG enhancement of MIANS reactivity (Wu et al., 1994). Clearly, at first approximation, the differences observed in the apparent K_Ds for ligand correspond to the high- and low-affinity sites on the periplasmic and cytoplasmic surfaces of the membrane, respectively, described here. As discussed by Wu et al. (1994), the findings as a whole have important potential implications with respect to the mechanism of transport. If the high- and low-affinity sites are on opposite sides of the membrane, as indicated by these studies, and both sites can be occupied simultaneously, the generally accepted model of transporters with a single site that is accessible alternatively from either side of the membrane and exhibits strong negative cooperativity should be questioned. However, it should be emphasized that although the present studies localize the high- and low-affinity sites to opposite sides of the membrane, it is not possible at present to determine with certainty whether or not the two sites can or cannot be occupied simultaneously.

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