Identification of the Epitope for Monoclonal Antibody 4B1 Which Uncouples Lactose and Proton Translocation in the Lactose Permease of *Escherichia coli*

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ABSTRACT: Monoclonal antibody 4B1 binds to a conformational epitope on the periplasmic surface of the lactose permease of *Escherichia coli*, uncoupling lactose and H^+ translocation in a manner indicating that it blocks deprotonation [Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984) *Biochemistry 23*, 3681; Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688]. In this paper, 4B1 binding to purified lactose permease is shown to exhibit a K_D of about 5×10^{-10} M by surface plasmon resonance. Furthermore, the combined use of mutants containing 6 contiguous His residues in each periplasmic loop in the permease and Cys-scanning mutagenesis in conjunction with chemical labeling demonstrates that 4B1 binds specifically to the periplasmic loop between helices VII and VIII and that Phe247 and Gly254 are the primary determinants. Remarkably, although 4B1 binding uncouples lactose and H^+ translocation, none of the amino acid residues in periplasmic loops, particularly Phe247 or Gly254, play an important role in the transport mechanism. Moreover, binding of avidin to biotinylated Glu255→Cys in the loop containing the epitope has no effect on transport activity. Therefore, the uncoupling effect of 4B1 involves highly specific interactions which in all likelihood exert a torsional effect on the loop, resulting in a conformational change in helix VII and/or VIII that alters the pK_as of residues involved in lactose-coupled H^+ translocation.

The lactose (lac)[1] permease of *Escherichia coli* is a paradigm for secondary transport proteins from archaebacteria to the mammalian central nervous system that transduce the energy stored in an electrochemical ion gradient into work in the form of a concentration gradient (reviewed in Kaback, 1983, 1989, 1992, 1995; Poolman & Konings, 1993). This hydrophobic, polytopic membrane protein catalyzes the coupled translocation of β-galactosides and H^+ (i.e., symport or cotransport) with a stoichiometry of unity. Encoded by the *lacY* gene, the permease has been solubilized from the membrane, 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 dichroic studies and hydropathy analysis of the primary amino acid sequence, a secondary structure was proposed (Foster et al., 1983) in which the permease has 12 putative transmembrane helices connected by hydrophilic loops (Figure 1). Evidence favoring general features of the model has been obtained from a variety of approaches (reviewed in Kaback, 1992), and analysis of an extensive series of lac permease−alkaline phosphatase (*lacY-phoA*) fusions (Calamia & Manoil, 1990) has provided unequivocal support for the 12-helix motif. Based on second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence, a model describing helix packing in the C-terminal half of the permease has been proposed (Jung et al., 1993; reviewed in Kaback et al., 1994). Evidence confirming and extending the model has been obtained by engineering divalent metal binding sites (bis-His residues) into the transmembrane domains of the molecule (He et al., 1995a,b; Jung et al., 1995b) and through the use of site-directed chemical cleavage (Wu et al., 1995b).

Immunological methods have provided an important tool for studying aspects of lac permease. Use of site-directed polyclonal antibodies demonstrates that the C-terminus, and the loops between helices IV and V and between VI and VII, are on the cytoplasmic surface of the membrane (Carrasco et al., 1984a, 1986b; Danho et al., 1985; Seckler et al., 1983, 1986). Moreover, monoclonal antibodies (mAbs) against lac permease have been prepared and characterized (Carrasco et al., 1982). Out of more than 60 mAbs, only one from hybridoma 4B1 strongly inhibits active transport. Binding studies demonstrate that 4B1 binds to the permease in right-side-out (RSO) membrane vesicles and proteoliposomes containing purified permease with 1:2 molar ratio, but not in inside-out vesicles, and Fab fragments bind with a stoichiometry of unity (Herzlinger et al., 1984). Since inhibition of transport is observed both with 4B1 IgG and with Fab fragments, aggregation of permease molecules in the plane of the membrane is not responsible for inhibition, even though the intact IgG may cause the permease to aggregate (Costello et al., 1984).

Functional studies (Carrasco et al., 1984b) reveal that 4B1 inhibits H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)-driven active

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; mAb, monoclonal antibody; EDTA, ethylenediaminetetracetic acid; DM, *n*-dodecyl β,D-maltopyranoside; $\Delta\bar{\mu}_{H^+}$, the H^+ electrochemical gradient across the membrane; NEM, *N*-ethylmaleimide; MPB, 3-(*N*-maleimidylpropionyl)biocytin; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; BSA, bovine serum albumin; RSO, right-side out; PMS, phenazine methosulfate.

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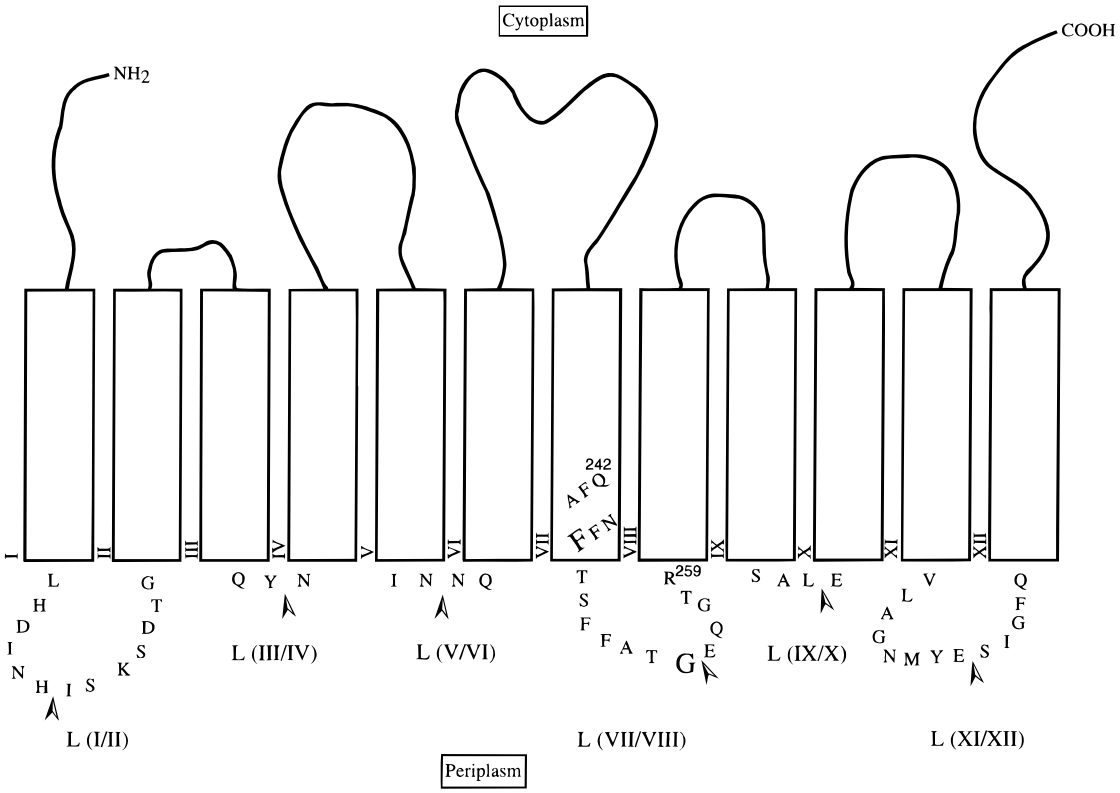

FIGURE 1: Secondary structure model of lac permease showing the 6-His insertion sites within the periplasmic loops. The single-letter amino acid code is used, and the 12 hydrophobic transmembrane helices are depicted as rectangles. Each of the amino acid residues shown was replaced individually with a Cys in Cys-less permease. Loops are designated by the letter L, followed in parentheses by the two connected helices separated by a slash. The arrowheads indicate the positions of either 6-His inserts or 3 tandem factor X_a protease sites in L(VII/VIII).

transport, as well as lactose/H^+ symport and lactose efflux under nonenergized conditions. Remarkably, however, ligand binding and translocation reactions that do not involve net H^+ translocation (i.e., equilibrium exchange and counterflow) are unaffected by 4B1 binding, and counterflow is stimulated by the mAb when the external lactose concentration is below the apparent K_m. The findings are consistent with a kinetic model for lactose translocation down a concentration gradient (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) in which dissociation of lactose occurs prior to dissociation of H^+ and indicate that the mAb specifically blocks deprotonation of the permease. Clearly, therefore, identification of the 4B1 epitope is important. However, the mAb is directed against a conformational epitope, and denatured permease no longer binds. In addition, synthetic peptides corresponding to periplasmic loops do not inhibit 4B1 binding to RSO vesicles or proteoliposomes reconstituted with purified permease, and numerous attempts to identify the epitope by fragmentation and fractionation have been unsuccessful.

By applying site-directed mutagenesis to wild-type permease and Cys-scanning mutagenesis to a functional permease molecule devoid of Cys residues, individual amino acid residues that are essential for transport and/or substrate binding have been identified (reviewed in Kaback, 1994, 1995). Single Cys residues have been placed at more than 350 out of the 417 different positions, and remarkably, less than a half-dozen residues have been shown to play a critical mechanistic role thus far. Specifically, only Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) have been shown to be essential for active lactose transport. On the other hand, the activity of many active Cys replacement mutants is altered by alkylation, and these mutants appear in clusters, suggesting that surface contours within the permease are important. In addition, site-directed spectroscopic and chemical labeling studies demonstrate that the reactivity and environment of various Cys residues are altered as a result of ligand binding or imposition of $\Delta\bar{\mu}_{H^+}$ (Jung, H., et al., 1994; Jung, K., et al., 1994; Sahin-Tóth & Kaback, 1993; Wu et al., 1994, 1995a; Wu & Kaback, 1994). Taken together, the findings indicate that permease turnover results from relatively simple chemistry involving a small number of critical side chains, coupled to widespread conformational changes in which the transmembrane helices move relative to one another, thereby re-emphasizing the importance of identifying the 4B1 epitope.

Since Ala-scanning mutagenesis has been used elegantly for identifying and studying mAb epitopes in human growth hormone (Jin et al., 1992, 1994), a mutational approach has now been applied in order to identify the 4B1 epitope in lac permease. Initially, by using His-insertion mutants in periplasmic loops (McKenna et al., 1992), the 4B1 epitope is localized to the loop between helices VII and VIII in the C-terminal half of the permease. Subsequently, by using Cys-scanning mutagenesis and studies with *N*-ethylmaleimide (NEM), Phe247 and Gly254 are identified as the primary determinants for 4B1 binding, and other experiments with 3-(*N*-maleimidylpropionyl)biocytin (MPB) suggest that the epitope may be in α-helical conformation. Although 4B1 binding to the loop between helices VII and VIII uncouples lactose from H^+ translocation, none of the residues in the periplasmic loops, particularly Phe247 or Gly254, play a direct role in the transport mechanism. These and other findings lead to the conclusion that the mechanism of 4B1

inhibition of active lactose transport involves conformational changes in helices VII and/or VIII that alter the pK_as of residues that are important for lactose-coupled H^+ translocation.

EXPERIMENTAL PROCEDURES

Materials. Deoxyoligonucleotides were synthesized on an Applied Biosystem 391 DNA synthesizer. All restriction endonucleases, T4 DNA ligase, and Taq DNA polymerase were from New England Biolabs. DNA Sequenase was from United States Biochemical. Rabbit polyclonal antiserum against C-terminus of lac permease (Carrasco et al., 1984a) was prepared by Babco. [1-^{14}C]Lactose, [α-^{35}S]dATP and ^{125}I-protein A were from Amersham. 3-(*N*-Maleimidylpropionyl)biocytin (MPB) was obtained from Molecular Probes. All other materials were reagent grade and were obtained from commercial sources.

Mutant Construction. Single-Cys mutants were constructed by site-specific mutagenesis of the Cys-less version of cassette *lacY* gene (EMBL X-56095) in plasmid pT7-5 (van Iwaarden et al., 1991) by using 1- or 2-stage polymerase chain reaction (PCR) (Ho et al., 1989). Mutations were verified by sequencing the length of the PCR-generated segment using dideoxynucleotide termination method and synthetic sequencing primers (Sanger et al., 1977) after alkali denaturation (Hattori & Sakaki, 1986). Construction of the His-insertion mutants has been described (McKenna et al., 1992), as well as that of a mutant containing 3 tandem factor X_a protease sites inserted at position E255 in the periplasmic loop between helices VII and VIII (Sahin-Tóth et al., 1995).

Growth of Cells. E. coli T184 (*lacZ*$^-$*Y*$^-$) cells transformed with plasmid pT7-5 encoding a given mutant was grown aerobically at 37 °C in Luria-Bertani broth with ampicillin (100 μg/mL) and streptomycin (10 μg/mL). Overnight cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 0.5 mM isopropyl 1-thio-β,D-galactopyranoside (IPTG). After additional growth for 2 h at 37 °C, cells were harvested by centrifugation.

Spheroplast Preparation. Spheroplasts were prepared as described (Herzlinger et al., 1984). Cells were suspended in 2 mL of 0.75 M sucrose/10 mM Tris-HCl (pH 8.0) at room temperature. Lysozyme and Pefabloc (Boehringer Mannheim) were added to final concentrations of 0.1 mg/mL and 1 mM, respectively, and the samples were incubated for 10 min. Four milliliters of 2 mM ethylenediaminetetraacetate (EDTA; potassium salt; pH 7.0) containing 0.1 mg/mL lysozyme was added, and incubation was continued for about 20 min. Spheroplast formation was confirmed by phase contrast microscopy, 10 mM $MgSO_4$ and 50 μg/mL DNase I were added, and the spheroplasts were harvested by centrifugation at room temperature. The pellet was resuspended and washed once with 100 mM potassium phosphate (KP_i; pH 7.0) containing 0.5 M sucrose. Finally, the spheroplasts were resuspended in 100 mM KP_i (pH 7.0)/0.5 M sucrose (suspension buffer).

Preparation of RSO Membrane Vesicles. RSO membrane vesicles were prepared by osmotic lysis of spheroplasts prepared by lysozyme−EDTA treatment (Kaback, 1971; Short et al., 1975).

Purification of mAb 4B1. Purification was carried out according to Carrasco et al. (1982) with minor modifications. Ascites fluid containing 4B1 IgG was applied to a protein A−Sepharose CL 4B column and washed with 0.15 M NaCl/0.1 M KP_i (pH 7.5) until no protein was detected in the eluant. Bound IgG was then eluted with 0.1 M Gly-HCl (pH 3.0). The protein peak was dialyzed overnight against 0.1 M KP_i (pH 7.5), and aliquots of the purified mAb were frozen and stored at −80 °C.

4B1 Binding to Purified Lac Permease. Cys-less lac permease with a biotin acceptor domain in the middle cytoplasmic loop was purified by avidin affinity chromatography as described (Wu & Kaback, 1994). The free *d*-biotin in the purified permease sample was removed by dialysis against 100-fold excess of 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% *n*-dodecyl β,D-maltoside (DM) (buffer A) with three changes. Real time detection of interaction of 4B1 mAb with purified lac permease was monitored by measuring surface plasmon resonance using the BIAcore system (Pharmacia). Streptavidin was covalently coupled to a sensor chip CM5 according to the manufacturer's instructions (Pharmacia Biosensor). Purified biotinylated lac permease (20 μg/mL) in buffer A was captured at a flow rate of 5 μL/min. After 10 min continuous flow, the sensor chip was washed with buffer A. Binding of mAb 4B1 to the streptavidin−permease sensor chip surface was carried out in buffer A containing 0.5 mg/mL of antibody. Association was monitored over 6 min at a flow rate of 5 μL/min; dissociation was followed for 4 min in free buffer flowing at a rate of 10 μL/min.

4B1 Binding to Spheroplasts. Aliquots of spheroplasts (0.5 mL containing 0.3 mg/mL protein in suspension buffer containing 5% BSA) were mixed with 5 μL of affinity purified 4B1 (5 mg/mL), incubated at room temperature for 1 h, centrifuged, washed once in suspension buffer, and resuspended in 0.4 mL of suspension buffer containing 5% BSA. Two microliters of ^{125}I-protein A (30 mCi/mg; 100 μCi/mL) was added, and incubation was continued for 45 min. The spheroplasts were then centrifuged, washed once by centrifugation, and resuspended to 50 μL of suspension buffer. Bound radioactivity was measured by liquid scintillation spectrometry using Scintsafe Econo 1 cocktail buffer.

Lactose Transport. RSO membrane vesicles were suspended in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ buffer to a concentration of 2 mg/mL total protein. Transport of [^{14}C]-lactose (10 mCi/mmol) at a final concentration of 0.4 mM was assayed under oxygen in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) (Konings et al., 1971; Kaback, 1974).

Quantitation of Lac Permease. Spheroplasts from each mutant were dissolved in 3% DM and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Newman et al., 1981). The proteins were electroblotted to a poly(vinylidene difluoride) membrane (PVDF-Immobilon, Millipore). Blots were then blocked in 5% BSA in 10 mM Tris-HCl (pH 7.6)/0.15 M NaCl/0.2% Triton X-100 (TBST) for 1 h. Anti-C-terminal antibody (Carrasco et al., 1984a) was added at a final dilution of 1:2000, and incubation was continued for 2 h. After washing with TBST, ^{125}I-protein A was added at a final dilution of 1:10 000, and incubation was continued for 1 h. After washing thoroughly, the membrane was exposed to a Phosphoimager screen. By using known amounts of purified lac permease, a standard curve was constructed. The amount of permease in each spheroplast preparation was then quantitated by comparison to the standard curve.

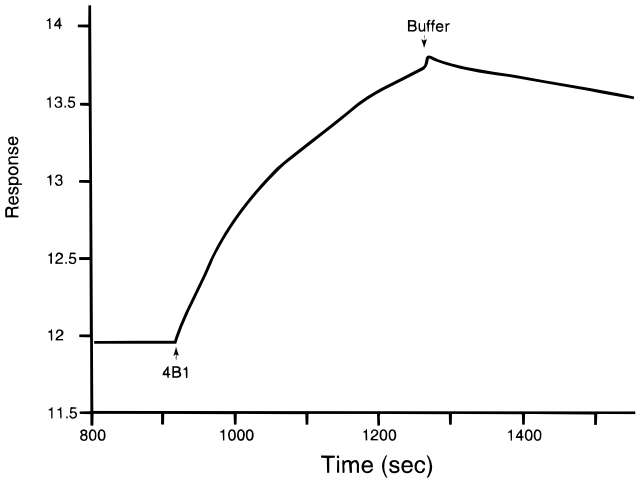

FIGURE 2: Sensogram of 4B1 binding to purified lac permease. Purified biotinylated lac permease in DM was immobilized on a sensor chip surface coated with streptavidin as described in the Experimental Procedures. mAb 4B1 at a concentration of 0.5 mg/mL was injected over the surface at a flow rate of 5 μL/min. The first arrow indicates the injection of buffer containing 4B1, and the second arrow indicates injection of buffer alone.

Protein Determinations. Protein was assayed as described (Peterson, 1977) with BSA as standard.

RESULTS

4B1 Binding to Purified Lac Permease. Site-directed fluorescence studies (Wu et al., 1994, 1995a; Wu & Kaback, 1994) indicate that purified lac permease maintains close to native conformation in DM. To further strengthen this conclusion, conformation-dependent binding of mAb 4B1 to purified permease in DM was tested by using surface plasmon resonance. After conjugating purified biotinylated permease to a Streptavidin-coated chip, 4B1 clearly exhibits time-dependent association with the permease (Figure 2). Analysis of the binding phase yields a relatively rapid k_{assoc} of 1.22×10^5 M^{-1} s^{-1}, and analysis of the dissociation phase exhibits a remarkably slow k_{dissoc} of 5.97×10^{-5} M^{-1} s^{-1}. Thus, 4B1 binds to purified permease with an equilibrium binding constant of about 5×10^{-10} M.

4B1 Binding to His-Insertion Mutants. Since 4B1 binds to an epitope on the periplasmic surface of the protein (Carrasco et al., 1982, 1984b; Herzlinger et al., 1984), studies were initiated by testing binding of 4B1 to mutants in the lac permease containing 6 contiguous His residues in each periplasmic loop (McKenna et al., 1992). The only periplasmic loop important for activity based on this approach is the short loop connecting helices IX and X (Figure 1), but use of Cys-scanning mutagenesis demonstrates that none of the individual residues are important for activity (Sahin-Tóth & Kaback, 1993). As shown in Figure 3, only the mutant with 6 His residues inserted immediately after Gly254 in the loop between helices VII and VIII exhibits dramatic reduction in 4B1 binding. The other mutants bind 4B1 at least 70% as well as wild-type permease. In addition, a functional mutant with 3 tandem factor X_a protease sites inserted at the same site (Sahin-Tóth et al., 1995) exhibits insignificant binding of 4B1. Based on these results, it seems likely that the 4B1 epitope is contained within the loop connecting helices VII and VIII.

Single-Cys Mutants. In order to determine which amino acid residues comprise the 4B1 epitope, single-Cys replacement mutants at each residue in the loop were tested for 4B1 specific binding activity relative to Cys-less permease before and after alkylation with NEM (Table 1). When binding assays are carried out in the absence of NEM treatment (Figure 4A), mutant F247C exhibits essentially no binding, mutants T253C and G254C bind about 25% as much 4B1 as control spheroplasts, and mutants F251C, A252C, and G257C bind 4B1 30−50% as well.[2] The remaining mutants exhibit specific binding activities that range from 60% to 110% of control. After treatment of the spheroplasts with NEM (Figure 4B and Table 1), it becomes apparent that the primary determinants for 4B1 binding are Phe247 and Gly254. Thus, 4B1 binding observed with F247C permease remains negligible, the binding activity of G254C is abolished, and binding by mutants F251C, A252C, T253C, or G257C is stimulated by alkylation. Although no clear pattern is observed for the effects of NEM treatment on 4B1 binding with single-Cys mutants in the loop containing the epitope, alkylation of the mutants with MPB which is considerably bulkier exhibits a periodicity consistent with a short α-helical configuration between positions 247 and 254 (Figure 5A). These two positions, as well as position 250, fall on the same face of a helical wheel plot (Figure 5B), thereby suggesting that 4B1 binds to this face. It may also be significant that this short, putative helical region is clearly amphipathic such that the face opposite to that where the mAb binds is much more hydrophilic.

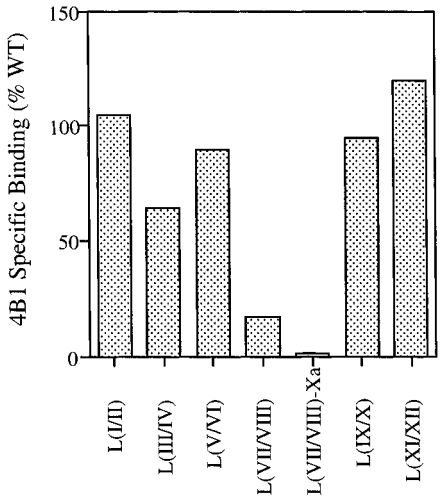

FIGURE 3: Binding of mAb 4B1 to *E. coli* spheroplasts harboring plasmid pT7-5/*lacY* with insertions in periplasmic loops of lac permease. Plasmids encoding mutants with 6 contiguous His residues inserted into given periplasmic loops or 3 tandem factor X_a protease sites in L(VII/VIII) were constructed as described by McKenna et al. (1992) or Sahin Tóth et al. (1995), respectively, transformed into *E. coli* T184, and overexpressed. Spheroplasts were prepared and incubated with 4B1 mAb and ^{125}I-protein A as described in the Experimental Procedures. The amount of permease expressed in each instance was quantitated by immunoblotting and comparison to a standard curve constructed from immunoblots with known amounts of purified lac permease as described in the Experimental Procedures. 4B1 specific binding (i.e., nmol of 4B1 bound per mole of permease) is expressed as a percentage of that observed with spheroplasts expressing wild-type permease. All data were corrected for 4B1 binding to spheroplasts harboring plasmid pT7-5 without a *lacY* insert.

[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.

Table 1: Lactose Transport Activity and 4B1 Binding of Single-Cys Mutants

	mutant	initial rate of lactose transport[a] (% C-less)	4B1 specific binding[b] (% C-less)	4B1 specific binding after NEM[c] (% C-less)		mutant	initial rate of lactose transport[a] (% C-less)	4B1 specific binding[b] (% C-less)	4B1 specific binding after NEM[c] (% C-less)
L(I/II)	L34C	66	85	105	L(VII/VIII)	F250C	70[e]	60	71
	H35C	10	128	130		F251C	37[e]	48	52
	D36C	32	100	98		A252C	95[e]	45	73
	I37C	62	113	100		T253C	90[e]	25	48
	N38C	100	86	80		G254C	80[e]	23	1
	H39C	96	91	117		E255C	115[e]	72	80
	I40C	64	116	130		Q256C	58	73	80
	S41C	82	109	117		G257C	74[g]	37	62
	K42C	77	101	105		T258C	34	67	96
	S43C	136	109	107		R259C	100	95	92
	D44C	88	97	117	L(IX/X)	S311C	115[h]	100	101
	T45C	117	100	95		A312C	110[h]	138	140
	G46C	82	80	85		L313C	85[h]	102	105
L(III/IV)	Q100C	75[d]	103	105		E314C	150[h]	101	124
	Y101C	85[d]	128	130	helix XI	S355C	180[i]	105	115
	N102C	40[d]	125	120		Q359C	90[i]	91	80
L(V/VI)	I164C	95[d]	105	126		M362C	150[i]	74	86
	N165C	80[d]	84	83		S366C	115[i]	88	102
	N166C	83[d]	108	125	L(XI/XII)	V367C	128	75	70
	Q167C	135[d]	125	130		L368C	115	94	88
helix VII	Y236C	33[e]	91	105		A369C	122	88	88
	D237C	20[e]	78	75		G370C	103	112	114
	D240C/K319A	45[f]	106	103		N371C	63	70	69
L(VII/VIII)	Q242C	58[e]	85	91		M372C	55	105	101
	F243C	42[e]	112	114		Y373C	120	106	140
	A244C	33[e]	110	114		E374C	130	80	118
	N245C	68[e]	68	83		S375C	73	86	92
	F246C	77[e]	98	108		I376C	97	90	94
	F247C	47[e]	1	1		G377C	40	82	90
	T248C	74[e]	104	95		F378C	71	102	84
	S249C	87[e]	83	65		Q379C	126	94	75

[a] Initial rates of transport were assayed in intact cells as described (Kaback, 1974). [b] Spheroplast preparation and 4B1 binding assay were carried out as described in the Experimental Procedures. [c] Spheroplasts were incubated with 1 mM NEM at 25 °C for 30 min, 10 mM dithiothreitol was added, and the spheroplasts were washed with 100 mM KP_i (pH 7.0)/0.5 M sucrose. Binding of 4B1 was assayed as described for the untreated samples. [d] Transport data from S. Frillingos, and H. R. Kaback (manuscript in preparation). [e] Data from Frillingos et al. (1994). [f] Data from Sahin-Tóth et al. (1992). [g] Data from Jung et al. (1995a). [h] Data from Sahin-Tóth and Kaback (1993). [i] Data from Dunten et al. (1993).

Initial rates of lactose transport and specific binding of 4B1 before and after NEM treatment in spheroplasts expressing single-Cys replacement mutants for each amino acid residue on the periplasmic face of the permease are tabulated in Table 1. Essentially all of the mutants exhibit highly significant activity, demonstrating that none of the individual amino acid residues in periplasmic loops play an essential role in the transport mechanism. Although mutant H35C exhibits low activity, replacement of His35 with Arg has essentially no effect on activity (Padan et al., 1985), and replacement with Cys impairs expression of the permease (data not shown). It is also apparent that, with the exception of F247C or G254C permease, each Cys-replacement mutant exhibits highly significant or, with the great majority, normal binding of 4B1 before or after treatment with NEM. In addition, several mutants in transmembrane helices bind 4B1 at essentially the control level.

Effect of 4B1 Binding on Activity of Cys-less, F247C, or G254C Permease. As shown previously with wild-type permease (Carrasco et al., 1982, 1984b), mAb 4B1 inhibits the activity of Cys-less permease in RSO membrane vesicles; however, Cys-less permease is unaffected by NEM (van Iwaarden et al., 1991; Sahin-Tóth et al., 1994) (Figure 6A). As expected, 4B1 does not inhibit transport in vesicles containing F247C permease, since the mAb does not bind to this mutant (Figure 6B). In contrast, 4B1 inhibits transport significantly in G254C vesicles, but inhibition is essentially completely blocked when the vesicles are treated with NEM prior to exposure to the mAb (Figure 6C). The findings are the inverse of those observed for binding. That is, G254C permease exhibits significant binding of 4B1 which inhibits transport. When binding is abolished by treatment with NEM, the mAb no longer inhibits.

Effect of Avidin Binding to the 4B1 Epitope. In order to determine if inhibition of permease activity by 4B1 is related simply to increased mass at the binding site or to a more specific interaction, transport activity of RSO vesicles containing E255C permease was tested after treatment with MPB alone or MPB and avidin (Figure 7A). Clearly, neither reagent alone or in combination has a significant effect on the rate of transport. On the other hand, MPB treatment alone has little effect on 4B1 inhibition of transport (Figure 7B) or 4B1 binding (Figure 7C), while exposure to avidin after treatment with MPB completely abolishes inhibition of transport by 4B1 and binding of the mAb (Figure 7B and Figure 7C, respectively). Therefore, it is highly unlikely that increased mass *per se* at the site of the epitope accounts for 4B1 inhibition of permease activity.

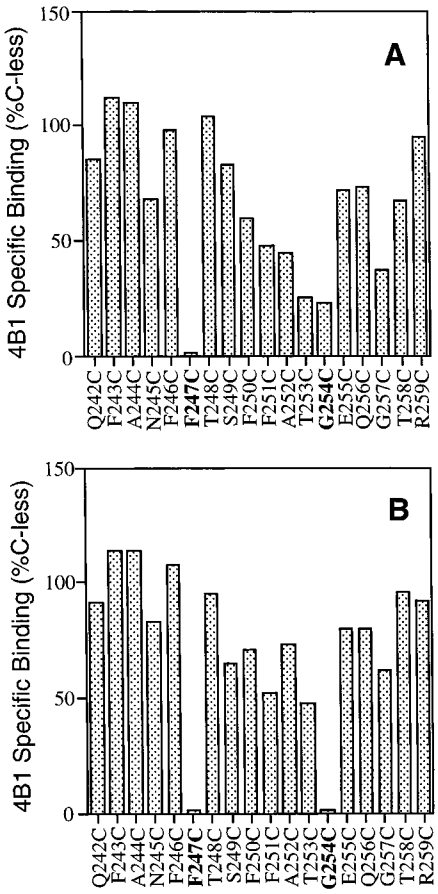

FIGURE 4: Binding of 4B1 to spheroplasts expressing single-Cys mutants in L(VII/VIII) and the effect of NEM. (A) Untreated spheroplasts expressing given single-Cys mutants were prepared and assayed for 4B1 binding as described in the Experimental Procedures. (B) Spheroplasts expressing given single-Cys mutants were incubated with 1 mM NEM at 25 °C for 30 min, 10 mM DTT was added, and the samples were washed with 100 mM KP_i (pH 7.0)/0.5 M sucrose. Binding of 4B1 was assayed as described for the untreated samples. 4B1 specific binding (i.e., nmol of 4B1 bound per mole of permease) is expressed as a percentage of that observed with spheroplasts expressing C-less permease. All data were corrected for 4B1 binding to spheroplasts harboring plasmid pT7-5 without a *lacY* insert.

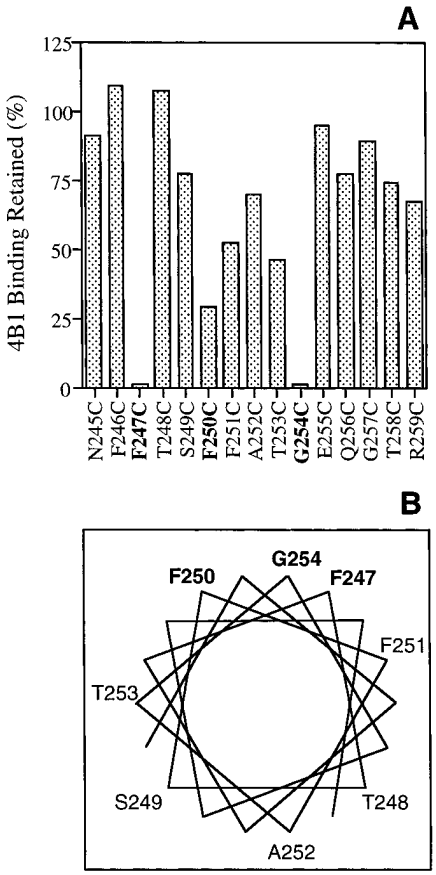

FIGURE 5: Effect of MPB on binding of 4B1 to spheroplasts expressing single-Cys mutants in L(VII/VIII). (A) Untreated spheroplasts expressing given single-Cys mutants were assayed directly for 4B1 binding as described in the Experimental Procedures. Identical samples were also treated with 1 mM MPB at 25 °C for 30 min, 10 mM DTT was added, and the samples were washed with 100 mM KP_i (pH 7.0)/0.5 M sucrose prior to assaying 4B1 binding. The results for the MPB-treated samples are expressed as a percentage of the untreated samples. All data were corrected for 4B1 binding to spheroplasts harboring plasmid pT7-5 without a *lacY* insert. (B) Helical wheel plot of residues 247−254.

DISCUSSION

Almost 15 years ago, mAbs against purified lac permease were prepared, and out of over 60 hybridoma lines cloned, only hybridoma 4B1 was found to secrete an IgG2a that inhibits active lactose transport in RSO membrane vesicles (Carrasco et al., 1982). Subsequent studies (Carrasco et al., 1984b; Herzlinger et al., 1984) demonstrate that 4B1 binds exclusively to an epitope on the periplasmic surface of the permease, that the intact antibody as well as Fab fragments inhibit transport, and that 4B1 uncouples lactose and H^+ translocation in such a manner that all translocation reactions involving net H^+ translocation are inhibited. On the other hand, ligand binding is not altered. Remarkably, moreover, equilibrium exchange and counterflow, which are thought to involve translocation of the ternary complex across the membrane without loss of H^+ (Carrasco et al., 1984b, 1986a, 1989; Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983), remain unaffected or are enhanced, respectively. The findings suggest that 4B1 binding inhibits deprotonation of the permease, thereby allowing the ternary complex to oscillate back and forth across the membrane, binding and releasing substrate on either face, but unable to lose H^+. Other experiments demonstrating that the effect of 4B1 is mimicked by D_2O (Viitanen et al., 1983) and particularly by a variety of amino acid substitutions for Glu325 (helix X) (Carrasco et al., 1986a, 1989) lend support to this idea. Since 4B1 clearly alters coupling between substrate and H^+ translocation in a highly specific manner, a variety of approaches were attempted to identify the epitope, all of which met with frustration. In this paper, we utilize insertional mutagenesis to localize the 4B1 epitope to the periplasmic loop between helices VII and VIII and Cys-scanning mutagenesis in conjunction with chemical modification to demonstrate that the primary determinants for 4B1 binding are Phe247 and Gly254.

In addition to the obvious conclusion that the findings provide direct support for *lacY-phoA* fusion analysis (Calamia & Manoil, 1990), indicating that this region of the permease is on the periplasmic surface of the membrane, there are a number of other important points raised by the study:

Although the 4B1 epitope is not discontinuous, 4B1 binding is conformationally dependent. Therefore, the epitope must contain a defined structure. Although it is impossible to draw a definitive conclusion in this regard from

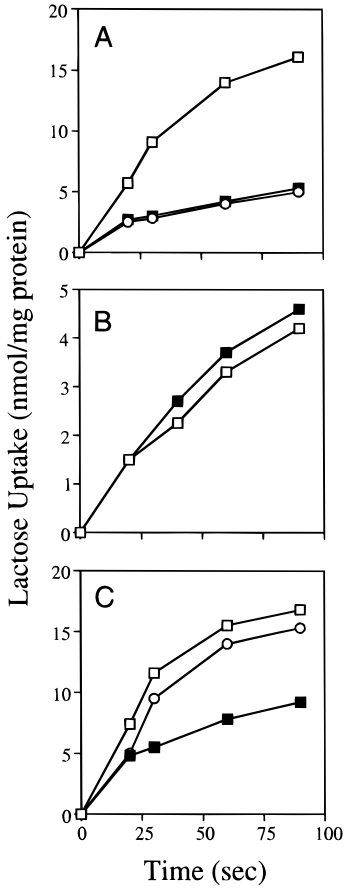

FIGURE 6: Effect of mAb 4B1 on lactose transport by RSO membrane vesicles containing Cys-less, G254C, or F247C permease. RSO vesicles from *E. coli* T184 expressing a given permease mutant were prepared and either assayed with no further treatment or incubated at 25 °C for 30 min with a 2-fold molar excess of 4B1 relative to permease. Where indicated, vesicles were also incubated with 1 mM NEM at 25 °C for 30 min, 10 mM DTT was added, and the samples were washed prior to assaying in the absence or presence of 4B1. [1-^{14}C]Lactose transport in the presence of ascorbate and PMS was assayed under oxygen as described in the Experimental Procedures. (A) Cys-less permease: □, no additions; ■, 4B1; ○, NEM treated plus 4B1. (B) F247C permease: □, no additions; ■, 4B1. (C) G254C permease: □, no additions; ■, 4B1; ○, NEM treated plus 4B1.

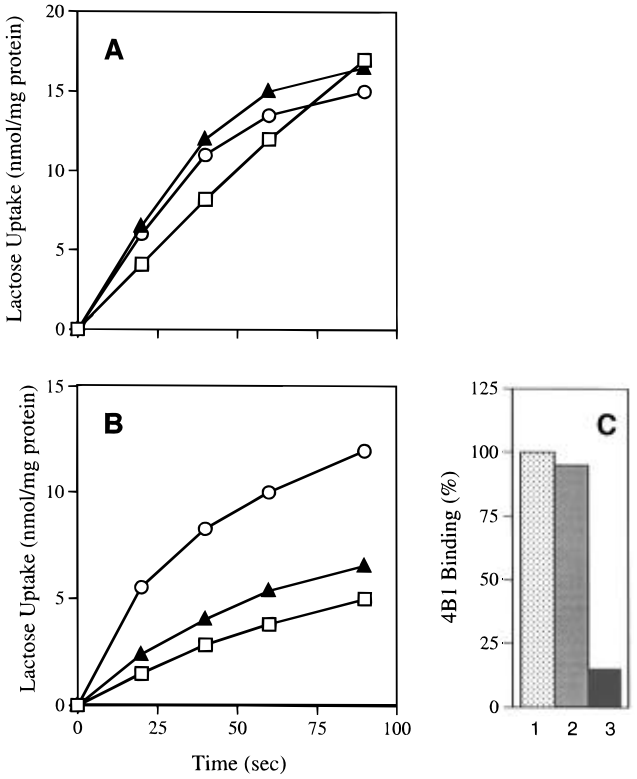

FIGURE 7: Effect of avidin binding to E255C permease on 4B1 inhibition of active transport and 4B1 binding. RSO vesicles from *E. coli* T184 expressing E255C permease mutant were prepared and assayed for [1-^{14}C]lactose transport as described in Figure 6 and *Experimental Procedures* under the following conditions. (A) No treatment (□); vesicles were incubated with 1 mM MPB at 25 °C for 30 min, 10 mM DTT was added, and the sample was washed once with 0.1 M KP_i (pH 7.5)/10 mM $MgSO_4$ prior to assay (▲); vesicles pretreated with MPB were assayed in the presence of a 5-fold molar excess of avidin relative to permease (○). (B) As described in (A) with a 2-fold molar excess of 4B1 relative to permease in addition. (C) 4B1 binding to spheroplasts expressing E255C permease. Binding of 4B1 was assayed as described in the Experimental Procedures and in Figure 3. Where indicated, the spheroplasts were assayed without treatment (**1**); after incubation with 1 mM MPB at 25 °C for 30 min and addition of 10 mM DTT, followed by one wash with 0.1 M KP_i (pH 7.0)/0.5 M sucrose (2); or after treatment with MPB as described above and in the presence of 50 μg/mL avidin (3).

the data at hand, the effect of MPB on 4B1 binding (Figure 5) exhibits a periodicity suggestive of α-helical structure. Furthermore, replacement of Gly254 with Cys does not abolish 4B1 binding, but causes about 75% inhibition, and alkylation abolishes binding. Although L(VII/VIII) does not contain a typical β-turn signature (Wilmot & Thornton, 1988), Gly is an amino acid commonly found in β-turns, and it is possible that the 4B1 epitope consists of a short, amphipathic helix (Figure 5B) lying parallel to the surface of the membrane, followed by a β-turn.

It is particularly interesting that Phe247 has been found to be the primary determinant in the 4B1 epitope, as this residue is depicted to be the C-terminal residue in helix VII (Figure 1), a transmembrane domain where there is ongoing discussion regarding the precise periplasmic boundary. Initial hydropathy profiling of the permease indicated that this transmembrane helix extends from Cys235 on the periplasmic surface to Leu210 on the cytoplasmic surface (Foster et al., 1983). Subsequently, a variety of findings (reviewed in Kaback, 1994; see Zen et al., 1994, and He et al., 1995a, in addition) demonstrate that Asp237 and Asp240 (helix VII) interact with Lys358 (helix XI) and Lys319 (helix X), respectively, and it was postulated that Asp237 and Asp240 are in the middle of helix VII with Phe247 at the N-terminus and Leu222 at the C-terminus (Figure 1). However, evidence has been presented (Jones et al., 1994; Ujwal et al., 1995) that these residues may be nearer to the periplasmic end of the helix than to the middle. The finding that Phe247 is the primary determinant in the 4B1 epitope makes it unlikely that this residue is within the membrane, as it would then be inaccessible to the mAb. Clearly, however, in order to resolve the periplasmic boundary of helix VII, more precise structural information is essential.

It was demonstrated recently (Sahin-Tóth et al., 1995) that, in order to construct an effective protease site in the loop containing the 4B1 epitope, insertion of a minimum of 3 factor Xa protease sites in tandem (a total of 12 amino acid residues) is necessary. In contrast, a single factor Xa site is sufficient when placed at the N-terminus of a biotin acceptor domain inserted into the middle cytoplasmic loop between helix VI and VII. The findings indicate that the loop

containing the epitope to 4B1 is probably buried within the tertiary structure of the permease. It is noteworthy, therefore, that 4B1 is directed to this region of the permease. Since the mAb binds with extremely high affinity, exhibiting a K_D of about 5×10^{-10} M, although the loop may be relatively inaccessible from the periplasmic surface, dynamic aspects of the structure can account for accessibility to a high-affinity mAb.

Perhaps the most interesting aspect of the findings is the observation that although 4B1 uncouples lactose and H^+ translocation, no amino acid residue in the loop between helices VII and VIII is essential for activity. Thus, each single-Cys replacement mutant in the loop catalyzes active lactose transport at highly significant initial rates and steady-state levels of accumulation (Table 1; see Frillingos et al., 1994, in addition). Furthermore, binding of avidin to a single biotinylated Cys residue at position 255 has no effect on transport. Since avidin is ca. 60 kDa, the mass of the mAb *per se* cannot be responsible for uncoupling. Rather, it seems more reasonable to suggest the mAb acts via secondary torsional effects on helix VII and/or VIII which in turn alter the pK_a(s) of a residue(s) involved in lactose-coupled H^+ translocation. In this respect, it is particularly interesting that helix VIII contains Glu269, a residue that is essential for transport and interacts with another essential residue, His322 (helix X), while helix VII contains Asp237 which interacts with Lys358 (helix XI) and Asp240 which interacts with Lys319 (helix X) (reviewed in Kaback, 1994; see He et al., 1995a, in addition). Although Asp237 and Asp240 do not play an essential role in the transport mechanism, as permease mutants with double neutral substitutions at positions 237 and 358 or 240 and 319 catalyze lactose accumulation, neutral substitution for either Asp residue alone causes markedly diminished activity. In any event, it is reasonable to postulate that torsional effects on either of these helices change the pK_a of one or more of these residues and that this change is reflected throughout the H-bonded network in the C-terminal half of the permease. In this respect, it is particularly interesting that 4B1 binding markedly alters the reactivity of V331C permease (Wu et al., 1994), as well as the fluorescence of this mutant after labeling with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) (J. Wu, S. Frillingos, and H. R. Kaback, unpublished observations).

ADDED IN PROOF

During the time this paper was in press, the following peptide containing residues 245−259 from L(VII/VIII) which encompasses the sequence of the 4B1 epitope with an additional C-terminal Cys residue was synthesized by J. Reeve in the Peptide Synthesis Facility at UCLA: AsnPhePheThrSerPhePheAlaThrGlyGluGlnGlyThrArgCys. Over a wide range of concentrations, the peptide exhibits no inhibitory effect whatsoever on 4B1 binding to spheroplasts expressing lac permease. Furthermore, when the C-terminal Cys residue is derivatized with the thiol-specific nitroxide spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate, no binding to mAb 4B1 is detected by electron paramagnetic resonance spectroscopy.

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