The Last Two Cytoplasmic Loops in the Lactose Permease of *Escherichia coli* Comprise a Discontinuous Epitope for a Monoclonal Antibody

Jianzhong Sun,[‡] Jun Li,[§] Nancy Carrasco,[||] and H. Ronald Kaback*,[‡]

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90095-1662, Molecular Immunology Department, Mail Code UE 0548, SmithKline Beecham, 709 Swedeland Road, King of Prussia, Pennsylvania 19406, and Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received September 11, 1996; Revised Manuscript Received October 31, 1996[⊗]

ABSTRACT: Monoclonal antibody (mAb) 4B11 binds to a conformational epitope in the lactose permease that is exposed on the cytoplasmic face of the membrane with a K_D of 2.8×10^{-7} M. By studying binding of 4B11 to permease mutants containing six contiguous His residues in each cytoplasmic loop, inserted factor Xa protease sites, or a C-terminal deletion, the cytoplasmic loops between helices VIII and IX (loop VIII/IX) and between helices X and XI (loop X/XI) are shown to comprise the epitope. Subsequently, Cys-scanning mutagenesis in conjunction with thiol modification was carried out in order to identify specific residues involved in 4B11 recognition. Glu342 and Arg344 in loop X/XI are primary determinants for 4B11 binding, while Ile283 in loop VIII/IX and Phe334 and Lys335 in loop X/XI are secondary determinants. Consistently, binding of avidin to biotinylated single-Cys replacements in loop VIII/IX or loop X/XI blocks 4B11 binding, but avidin binding to biotinylated Cys residues in other cytoplasmic loops or insertion of cytochrome b_{562} into cytoplasmic loop VI/VII has no significant effect. The studies demonstrate that the last two cytoplasmic loops in lactose permease comprise a discontinuous epitope for monoclonal antibody 4B11 and thereby provide independent evidence for the conclusion that helices VIII−XI are in close proximity.

The lactose (lac)[1] permease of *Escherichia coli* is a paradigm for secondary transport proteins from archaea to the mammalian central nervous system that transduce the energy stored in electrochemical ion gradients into concentration gradients [reviewed in Kaback (1983, 1989, 1992a) and Poolman and Konings (1993)]. This integral membrane protein catalyzes the coupled translocation of β-galactosides and H^+ with a stoichiometry of unity (i.e., symport or cotransport). Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted, 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 (Foster et al., 1983) in which the permease is composed of 12 α-helical rods that traverse the membrane in zig-zag fashion with the N and C termini on the cytoplasmic face (Figure 1). Evidence favoring general aspects of the model has been obtained from various approaches [reviewed in Kaback (1992b)], and analysis of a large number of lac permease−alkaline phosphatase (*lacY*−*phoA*) fusions has provided unequivocal support for the 12-helix motif (Calamia & Manoil, 1990). 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*- or *tris*-His residues) into transmembrane domains of the molecule (Jung et al., 1995; He et al., 1995a,b), through use of site-directed chemical cleavage (Wu et al., 1995b), site-directed spin-labeling, and thiol cross-linking [Wu et al., 1996; reviewed in Kaback (1996)].

Immunological approaches have provided a useful tool for studying lac permease. Use of site-directed polyclonal antibodies demonstrates that the second and the third cytoplasmic loops and the C terminus are on the cytoplasmic surface of the membrane (Seckler et al., 1983, 1986; Carrasco et al., 1984a, 1986; Danho et al., 1985; Herzlinger et al., 1985). Furthermore, a battery of monoclonal antibodies against the permease has been prepared (Carrasco et al., 1982), and one mAb from hybridoma 4B1 binds to a structurally dependent epitope on the periplasmic surface of the permease (Carrasco et al., 1982; Herzlinger et al., 1984) and uncouples lactose and H^+ translocation (Carrasco et al., 1982, 1984b; Frillingos & Kaback, 1996a). Recently, by using insertional mutagenesis and Cys-scanning mutagenesis with thiol modification, the epitope for 4B1 was localized to the periplasmic loop between helices VII and VIII (loop VII/VIII) and shown to comprise one face of a short amphipathic helix followed by a β-turn (Sun et al., 1996; J. Sun, S. Frillingos, and H. R. Kaback, submitted for publication).

* Address correspondence to this author at HHMI/UCLA 5-748 MacDonald Research Labs, Box 951662, Los Angeles, CA 90095-1662. Telephone: (310) 206-5053. Telefax: (310) 206-8623. E-mail: RonaldK@HHMI.UCLA.EDU.

[‡] University of California, Los Angeles.

[§] SmithKline Beecham.

[||] Albert Einstein College of Medicine.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; mAb, monoclonal antibody; DM, *n*-dodecyl β-D-maltopyranoside; NEM, *N*-ethylmaleimide; MPB, 3-(*N*-maleimidylpropionyl)biocytin; DTT, dithiothreitol; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate.

S0006-2960(96)02292-1 CCC: $14.00

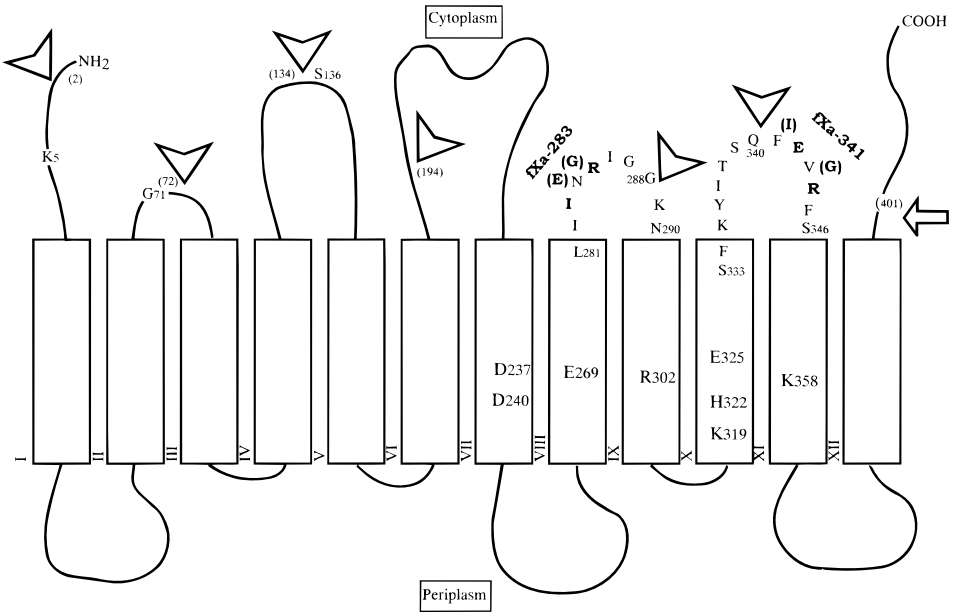

FIGURE 1: Secondary structure of lac permease showing the 6-His insertion sites and factor Xa protease sites within the cytoplasmic loops. The single-letter amino acid code is used, and the 12 hydrophobic transmembrane helices are depicted as rectangles. Each of the amino acid residues shown was replaced individually with a Cys in Cys-less permease. Arrowheads indicate the positions of either 6-His inserts (open arrowhead) or a C-terminal deletion at position 401 (open arrow). Factor Xa protease sites (IEGR) engineered into hydrophilic loops VIII/IX and X/XI are indicated. Cytochrome b_{562} was inserted at the same position as the 6-His insertion in the middle cytoplasmic loop.

In this paper, the same approach is utilized to characterize the recognition site for mAb 4B11 (Carrasco et al., 1982) which is shown to be a structurally dependent epitope on the cytoplasmic surface of the membrane composed of residues in the last two cytoplasmic loops.

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 the C terminus of lac permease (Carrasco et al., 1984a) was prepared by Babco. [α-^{35}S]dATP and [^{125}I]-protein A were from Amersham. 3-(*N*-Maleimidylpropionyl)biocytin was obtained from Molecular Probes. All other materials were reagent grade and obtained from commercial sources.

Mutant Construction. Single-Cys mutants were constructed by site-specific mutagenesis of the Cys-less version of cassette *lac Y* gene (EMBL X-56095) in plasmid pT7-5 (van Iwaarden et al., 1991) by using one- or two-stage polymerase chain reaction (PCR) (Ho et al., 1989). Mutations were verified by sequencing the length of the PCR-generated segment by using dideoxynucleotide termination and synthetic sequencing primers (Sanger et al., 1977) after alkali denaturation (Hattori & Sakaki, 1986). His-insertion mutants (McKenna et al., 1992), mutants containing factor Xa protease sites inserted at given positions in cytoplasmic loop VIII/IX or loop X/XI (Sahin-Tóth et al., 1995), and the C-terminal deletion mutant (Δ401; McKenna et al., 1991) were constructed as described (Figure 1).

Growth of Bacteria. E. coli T184 (*lacZ*$^-$*Y*$^-$) 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.

Membrane Preparation. Disrupted membrane fractions from T184 cells were prepared as described (Frillingos & Kaback, 1996b). Briefly, cells were suspended in ice-cold osmotic shock buffer [25 mM Tris-HCl (pH 8.0)/45% sucrose/1.0 mM ethylenediaminetetraacetate], centrifuged, resuspended in ice-cold water, allowed to stand for 10 min on ice, and then incubated with lysozyme (0.1 mg/mL) for 30 min. Suspensions were sonified, and after removal of unlysed cells, membranes were harvested by centrifugation.

Purification of mAb 4B11. Purification was carried out as described (Carrasco et al., 1982; Sun et al., 1996). The protein peak was dialyzed overnight against 0.1 M potassium phosphate (KP_i; pH 7.5), and aliquots of purified mAb were frozen and stored at −80 °C.

4B11 Binding to Purified Lac Permease. Wild-type lac permease or a mutant with cytochrome b_{562} inserted into loop VI/VII (Privé et al., 1994; Privé & Kaback, 1996), both containing six contiguous His residues at the C terminus, was purified by nickel chelate chromatography as described. mAb 4B11 was covalently coupled to a sensor chip (CM5) according to the manufacturer's instructions (Pharmacia Biosensor). Real-time detection of the interaction of 4B11 with the permease was monitored by measuring surface plasmon resonance using the BIAcore system (Pharmacia). Lac permease (80 μg/mL) in 50 mM KP_i (pH 7.5)/150 mM NaCl/0.1% *n*-dodecyl-β-D-maltoside (DM) was captured at a flow rate of 15 μL/min. Dissociation was followed by introducing the same solution free of permease at a flow rate of 15 μL/min.

4B11 Binding to Membranes. Aliquots of membrane preparations (0.2 mL containing 0.5 mg/mL protein) suspended in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$/10% bovine serum albumin (BSA)/1.0 M sucrose were mixed with 10 μL of affinity-purified 4B11 (0.75 mg/mL) and incubated

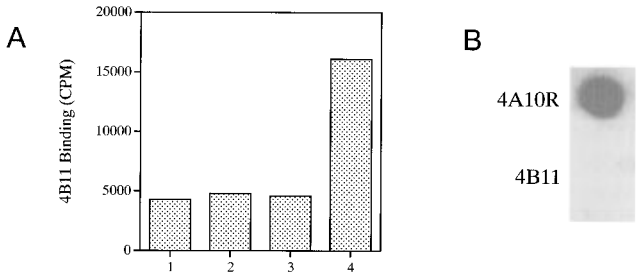

FIGURE 2: (A) Binding of mAb 4B11 to spheroplasts or sonified membranes with or without lac permease. Binding to spheroplasts was carried out as described previously (Sun et al., 1996). Sonified membranes were prepared and incubated with 4B11 and [^{125}I]protein A as described under Experimental Procedures. 1, spheroplasts without lac permease; 2, spheroplasts with lac permease; 3, membranes without lac permease; 4, membranes with lac permease. (B) Dot blot assay of mAb 4A10R or 4B11 to lac permease. An aliquot of a spheroplast suspension (10 μg total protein) was dissolved in 50 μL of 2% DM solution and blotted onto a nitrocellulose membrane by using a Bio-Dot microfiltration apparatus (Bio-Rad). After blocking with 10 mM Tris-HCl (pH 7.4)/ 150 mM NaCl/0.2% Triton X-100 (TBST) containing 5% BSA, the blots were transferred into a solution of TBST containing 5% BSA and 8 μg/mL mAb 4A10R or 4B11 and incubated for 1 h. The sheets were washed 3 times with TBST for 15 min each and incubated with a 1:1000 dilution of peroxidase-labeled protein A in TBST buffer containing 5% BSA for 1 h followed by three washes with TBST. The sheets were then developed with an ECL detection kit (Amersham).

at room temperature for 30 min. The membranes were harvested by centrifugation, washed once in the suspension buffer without BSA, and resuspended in 0.15 mL of suspension buffer. Two microliters of [^{125}I]protein A (30 mCi/mg; 100 μCi/mL) was added, and incubation was continued for 30 min. The membranes were then centrifuged, washed once, and resuspended to 50 μL of suspension buffer without BSA. Bound radioactivity was measured by liquid scintillation spectrometry using Scintsafe Econo 1 cocktail buffer.

Quantitation of Lac Permease. Permease was quantitated by PhosphorImager screening as described previously (Sun et al., 1996). Briefly, membrane fractions from *E. coli* T184 harboring plasmid with a given mutant were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Newman et al., 1981), and immunoblot analysis was carried out by using rabbit polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984a). The amount of permease was then quantitated with a PhosphorImager Model 425F (Molecular Dynamics).

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

RESULTS

4B11 Binds to a Conformational Epitope on the Cytoplasmic Surface of the Membrane. As shown in Figure 2A, 4B11 binds to disrupted membranes containing lac permease, but does not bind to membranes devoid of permease or more importantly to spheroplasts with or without the permease. Therefore, it is apparent that the 4B11 epitope is exposed from the cytoplasmic face of the membrane.

Dot blot analysis demonstrates that 4B11 does not bind to solubilized permease bound to nitrocellulose (Figure 2B). In contradistinction, mAb 4A10R which binds primarily to the C terminus in a manner that is not structurally dependent (Carrasco et al., 1982; Herzlinger et al., 1985) exhibits strong

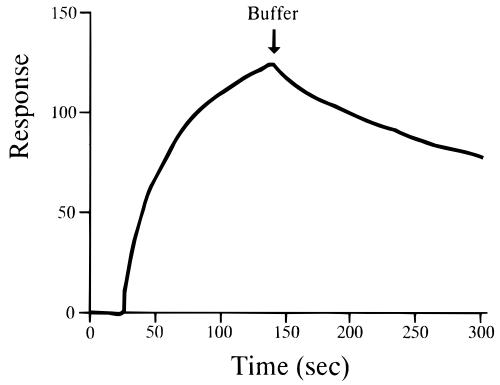

FIGURE 3: Sensograms of lac permease binding to immobilized mAb 4B11. Purified mAb 4B11 was immobilized on a sensor chip surface as described under Experimental Procedures. Purified lac permease with a 6-His tag at the C-terminus at a concentration of 80 μg/mL in 50 mM KP_i, pH 7.4/150 mM NaCl/ 0.1% DM solution was injected over the surface at a flow rate of 15 μL/min. The association phase and dissociation phase of the interaction were monitored. The arrow indicates the injection of buffer without lac permease.

binding under the same conditions, thereby indicating that the 4B11 epitope is sensitive to the conformation of the permease.

4B11 Binding to Purified Lac Permease. A variety of experimental approaches demonstrate that purified lac permease maintains close to native conformation in DM (Wu et al., 1994, 1995a; Wu & Kaback, 1994; Sun et al., 1996). Consistent with this conclusion, mAb 4B11 binds to purified permease in DM as determined by surface plasmon resonance (Figure 3). With mAb 4B11 conjugated to the chip, addition of purified wild-type permease in DM exhibits time-dependent association. Analysis of the binding phase yields a k_{assoc} of 1.0×10^4 M^{-1} s^{-1}, and analysis of the dissociation phase exhibits a relatively slow k_{dissoc} of 2.8×10^{-3} s^{-1}, yielding an equilibrium binding constant of 2.8×10^{-7} M. With a mutant chimeric permease containing cytochrome b_{562} in the middle cytoplasmic loop (Privé et al., 1994; Privé & Kaback, 1996), an equilibrium binding constant of about 5×10^{-7} M is observed (data not shown).

4B11 Binding to Insertion Mutants in Cytoplasmic Loops or a C-Terminal Deletion Mutant. Since 4B11 binds to an epitope on the cytoplasmic surface of the protein, mAb binding was determined with mutants containing six contiguous His residues in each cytoplasmic loop (McKenna et al., 1992), mutants with a factor Xa protease site in loop VIII/ IX or loop X/XI (Sahin-Tóth et al., 1995), or a C-terminal deletion mutant (Δ401; McKenna et al., 1991) (Figure 4). Clearly, only the mutants with a 6-His insert or a factor Xa protease site in loop VIII/IX (after Gly288 or at position 283, respectively; see Figure 1) and a 6-His insert or a factor Xa protease site in loop X/XI (after Gln340 or at position 341, respectively; see Figure 1) exhibit significant reduction in 4B11 binding. The other 6-His insertion mutants and Δ401 permease bind 4B11 as well as C-less or wild-type permease. Based on these results, it is likely that the 4B11 recognizes the last two cytoplasmic loops in the permease (i.e., loops VIII/IX and X/XI).

Binding by Single-Cys Mutants. In order to determine which amino acid residues comprise the 4B11 epitope, single-Cys replacement mutants at each position in loops VIII/IX

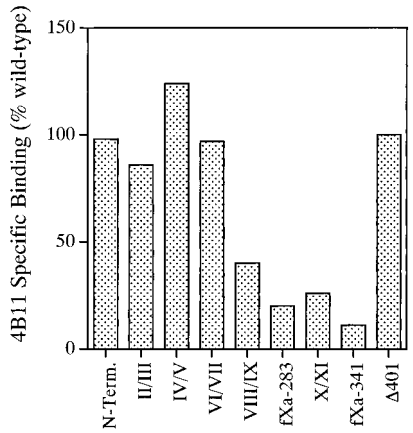

FIGURE 4: Binding of mAb 4B11 to sonified membrane preparations containing lac permease mutants with 6-His residues inserted into the cytoplasmic loops, factor Xa sites inserted at position 283 (loop VIII/IX) or 341 (loop X/XI), or deletion of the C terminus (Δ401). Plasmids encoding given mutants were constructed and overexpressed, and sonified membranes were prepared and incubated with 4B11 and [^{125}I]protein A as described under Experimental Procedures. The amount of permease expressed in each mutant was quantitated by immunoblotting with anti-C-terminal antibody as described under Experimental Procedures, except for the Δ401 mutant which was quantitated with mAb 4B1 (Sun et al., 1996). 4B11 specific binding (i.e., moles of 4B11 bound per mole of permease) is expressed as a percentage of that observed with membranes expressing wild-type permease. All data were corrected for 4B11 binding to membrane harboring plasmid pT7-5 without a *lacY* insert.

and X/XI (with the exceptions of G287C[2] and Y336C which are poorly expressed) were tested for 4B11 binding before and after alkylation with N-ethylmaleimide (NEM) or 3-(*N*-maleimidylpropionyl)biocytin (MPB) (Table 1; Figure 5A,B). Strikingly, mutant E342C or mutant R344C exhibits essentially no binding, indicating that Glu342 and Arg344 are primary epitope determinants. The remaining mutants exhibit specific binding activities that range from 70 to 130% of control.

After alkylation of each mutant with NEM or MPB, it becomes apparent that Ile283 (loop VIII/IX), Phe334, and Lys335 (loop X/XI) are probably secondary epitope determinants, since NEM or MPB treatment reduces 4B11 binding by 50−70% or 70−80%, respectively. In addition, binding by mutants K289C, V343C, or F345C is reduced by about 40% after alkylation with NEM and/or MPB. Therefore, Lys289 (loop VIII/IX), Val343, and Phe345 (loop X/XI) may also play a minor role in 4B11 recognition.

Effect of Avidin Binding to Cytoplasmic Loops on 4B11 Binding. In order to provide further evidence that the 4B11 epitope is located specifically in loops VIII/IX and X/XI, single-Cys mutants in the N terminus (K5C) and cytoplasmic loop II/III (G71C), IV/V (S136C), VIII/IX (G288C), and X/XI (K335C) were biotinylated with MPB, and 4B11 binding was tested after exposure of disrupted membranes to avidin [see Sun et al. (1996)]. As shown in Figure 6, only mutants G288C and K335C exhibit decreased 4B11 binding after exposure to MPB and avidin. Furthermore, as discussed above, permease with cytochome b_{562} in the middle cytoplasmic loop exhibits an equilibrium binding constant

[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 a second letter denoting the amino acid replacement at this position.

Table 1: Expression and 4B11 Binding of Single-Cys Mutants

		4B11 specific binding (% C-less)		
mutant	expression[a]	no treat[b]	NEM[c]	MPB[d]
L281C	+++	111	102	86
I282C	+++	114	103	79
I283C	+++	104	50	22
N284C	+++	103	130	90
R285C	+++	118	105	101
I286C	+++	105	106	91
G287C	+	ND	ND	ND
G288C	+++	70	66	54
K289C	+++	96	61	60
N290C	+++	130	99	117
S333C	+++	85	82	70
F334C	+++	75	35	30
K335C	+++	90	50	20
Y336C	+	ND	ND	ND
I337C	+++	101	82	81
T338C	+++	115	108	103
S339C	+++	100	112	68
Q340C	+++	117	101	111
F341C	+++	102	110	100
E342C	+++	1	1	1
V343C	+++	90	104	62
R344C	+++	1	1	1
F345C	+++	120	120	62
S346C	+++	95	95	71

[a] Expression of the mutants as judged from Western blot analysis: +++, 70−130%; +, 5−10%. Boldfaced mutants are those showing decreased binding to 4B11. [b] Membrane preparations and 4B11 binding assays were performed as described under Experimental Procedures. Binding to mutants G287C and Y336C could not be measured due to the low expression. [c] Membrane preparations were incubated with 1.0 mM NEM at 25 °C for 30 min, 10 mM dithiothreitol was added, and binding of 4B11 was assayed as described for the untreated samples. [d] Membrane preparations were incubated with 1.0 mM MPB at 25 °C for 30 min, 10 mM dithiothreitol was added, and 4B11 binding was assayed as described for the untreated samples.

for 4B11 that is comparable to wild-type, and Δ401 permease which is devoid of the C-terminus binds 4B11 normally (Figure 4). Clearly, therefore, the epitope for mAb 4B11 is restricted to loops VIII/IX and X/XI.

DISCUSSION

As demonstrated here, mAb 4B11, an IgG_{2a} mAb described almost 15 years ago (Carrasco et al., 1982), binds to a structurally dependent epitope in lac permease that is exposed on the cytoplasmic face of the membrane. Furthermore, by utilizing a recently developed approach for characterizing discontinuous epitopes (Sun et al., 1996), we show that the 4B11 epitope is composed of amino acid residues in the last two cytoplasmic loops of the permease. Thus, use of mutants with insertions in each cytoplasmic loop or a C-terminal deletion allows relatively gross localization of the epitope to loops VIII/IX and X/XI, and Cys-scanning mutagenesis in conjunction with thiol modification highlights Glu342 and Arg344 (loop X/XI) as primary determinants and Ile283 (loop VIII/IX), Phe334, and Lys335 (loop X/XI) as secondary determinants. In addition, evidence is presented suggesting that Lys289 (loop VIII/IX), Val343, and Phe345 (loop X/XI) also play a minor role. In contradistinction, the N terminus, the other cytoplasmic loops, or the C terminus plays no role whatsoever in 4B11 recognition, as evidenced by the findings that insertion of six His residues into the N terminus and cytoplasmic loops other than VIII/IX or X/XI, deletion of the C terminus, or

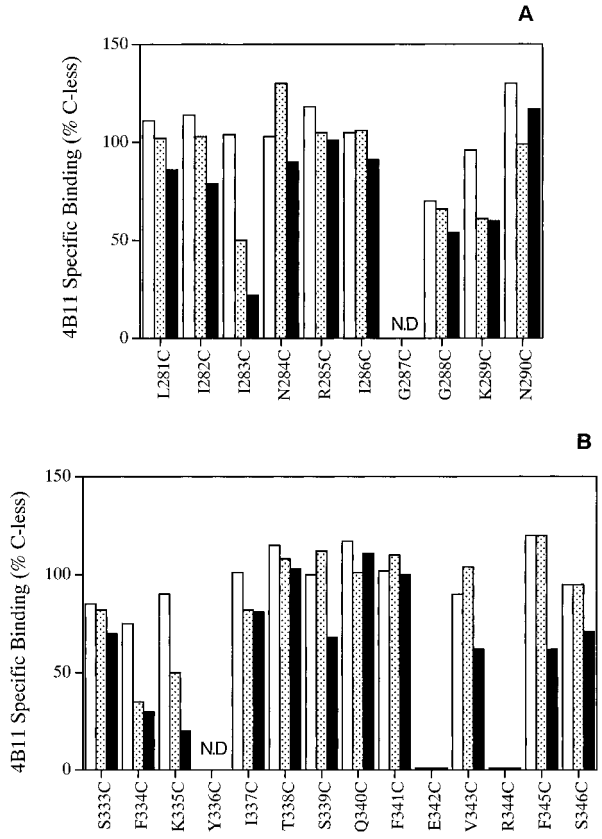

FIGURE 5: Binding of mAb 4B11 to sonified membranes expressing given single-Cys mutants in loop VIII/IX (A) or loop X/XI (B). Given mutants were overexpressed, and sonified membranes were prepared and incubated with 4B11 and [^{125}I]protein A as described under Experimental Procedures. The amount of permease expressed in each instance was quantitated by immunoblotting as described under Experimental Procedures. 4B11 specific binding (i.e., moles of 4B11 bound per mole of permease) is expressed as a percentage of that observed with membranes expressing C-less permease. All data were corrected for 4B11 binding to membrane harboring plasmid pT7-5 without a *lacY* insert. Open bars, no treatment; stippled bars, samples incubated with 1.0 mM NEM at 25 °C for 30 min and quenched with 10 mM DTT; solid bars, samples incubated with 1.0 mM MPB at 25 °C for 30 min and quenched with 10 mM DTT.

insertion of cytochrome b_{562} into loop VI/VII have no significant effect on 4B11 binding.

In addition to the obvious conclusion that the findings provide direct support for *lacY*−*phoA* fusion analysis (Calamia & Manoil, 1990), indicating that loops VIII/IX and X/XI are on the cytoplasmic surface of the membrane, there are a number of other important points raised by the study:

Purified lac permease binds 4B11 in DM, as determined by surface plasmon resonance, thereby providing additional support for the contention that the permease maintains near-native structure in this detergent (Wu et al., 1994, 1995a; Wu & Kaback, 1994; Sun et al., 1996). However, unlike the situation with the 4B1 epitope which is in periplasmic loop VII/VIII and appears to be on one face of a short helix (Sun et al., 1996), it is not possible to speculate further about the structure of the 4B11 epitope because the binding observed with the Cys-replacement mutants before or after alkylation does not exhibit a regular pattern.

Replacement of any residue in loops VIII/IX and X/XI with Cys except for Glu342 and Arg344 causes little or no reduction in 4B11 binding. In marked contrast, E342C or R344C permease binds essentially no 4B11. Thiol modification with NEM or MPB inhibits 4B11 binding to mutants I283C, K289C, F334C, and K335C, and in most instances, MPB with a bulkier alkylating group is more effective than NEM. Finally, binding by mutants V343C and F345C is reproducibly inhibited by MPB, albeit by only about 40%, but not by NEM. Therefore, it seems clear that Glu342 and Arg344 (loop X/XI) are the primary determinants for 4B11 recognition. In contrast, none of the other residues appears to be directly involved in mAb recognition, since the Cys-replacement mutants bind in a manner comparable to the control. However, since binding by mutants I283C, K289C, F334C, and K335C or mutants V343C and F345C, respectively, is significantly inhibited by NEM or MPB or by MPB exclusively, we suggest that these positions are close to the primary epitope determinants, Glu342 and Arg344 (Figure 7). By this means, Cys replacement in itself causes little or no effect on 4B11 binding, but increasing the bulk of the Cys side chain at these positions results in steric inhibition, and a more marked effect is observed with MPB than NEM depending upon the proximity of the particular position to the primary determinants. In any case, the finding that Phe334 is a secondary determinant makes it unlikely that this position is within the membrane, as it would then be inaccessible to the mAb (Figure 1).

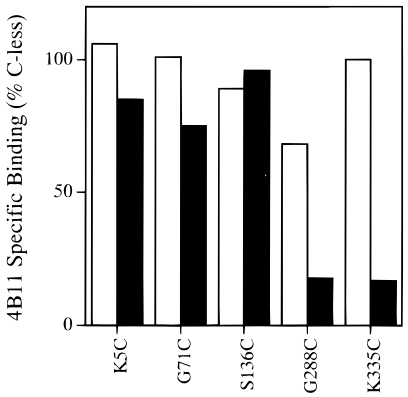

FIGURE 6: Effect of avidin attachment to biotinylated Cys residues in cytoplasmic loops on 4B11 binding. Open bars, sonified membrane preparations expressing given single-Cys mutants were prepared and assayed in the presence of 0.1 mg/mL avidin for 4B11 binding as described under Experimental Procedures. Solid bars, sonified membrane preparations expressing given single-Cys mutants were incubated with 1.0 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. Avidin was then added to a final concentration of 0.1 mg/mL, and binding of 4B11 was assayed as described for the untreated samples. 4B11 specific binding (moles of 4B11 bound per mole of permease) is expressed as a percentage of that observed with membranes expressing C-less permease.

Since the mutants used in this study exhibit significant ability to catalyze lactose accumulation against a concentration gradient, it seems unlikely that the mutations cause gross changes in the folding of the mutant proteins. Furthermore, inhibition of 4B11 binding by alkylation of certain single-Cys replacement mutants appears to correlate with the bulk of the adduct (i.e., MPB is generally more effective than NEM). In addition, avidin binding to biotinylated single-Cys replacements in loop VIII/IX or X/XI blocks 4B11 binding, but binding of avidin to biotinylated Cys residues in other cytoplasmic loops or insertion of cytochrome b_{562} into cytoplasmic loop VI/VII has no significant effect. Taken as a whole, therefore, the results suggest that reduction of binding results from direct steric effects rather than indirect

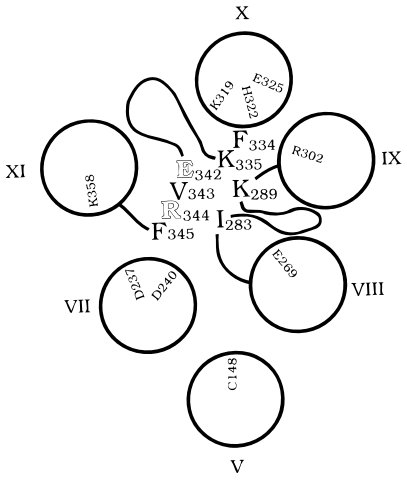

FIGURE 7: Helix packing model of helices V and VII−XI in lac permease viewed from the cytoplasm showing loops VIII/IX and X/XI which comprise a discontinuous epitope for 4B11. The transmembrane helices are depicted as circles. Irreplaceable residues and interacting Asp-Lys pairs are given. Primary determinants are represented by enlarged and open amino acid letters; secondary determinants by enlarged ones.

effects caused by conformational alterations in the loops. However, in either case, it is apparent that loops VIII/IX and X/XI must be in close proximity.

The packing of helices V and VII−XI (Figure 7) is based on site-directed excimer fluorescence (Jung et al., 1993) which demonstrates that helix VIII (Glu269) is close to helix X (His322), that helix IX (Arg302) is close to helix X (Glu325), and that helix X is in an α-helical conformation. The presence of two pairs of charged residues that interact functionally—Asp237 (helix VII) with Lys358 (helix XI) and Asp240 (helix VII) with Lys319 (helix X)—demonstrates that helix VII is close to helices X and XI (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos & Kaback, 1996c). These proximity relationships have been confirmed by engineering divalent metal-binding sites (*bis*- or *tris*-His residues) within the permease (Jung et al., 1995; He et al., 1995a,b). In addition, site-directed chemical cleavage supports the positioning of helix X next to helices VII and XI and indicates further that helix V is in close proximity to helices VII and VIII (Wu et al., 1995b). The relationship between helices V, VII, and VIII has been confirmed by site-directed spin-labeling and thiol cross-linking experiments (Wu et al., 1996). The findings presented here are particularly important in the context of the helix packing model shown, as they demonstrate that the 4B11 epitope is comprised of amino acid residues in the last two cytoplasmic loops in the permease. Therefore, the studies provide strong, independent support for the contention that helices VIII, IX, X, and XI must be within close proximity.

ACKNOWLEDGMENT

We thank Kerstin E. Stempel for synthesizing oligodeoxynucleotides and Alberto Gonzales for excellent technical assistance.

REFERENCES

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937−4941.

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894−6898.

Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984a) *Biochemistry 23*, 3681−3687.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672−4676.

Carrasco, N., Herzlinger, D., Dechiara, S., Danho, W., & Kaback, H. R. (1985) *Ann. N.Y. Acad. Sci. 456*, 305−306.

Carrasco, N., Herzlinger, D., Danho, W., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 453−467.

Danho, W., Makofske, R., Humiec, F., Gabriel, T. F., Carrasco, N., & Kaback, H. R. (1985) in *Peptides: Structure and Function* (Deber, C. M., Hruby, V. J., & Kopple, K. D., Eds.) pp 59−62, Pierce Chemical Co., Rockford, IL.

Dunten, R. L., Sahin-Toth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 3139−3145.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31−34.

Frillingos, S., & Kaback, H. R. (1996a) *Biochemistry 35*, 10166−10171.

Frillingos, S., & Kaback, H. R. (1996b) *Biochemistry 35*, 3950−3956.

Frillingos, S., & Kaback, H. R. (1996c) *Biochemistry 35*, 13363−13367.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 1291−1297.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Biochemistry 34*, 15661−15666.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 15667−15670.

Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688−3693.

Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221−229.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51−59.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273−12278.

Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 6272−6277.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95−112.

Kaback, H. R. (1989) *Harvey Lect. 83*, 77−103.

Kaback, H. R. (1992a) *Int. Rev. Cytol. 137A*, 97−125.

Kaback, H. R. (1992b) *Biochim. Biophys. Acta 1101*, 210−213.

Kaback, H. R. (1996) in *Handbook of Biological Physics II: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., & Lolkema, J. S., Eds.) pp 203−227, Elsevier, Amsterdam.

Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Zen, K. (1994) *J. Exp. Biol. 196*, 183−195.

King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177−186.

Lee, J. L., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−20764.

McKenna, E., Hardy, D., Pastore, J. C., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2969−2973.

McKenna, E., Hardy, D., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954−11958.

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804−11808.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.

Poolman, B., & Konings, W. N. (1993) *Biochim. Biophys. Acta 1183*, 5−39.

Privé, G. G., & Kaback, H. R. (1996) *J. Bioenerg. Biomembr. 28*, 29−34.

Privé, G. G., Verner, G. E., Weitzman, C., Zen, K. H., Eisenberg, D., & Kaback, H. R. (1994) *Acta Crystallogr.* 375−379.

Sahin-Tóth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 10027−10035.

Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547−10551.

Sahin-Tóth, M., Lawrence, M. C., & Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5425.

Sahin-Tóth, M., Dunten, R. L., & Kaback, H. R. (1995) *Biochemistry 34*, 1107−1112.

Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817−10820.
Seckler, R., Moroy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403−2409.
Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−998.
van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595−9600.
Wu, J., & Kaback, H. R. (1994) *Biochemistry 33*, 12166−12171.
Wu, J., Frillingos, S., Voss, J., & Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.
Wu, J., Frillingos, S., & Kaback, H. R. (1995a) *Biochemistry 34*, 8257−8263.
Wu, J., Perrin, D., Sigman, D., & Kaback, H. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−9190.
Wu, J., Voss, J., Hubbell, W. L., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10123−10127.

BI962292F

Corrections

Solution NMR Evidence That the HIV-1 Protease Catalytic Aspartyl Groups Have Different Ionization States in the Complex Formed with the Asymmetric Drug KNI-272, by Yun-Xing Wang, Darón I. Freedberg, Toshimasa Yamazaki, Paul T. Wingfield, Stephen J. Stahl, Joshua D. Kaufman, Yoshiaki Kiso, and Dennis A. Torchia*, Volume 35, Number 31, August 6, 1996, Pages 9945−9950.

Page 9946. In the caption to Figure 1, H_{10} should read H_1. H_1 is the methylthioalanine H_α of KNI-272.

Page 9947. In Figure 3, H_{10} should read H_1 in the spectrum, and H_{23} should read H_{22} in the caption.

Page 9948. In Figure 4, H_{16}, H_{20}, and H_{21} should read H_1, H_{18}, and H_{19}, respectively, T_{12}/T_{112} should be in italic letters, methyl should read thiomethyl, the D29−R87 link should read D30−R87, and the D129−R187 link should be omitted.

Page 9950. Tables S2 and S3 (in Supporting Information) have also been corrected and are available as new Supporting Information.

The Asp assignments in the paper are correct, as are the results and conclusions regarding the Asp pK_a values and ionization states.

SUPPORTING INFORMATION AVAILABLE

Proton chemical shifts of inhibitor KNI-272 (Table S2) and chemical shift assignments of the ^1H, ^{13}C, and ^{15}N signals of the HIV-1 protease/KNI-272 complex (Table S3) (7 pages). Ordering information is given on any current masthead page.

BI965013X

S0006-2960(96)05013-1 CCC: $14.00 Published 1997 by the American Chemical Society