Binding of Ligand or Monoclonal Antibody 4B1 Induces Discrete Structural Changes in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: By using Cys-scanning mutagenesis with site-directed sulfhydryl modification *in situ* [Frillingos, S., & Kaback, H. R. (1996) *Biochemistry 35*, 3950−3956], conformational changes induced by binding of ligand or monoclonal antibody (mAb) 4B1 in the lactose permease of *Escherichia coli* were studied. Out of 31 single-Cys replacement mutants in helices I, V, VII, VIII, X, or XI, 4B1 binding alters the reactivity of Val238→Cys (helix VII), Val331→Cys (helix X), or single-Cys355 (helix XI) permease with *N*-ethylmaleimide (NEM) in right-side-out membrane vesicles. In addition, site-directed fluorescence spectroscopy shows that mAb 4B1 binding causes position 331 (helix X) in the permease to experience a more hydrophobic environment. In contrast, ligand binding elicits more widespread changes, as evidenced by enhancement of the NEM reactivity of Ala244→Cys, Thr248→Cys (helix VII), Thr265→Cys (helix VIII), Val315→Cys (helix X), Gln359→Cys, or Met362→Cys (helix XI) permease, none of which are altered by 4B1 binding. Furthermore, no effect of 4B1 is observed on the reactivity of Cys148 (helix V), Val264→Cys, Gly268→Cys, or Asn272→Cys (helix VIII), positions which probably make direct contact with substrate. With respect to the N-terminal half of the permease, 4B1 binding causes a small increase in the reactivity of mutants Pro28→Cys or Pro31→Cys (helix I), while ligand binding causes much greater increases in reactivity. The findings indicate that 4B1 binding induces a structural change in the permease that is much less widespread than that induced by ligand binding.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic membrane transport protein encoded by the *lacY* gene. The permease has been solubilized from the membrane, purified, reconstituted, and shown to be solely responsible for the coupled stoichiometric translocation of β-galactosides and H^+ as a monomer [reviewed in Kaback et al. (1994) and Kaback (1996)]. All available evidence indicates that the protein is composed of 12 α-helical rods that traverse the membrane in a zigzag fashion with both N and C termini in the cytosolic side. Moreover, site-directed excimer fluorescence, site-directed mutagenesis, and second-site suppressor studies have led to a helix-packing model [Figure 6; see Kaback et al. (1994) and Kaback (1996)]. The model has been confirmed and extended by engineering divalent metal-binding sites (bis- or tris-His residues) between transmembrane domains (Jung et al., 1995; He et al., 1995a,b), site-directed chemical cleavage (Wu et al., 1995a), site-directed spin-labeling, and thiol cross-linking (Wu et al., 1996b; Wu & Kaback, 1996), and by the demonstration that the last two cytoplasmic loops comprise a discontinuous epitope for monoclonal antibody (mAb) 4B11 (Sun et al., 1997).

Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) reveal that remarkably few residues are directly involved in the transport mechanism [reviewed in Kaback et al. (1994) and Kaback (1996)]. In addition, the activity of certain active Cys-replacement mutants is altered by alkylation, and these mutants appear in clusters, suggesting that interfaces within the permease are important for substrate binding and/or the conformational changes associated with turnover. Site-directed fluorescence spectroscopy (Jung et al., 1994a,b; Wu & Kaback, 1994; Wu et al., 1994, 1995b) and site-directed sulfhydryl modification *in situ* (Frillingos & Kaback, 1996c, 1997a; Frillingos et al., 1997c) also show that ligand binding alters the reactivity of individually placed Cys residues in helices I, V, VIII, or X, indicating that widespread conformational changes occur within the permease.

Many observations indicate that the C-terminal half of the permease plays a more direct role in the transport mechanism than the N-terminal half. Significant downhill transport activity is retained in mutants deleted of helices I−VI (Bibi et al., 1991; Wu et al., 1996a). With almost all of the residues in the permease mutagenized [see Kaback et al. (1994) and Kaback (1996)], the four charged residues found to be irreplaceable with respect to active transport are located in helices VIII, IX, and X. Furthermore, the epitope for mAb 4B1 which uncouples lactose from H^+ translocation has been localized to the periplasmic loop between helices VII and

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; mAb, monoclonal antibody; NEM, *N*-ethylmaleimide; MTSES, methanethiosulfonate ethylsulfonate; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; DM, *n*-dodecyl β-D-maltopyranoside; $NaDodSO_4$, sodium dodecyl sulfate.

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VIII (loop VII/VIII; Sun et al., 1996). On the other hand, the N-terminal 22 amino acid residues can be deleted from the permease without abolishing activity (Bibi et al., 1992), and no essential residues have been found thus far in the N-terminal half, as judged by site-directed and Cys-scanning mutagenesis [Kaback et al., 1994; Kaback, 1996; see Frillingos and Kaback (1996a) and Frillingos et al. (1997b) in addition].

Monoclonal antibody 4B1 blocks all reactions catalyzed by the permease which involve net H^+ translocation with no effect on equilibrium exchange or counterflow (Carrasco et al., 1982, 1984b; Herzlinger et al., 1984). None of the amino acid residues in periplasmic loops, particularly loop VII/VIII, play a direct role in the transport mechanism (Sun et al., 1996). Furthermore, avidin binding to a biotinylated Cys residue in loop VII/VIII has no effect on transport. Therefore, it was suggested that binding of the mAb exerts a torsional effect, causing a change in helices VII and/or VIII that alters the pK_as of residues critically involved in lactose-coupled H^+ translocation. In this regard, evidence has been presented (Frillingos & Kaback, 1996b) suggesting that 4B1 may alter the pK_a of an Asp residue in place of Glu325 (helix X), one of the four irreplaceable residues. Since equilibrium exchange and counterflow represent translocation reactions in which the permease is saturated with substrate on both sides of the membrane, and mAb 4B1 has no effect on these reactions, the possibility arises the permease might assume a similar conformation in the presence of ligand or mAb 4B1.

In this paper, Cys-scanning mutagenesis is used in conjunction with site-directed thiol modification in right-side-out (RSO) membrane vesicles (Frillingos & Kaback, 1996c) to examine dynamic changes induced by binding of ligand or mAb 4B1. The findings suggest that the permease is highly flexible and that ligand binding or binding of mAb 4B1 induces different conformational states.

MATERIALS AND METHODS

Materials. *N*-[*ethyl*-1-^{14}C]Ethylmaleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, MA). Methanethiosulfonate ethylsulfonate (sodium salt) was from Toronto Research Chemicals (Ontario, Canada). [^{125}I]Protein A was from Amersham (Arlington Heights, IL). Immobilized monomeric avidin was purchased from Pierce (Rockford, IL). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984a) 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 et al., 1980) harboring plasmid pKR35/*lacY*-L6XB or plasmid pKR35/*lacY*-CXB (Consler et al., 1993) encoding given single-Cys mutants with a biotin acceptor domain in the middle cytoplasmic loop (L6XB) or at the C terminus (CXB) 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 all single-Cys mutants. Mutants P28C,[2] P31C (Sahin-Tóth et al., 1994), single-Cys148 (Weitzman & Kaback, 1995), E255C (Frillingos et al., 1994), V315C, V316C, M323C, E325C, L330C, V331C (Sahin-Tóth & Kaback, 1993), F354C, C355, Q359C, M362C, or S366C (Dunten et al., 1993) were transferred to plasmid pKR35/*lacY*-L6XB, and mutants Y236C, V238C, F243C, A244C, F247C, T248C (Frillingos et al., 1994), G262C, V264C, T265C, G268C, E269C, N272C, A273C, M276C, F277C, or A279C (Frillingos et al., 1997b) were transferred to plasmid pKR35/*lacY*-CXB by restriction fragment replacement. Mutations were verified by sequencing the length of the inserted DNA fragment through the ligation junctions using dideoxynucleotide termination (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986).

Growth of Bacteria. *E. coli* T184 (*Z*$^-$*Y*$^-$) transformed with each plasmid was grown aerobically at 37 °C in Luria−Bertani medium 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 IPTG (0.3 mM). After additional growth for 2 h at 37 °C, cells were harvested and used for preparation of membranes.

Membrane Preparation. Right-side-out (RSO) membrane vesicles were prepared from *E. coli* T184 expressing a given mutant by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis (Kaback, 1971; Short et al., 1975).

Labeling with [^{14}C]NEM. Modification with [1-^{14}C]NEM was performed essentially as described (Frillingos & Kaback, 1996c). Briefly, RSO membrane vesicles (0.3 mg of protein in 50 μL) were incubated in 100 mM potassium phosphate (KP_i) (pH 7.5)/10 mM $MgSO_4$ containing 0.4 mM [^{14}C]NEM (40 mCi/mmol), in the absence or presence of 10 mM TDG at 25 °C. Labeling was terminated by addition of 5 mM DTT, and membranes were treated with 2.0% (w/v) *n*-dodecyl β-D-maltopyranoside (DM) for 5 min. The DM extract was then incubated with immobilized monomeric avidin to purify biotinylated permease. After extensive washing, biotinylated permease was eluted from the resin with 5 mM *d*-biotin and electrophoresed, and [^{14}C]NEM labeling was analyzed by autoradiography. Quantitation was performed with a Model 425F PhosphorImager (Molecular Dynamics).

To assess the effect of mAb 4B1 on NEM reactivity, RSO vesicles containing a given single-Cys mutant were diluted to a protein concentration of 0.6 mg/mL in 0.5 mL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ containing 50 μL of affinity-purified mAb 4B1 (5 mg/mL; Sun et al., 1996) and incubated at room temperature for 45 min. Control samples were incubated in 0.5 mL of 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ with no further additions. After incubation, the vesicles were washed with ice-cold buffer and resuspended to a protein concentration of 6 mg/mL before initiation of [^{14}C]NEM labeling. The samples were then treated as described above.

Western Blot Analysis. Fractions containing biotinylated lac permease were analyzed electrophoretically on sodium dodecyl sulfate ($NaDodSO_4$)−12% polyacrylamide gels

[2] For designation of single-Cys permease mutants, the first letter denotes the amino acid present in the wild-type permease at the numbered position, and the final letter denotes the Cys residue at the corresponding position of the mutant.

(Newman et al., 1981). Protein was electroblotted on 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., 1984a). 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 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 as described (Sun et al., 1996; Frillingos & Kaback, 1996c).

Purification of V331C Permease, Labeling with 2-(4-Maleimidoanilino)naphthalene-6-sulfonic Acid (MIANS), and Fluorescence Measurements. V331C permease with a biotin acceptor domain in the middle cytoplasmic loop (V331C-L6XB) was purified and labeled with MIANS (Molecular Probes, Inc.) as described (Wu et al., 1994). Emission spectra of MIANS-labeled V331C permease were recorded at 30 °C in an SLM 8000C spectrofluorometer (SLM-Aminco Instruments Inc., Urbana, IL) with an excitation wavelength of 330 nm and a 4-nm slit for both excitation and emission. Where indicated, mAb 4B1 was added at a molar ratio of 1:1 relative to the permease.

RESULTS

Helix X. Site-directed Cys modification and fluorescence studies with V331C permease (Wu et al., 1994; Frillingos & Kaback, 1996c) demonstrate that position 331 (helix X) participates in ligand-induced conformational changes. In order to test the possibility that 4B1 binding also perturbs V331C permease, the reactivity of V331C permease was examined after preincubation with mAb 4B1. As shown in Figure 1A, V331C permease is readily labeled after 10-min incubation with NEM, and addition of TDG or 4B1 elicits a 35% or 75% decrease in labeling, respectively (Table 1). In addition, when V331C permease is solubilized, purified in DM, and labeled with MIANS, mAb 4B1 causes an increase in fluorescence and a 5 nm blue-shift in the emission maximum (Figure 1B).

A dramatic increase (at least 6-fold) in the reactivity of V315C permease with NEM is observed in the presence of TDG [see Sahin-Tóth and Kaback (1993), Jung et al. (1994a), and Frillingos and Kaback (1996c)]; however, 4B1 causes no significant change in reactivity (Table 1). Furthermore, no effect of TDG or 4B1 is observed on the reactivity of L330C permease, while labeling of V316C, M323C, or E325C permease is negligible in the absence or presence of TDG or 4B1. On the other hand, E325C permease exhibits enhanced reactivity with pyrene maleimide in the presence of TDG [K. Jung and H. R. Kaback, unpublished observations; see Jung, K., et al. (1994)].

Helix XI. As shown for V315C permease, Q359C or M362C permease reacts with NEM very slowly in the absence of ligand, and reactivity is enhanced approximately 3-fold in the presence of TDG, while no significant effect of mAb 4B1 is observed (Figure 2; Table 1). In contrast, the reactivity of Cys355 permease is essentially unaffected by TDG, but inhibited by mAb 4B1, and S366C permease exhibits similar properties, but inhibition of labeling by 4B1 is less dramatic. Although F354C permease is labeled by

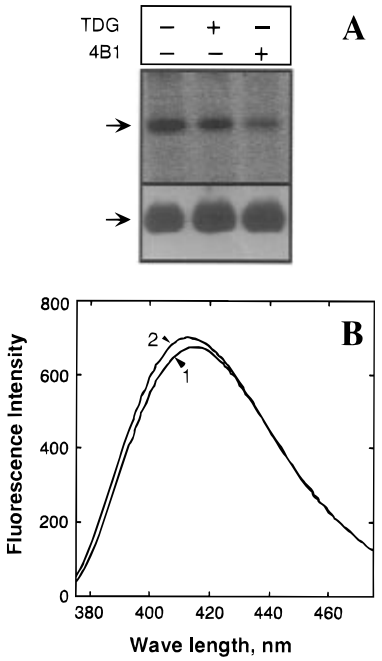

FIGURE 1: (A) Comparison of the effects of TDG and mAb 4B1 on the reactivity of V331C permease with [1-^{14}C]NEM in RSO membrane vesicles. RSO vesicles (0.3 mg of protein in 0.5 mL) prepared from *E. coli* T184 transformed with pKR35/V331C-L6XB were preincubated with or without mAb 4B1 (as indicated) for 45 min and, after removal of excess antibody and resuspension in 50 μL, incubated with [1-^{14}C]NEM (0.4 mM) in the absence or presence of TDG (10 mM) for 10 min, as indicated. The reactions were quenched with DTT, and biotinylated permease was solubilized and purified as described under Materials and Methods. Aliquots containing 5 μg of protein were separated on 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 (0.5 μg of protein) was analyzed on Western blot (lower panel). The arrow indicates the position of LacY-L6XB permease (ca. 43 kDa). (B) Effect of mAb 4B1 on the fluorescence emission spectrum of MIANS-labeled V331C permease. Purified V331C-L6XB permease was labeled with MIANS, and excess reagent was removed with dialysis as described (Wu et al., 1994). Fluorescence spectra were recorded at 30 °C with MIANS-labeled V331C permease (20 μg/mL). Curve 1, no addition; curve 2, mAb 4B1 added at a molar ratio of 1:1 to the permease.

NEM, reactivity is not altered significantly by either TDG or mAb 4B1.

Helix VII. Y236C permease reacts readily with NEM, but no significant effect of TDG or 4B1 is observed, while F243C or F247C permease does not react with NEM under any condition tested (Figure 3; Table 1). On the other hand, the reactivity of V238C permease is inhibited by either TDG or 4B1 to similar extents, while the reactivity of A244C or T248C permease is enhanced 1.5-fold and 3−4-fold, respectively, by TDG, but unaffected by mAb 4B1 (Figure 3; Table 1). E255C permease (loop VII/VIII) exhibits high reactivity with NEM which is not altered significantly by either TDG or 4B1 (Table 1).

Helix VIII. With all 10 NEM-sensitive single-Cys replacement mutants in helix VIII (Frillingos et al., 1997c; Frillingos & Kaback, 1997a), reactivity with NEM remains unaltered in the presence of 4B1 (Table 1). Strikingly, however, the reactivity of V264C, G268C, or N272C permease is blocked by TDG, while the reactivity of T265C permease is markedly enhanced by TDG (Frillingos & Kaback, 1997a; Figure 4). Pretreatment of T265C permease

Table 1: [^{14}C]NEM Labeling of Single-Cys Mutants

		[^{14}C]NEM specific labeling[a]		
	mutant	no addition	TDG[b]	mAb 4B1[c]
helix I	P28C	70	150	120
	P31C	35	155	69
helix V	C148	100	*nd*	98
helix VII	Y236C	170	148	160
	V238C	74	25	30
	F243C	*nd*	*nd*	*nd*
	A244C	60	95	70
	F247C[d]	*nd*	*nd*	*nd*[e]
	T248C[d]	45	155	38
loop VII/VIII	E255C	140	150	148
helix VIII	G262C	85	93	90
	V264C	105	31	101
	T265C	45	160	56
	G268C	55	21	52
	E269C	185	197	170
	N272C	75	26	78
	A273C	131	110	130
	M276C	125	115	131
	F277C	140	151	137
	A279C	111	101	95
helix X	V315C	32	225	35
	V316C	*nd*	*nd*	*nd*
	M323C	*nd*	*nd*	*nd*
	E325C	*nd*	*nd*	*nd*
	L330C	170	180	170
	V331C	165	96	54
helix XI	F354C	186	180	168
	C355	156	145	35
	Q359C	45	141	41
	M362C	26	80	20
	S366C	195	207	170

[a] RSO membrane vesicles (0.3 mg of protein in 50 μL) containing pKR35/*lacY* L6XB or CXB encoding each single-Cys mutant were incubated with [1-^{14}C]NEM (0.4 mM) for 10 min, at pH 7.5 and 25 °C, as described under Materials and Methods. Labeled permease was purified by avidin-affinity chromatography and analyzed electrophoretically followed by quantitative autoradiography and Western blot. Quantitation was performed with a Model 425F PhosphorImager (Molecular Dynamics). Specific [^{14}C]NEM labeling is calculated as the amount of the radioactive signal divided by the amount of the immunoreacting permease signal and expressed as a percentage of the specific labeling found for C148 permease (which is taken as 100%). Values of 10% or less are given as *nd* (not detectable). [b] Reactions with [1-^{14}C]NEM were performed in the presence of 10 mM TDG. [c] Vesicles were preincubated with mAb 4B1 for 45 min, and excess antibody was washed out before intiation of the [1-^{14}C]NEM reaction, as described under Materials and Methods. [d] Positions 247 and 248 are in the boundary between helix VII and loop VII/VIII (King et al., 1991; Sun et al., 1996). [e] F247C does not bind mAb 4B1 (Sun et al., 1996).

with the hydrophilic sulfhydryl reagent MTSES (Akabas et al., 1992; Stauffer & Karlin, 1994) has little or no effect on the weak reactivity of this mutant with NEM (Figure 4). However, in the presence of TDG, this position becomes accessible to MTSES, as evidenced by the decrease in the TDG-induced NEM reactivity. Thus, ligand binding causes position 265 to become more accessible to solvent. Binding of mAb 4B1 causes a very small enhancement in the NEM reactivity of T265C permease and a small increase in accessibility to MTSES.

C148 (Helix V), P28C, or P31C Permeases (Helix I). With respect to the N-terminal half of the permease (helices I−VI), incubation with mAb 4B1 does not affect the reactivity of the Cys148 in helix V, a residue that interacts sterically with substrate (Jung et al., 1994b; Wu & Kaback, 1994). In contrast, TDG completely abolishes the reactivity of C148 permease, as shown previously [Table 1; see Frillingos and Kaback (1996c) in addition]. On the other hand, the periplasmic end of helix I contains a conformationally active face, as indicated by previous findings (Wu et al., 1995a) that the reactivity of mutants P28C and P31C (helix I) with NEM or MIANS is markedly enhanced by TDG. Incubation with 4B1 (Figure 5) also enhances the NEM reactivity of P28C or P31C, but to a lesser extent than observed in the presence of TDG (Table 1).

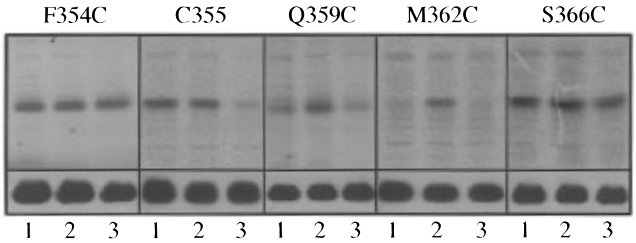

FIGURE 2: Reactivity of RSO membrane vesicles containing permeases with single-Cys replacements in helix XI with [1-^{14}C]NEM. Lanes 1, no addition; lanes 2, incubation in the presence of TDG (10 mM); lanes 3, incubation after pretreatment with mAb 4B1. RSO membrane vesicles (0.3 mg of protein in 0.5 mL) prepared from *E. coli* T184 transformed with pKR35/*lacY* L6XB encoding single-Cys mutants F354C, C355, Q359C, M362C, or S366C (as indicated) were preincubated with or without mAb 4B1 for 45 min and, after removal of excess antibody and resuspension in 50 μL, incubated with [1-^{14}C]NEM (0.4 mM) in the absence or presence of TDG (10 mM). The reactions were quenched with DTT for 10 min, and biotinylated permease was solubilized and purified as described under Materials and Methods. Aliquots containing 5 μg of protein were separated on 12% NaDodSO$_4$−polyacrylamide gel electrophoresis, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography (*upper panels*). A fraction of the eluted molecules (0.5 μg of protein) was analyzed on Western blot (*lower panels*).

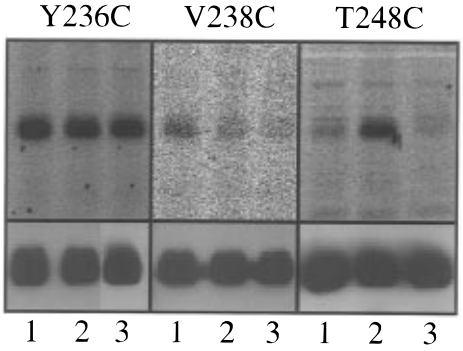

FIGURE 3: Reactivity of RSO membrane vesicles containing permeases with single-Cys replacements in helix VII with [1-^{14}C]NEM. Lanes 1, no addition; lanes 2, incubation in the presence of TDG (10 mM); lanes 3, incubation after pretreatment with mAb 4B1. RSO membrane vesicles (0.3 mg of protein in 0.5 mL) prepared from *E. coli* T184 transformed with pKR35/*lacY* CXB encoding single-Cys mutants Y236C, V238C, or T248C (as indicated) were preincubated with or without mAb 4B1 for 45 min and, after removal of excess antibody and resuspension in 50 μL, incubated with [1-^{14}C]NEM (0.4 mM) in the absence or presence of TDG (10 mM). The reactions were quenched with DTT at 10 min, and biotinylated permease was solubilized and purified as described under Materials and Methods. Conditions for autoradiography (*upper panels*) and Western blot (*lower panels*) were as in Figure 2.

DISCUSSION

Use of Cys-scanning mutagenesis to systematically replace each residue in lac permease with Cys has yielded a library

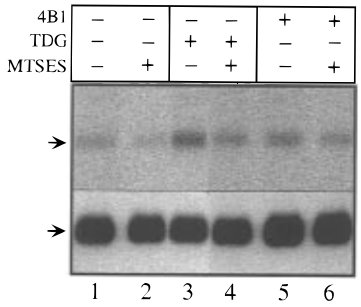

FIGURE 4: Effect of TDG or mAb 4B1 on the accessibility of T265C permease to MTSES from the periplasmic surface of the membrane. RSO membrane vesicles (0.3 mg of protein in 0.5 mL) prepared from *E. coli* T184 transformed with pKR35/*lacY* CXB encoding the single-Cys mutant T265C were incubated with or without MTSES (200 μM) for 3 min in the absence or presence of TDG (10 mM) or after a 45-min incubation with mAb 4B1. Samples were then washed twice with 1 mL of ice-cold buffer [100 mM KP_i (pH 7.5)/10 mM $MgSO_4$]. After removal of excess MTSES, antibody, and TDG, the vesicles were resuspended in 50 μL of the same buffer, TDG was added again to the samples originally treated with TDG (10 mM final concentration), and the vesicles were incubated with [1-^{14}C]NEM (0.4 mM) for 30 min, as described under Materials and Methods. Lanes 1, 3, 5, no MTSES (controls); lanes 2, 4, 6, with MTSES; lanes 1, 2, absence of TDG or 4B1; lanes 3, 4, presence of TDG (ca. 10 mM); lanes 5, 6, presence of 4B1. All other conditions were as described in Figure 3.

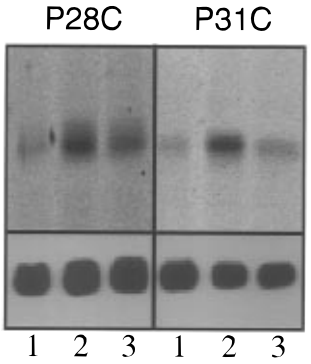

FIGURE 5: Reactivity of RSO membrane vesicles containing P28C or P31C permease with [1-^{14}C]NEM. Lanes 1, no addition; lanes 2, incubation in the presence of TDG (10 mM); lanes 3, incubation after pretreatment with mAb 4B1. All conditions were as in Figure 2.

of unique molecules that are being used for structure/function studies on this model symport protein [see Kaback et al. (1994) and Kaback (1996)]. In this context, the approach has demonstrated that remarkably few residues are directly involved in the transport mechanism. However, about 43 of the Cys-replacement mutants are inactivated by alkylation, and these mutants cluster in such a manner as to indicate that interfaces within the permease are important for the conformational changes associated with turnover. Furthermore, by studying substrate protection against NEM inactivation and/or labeling, residues at the interface between helices V and VIII which are likely to be in direct contact with ligand have been delineated (Jung et al., 1994b; Wu & Kaback, 1994; Frillingos & Kaback, 1996c, 1977a; Frillingos et al., 1997c). Finally, in addition to site-directed spectroscopy [Kaback, 1996; see Wu et al. (1996b) and Voss et al. (1996) in addition] and thiol cross-linking (Wu et al., 1996b; Wu & Kaback, 1996), Cys-scanning mutagenesis is particularly useful for characterizing discontinuous mAb epitopes (Sun et al., 1996, 1997).

In this paper, the reactivity of 31 single-Cys mutants is examined *in situ* with the intent of determining whether lac permease assumes the same conformational state in the presence of ligand or upon binding of mAb 4B1 which is thought to block deprotonation of the permease by binding specifically to periplasmic loop VII/VIII (Sun et al., 1996; Frillingos & Kaback, 1996b). This question is important because 4B1 blocks lactose/H^+ symport with no effect on equilibrium exchange or counterflow, and external lactose inhibits H^+ translocation induced by lactose efflux down a concentration gradient (Kaczorowski & Kaback, 1979). Therefore, it is possible that binding of ligand or 4B1 may induce a similar conformational change. The results provide preliminary documentation of the widespread nature of the conformational changes induced by ligand and indicate that the mAb induces a more subtle alteration.

Clearly, binding of the high-affinity β-galactoside analog TDG causes alterations in the reactivity of single-Cys replacements in helices I, VII, VIII, X, and XI (Figure 6A). Thus, changes in NEM reactivity that cannot be attributed to direct steric interaction are observed with single-Cys residues at positions 28 and 31 in helix I, positions 238, 244, and 248 in helix VII, position 265 in helix VIII, positions 315 and 331 in helix X, and positions 359 and 362 in helix XI. With the exceptions of V238C (helix VII) or V331C (helix X) permease, TDG elicits an increase in NEM reactivity which must be attributed to an indirect structural effect. Moreover, with V331C (Wu et al., 1994) or V238C permease (J. Voss and H. R. Kaback, unpublished results), where decreased reactivity is observed, site-directed fluorescence and electron paramagnetic resonance spectroscopy, respectively, indicate that in the presence of ligand these positions move into a more tightly packed, hydrophobic environment (see Figure 1B in addition). In contrast, inhibition of reactivity is observed for single-Cys148 and M145C (helix V) permease (Wu & Kaback, 1994; Frillingos & Kaback, 1996c), as well as for V264C, G268C, or N272C (helix VIII) permease (Frillingos et al., 1997c; Frillingos & Kaback, 1997a). Since positions 148 and 145 in helix V interact sterically with substrate and positions 264, 268, and 272 are on the same face of helix VIII and appear to be in close approximation to positions 148 and 145, it has been suggested that the latter residues also make direct contact with substrate and that the interface between helices V and VIII may form part of the substrate translocation pathway (Figure 6).

In contrast, changes in the reactivity of the Cys-replacement mutants observed in the presence of mAb 4B1 appear to be much less widespread (Figure 6B). Similar effects of TDG and 4B1 are seen at positions 331 (helix X), 238 (helix VII), and 28 and 31 (helix I) which lie outside of the putative interface between helices V, VII, VIII, X, and XI (compare Figure 6A and Figure 6B). Conversely, positions within the interface including 148 (helix V), 264, 268, and 272 (helix VIII), which probably interact directly with substrate, and positions 315 (helix X), 359, and 362 (helix XI), which are conformationally sensitive with respect to ligand binding, are not affected by mAb 4B1. However, mAb decreases the reactivity of single-Cys residues at positions 355 and 366, the effect with 355 being more dramatic. In the case of T265C permease, while TDG causes a dramatic increase in NEM reactivity and enhances the accessibility of this position to MTSES, 4B1 causes similar, but much less pronounced

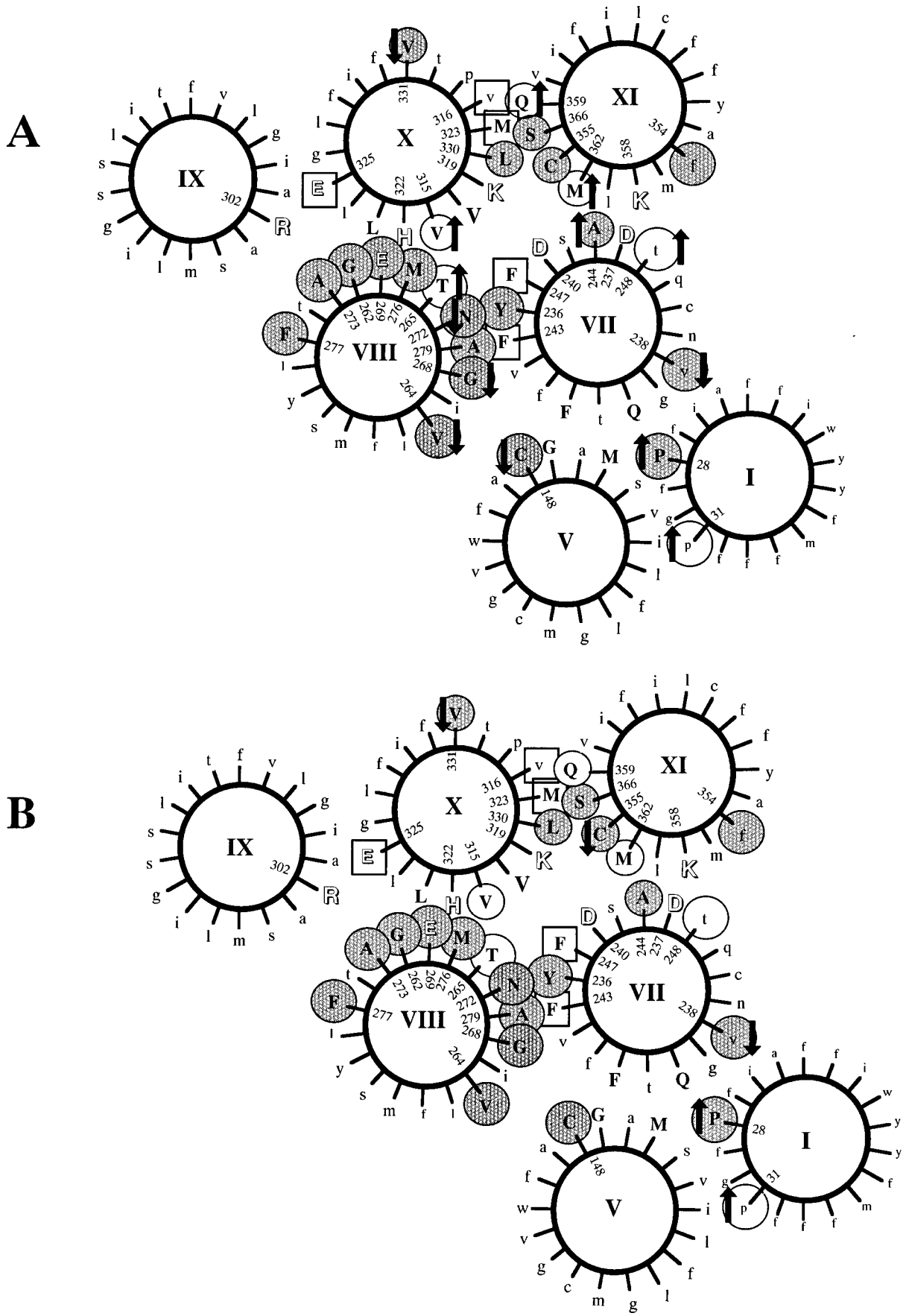

FIGURE 6: Helical wheel model of helices I, V, and VII−XI in lac permease highlighting positions of Cys-replacement mutants examined in this study. Emboldened capital letters represent positions where a Cys-replacement mutant is inactivated by treatment with NEM [Kaback, 1996; see Frillingos and Kaback (1996a) and Frillingos et al. (1997a,b) in addition]. Positions examined are numbered, and the corresponding residues are encircled. Open circles indicate positions where the reactivity of the Cys-replacement with [1-^{14}C]NEM is low (<50% of C148; Table 1). Filled circles indicate positions where the reactivity is high (>50% of C148; Table 1). Open squares indicate positions where reactivity is undetectable (*nd*; Table 1). Arrows indicate changes in reactivity in the presence of TDG (A) or mAb 4B1 (B).

effects. Therefore, although binding of 4B1 brings about less widespread changes in the reactivity of these single-Cys residues to sulfhydryl reagents, it is noteworthy that the direction of the changes (i.e., increase or decrease in reactivity) is similar to that produced by TDG.

Following the localization of the epitope for mAb 4B1 in periplasmic loop VII/VIII and the demonstration that avidin binding to a biotinylated Cys residue in the loop has no effect on transport (Sun et al., 1996), it was suggested that the uncoupling effect of 4B1 is mediated through a torsion-induced conformational change in helices VII and/or VIII which alters the pK_a of a residue(s) essential for coupling. In this regard, based on the properties of site-directed mutations in each of the four irreplaceable residues, it was suggested that Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X) participate in an H-bond network that is involved in lactose-coupled H^+ translocation (Kaback, 1987, 1996), and it was shown recently that binding of 4B1 alters the apparent pK_a of an Asp residue at position 325 (Frillingos & Kaback, 1996b). As shown here, 4B1 alters the reactivity of individually placed Cys residues in helices VII (238), X (331), and XI (355). In addition, a small but reproducible increase in reactivity is observed for Cys residues at positions 28 and 31 in helix I which can be readily explained by the recent finding that helix I is in close proximity to helix VII (Wu & Kaback, 1996). Taken together, the results indicate that mAb 4B1 binding to periplasmic loop VII/VIII elicits a relatively limited structural change leading to an alteration(s) in the pK_a of a residue(s) essential for coupling and that the change differs from that induced by substrate binding.

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