Purification and Functional Characterization of the C-Terminal Half of the Lactose Permease of *Escherichia coli*

Jianhua Wu, Jianzhong Sun, 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

Received January 10, 1996; Revised Manuscript Received February 15, 1996[⊗]

ABSTRACT: The lactose permease has been expressed in contiguous, non-overlapping polypeptide fragments containing the N-terminal (N6) and C-terminal (C6) transmembrane domains of the protein [Bibi, E., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4325; Zen, K., et al. (1994) *Biochemistry 33*, 8198]. When expressed individually, N6 and C6 are unstable and do not catalyze active transport. However, when expressed simultaneously, the polypeptides stabilize each other and form a complex that catalyzes active lactose transport. Moreover, a deletion construct containing the first transmembrane domain and the six C-terminal transmembrane domains mediates downhill lactose translocation [Bibi et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7271]. Here we report that C6 can be expressed independently in a relatively stable form that binds monoclonal antibodies 4B1 and 4B11, which interact with conformationally dependent epitopes on the periplasmic and cytoplasmic surfaces of the membrane, respectively. In addition, C6 retains the ability to catalyze lactose translocation down a concentration gradient in a specific manner. Finally, as observed with full-length Val331Cys permease, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside quenches the fluorescence of 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS)-labeled C6 with a single-Cys residue in place of Val331, exhibiting an apparent K_d of 0.4 mM. Unlike full-length Val331Cys permease, however, ligand does not induce a change in the position of the emission maximum of MIANS-labeled C6(Val331Cys) permease nor in the reactivity of C6(Val331Cys) permease with MIANS. The results indicate that C6 retains a conformation similar to that in the native permease and that most of the structure required for high-affinity binding and substate translocation is located in the C-terminal half of the molecule.

The lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic, plasma membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+. The permease is encoded by the *lacY* gene, which has been cloned and sequenced, and the *lacY* gene product has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport [reviewed in Kaback (1983, 1989, 1992) and Poolman and Konings (1993)] as a monomer [see Sahin-Tóth et al. (1994)]. On the basis of circular dichroism and hydropathy analysis of the primary amino acid sequence, a secondary-structure was proposed (Foster et al., 1983) in which the permease is composed of 12 hydrophobic α-helices that traverse the membrane in zig-zag fashion connected by hydrophilic loops with both N- and C-termini in the cytosolic side. Evidence favoring general aspects of the model and showing that the N- and C-termini, as well as the loops between helices IV and V and VI and VII, are on the cytoplasmic face of the membrane has been obtained from a variety of experimental approaches [see Kaback (1983, 1989, 1992)]. Moreover, analysis of a large number of lac permease−alkaline phosphatase (*lacY-phoA*) fusions has provided unequivocal support for the 12 transmembrane−helix motif (Calamia & Manoil, 1990). On the basis of 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; 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 (Jung et al., 1995; He et al., 1995a,b) and through the use of site-directed chemical cleavage [Wu et al., 1995; reviewed in Kaback (1996)].

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 II−VI (N_1C_6; Bibi et al., 1991). With over 95% of the residues in the permease mutagenized [reviewed in Kaback et al. (1994, 1996)], the four charged residues found to be mandatory for active transport thus far are located in transmembrane domains in the C-terminal half of the permease. Furthermore, the epitope for a monoclonal antibody (mAb) that uncouples lactose from H^+ translocation has been localized

* Corresponding author.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1996.

[1] Abbreviations: lac, lactose; N1C6, lac permease deleted of helices II−VI; C6, the C-terminal half of lac permease with transmembrane domains VII−XII; mAb, monoclonal antibody; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; RSO, right-side-out; IPTG, isopropyl 1-thio-β,D-galactopyranoside; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; DM, dodecyl β,D-maltopyranoside; KP_i, potassium phosphate; C-less permease, functional lac permease devoid of Cys residues.

[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 wild-type lac permease. The sequence is followed by a second letter denoting the amino acid replacement.

0006-2960/96/0435-5213$12.00/0

recently (Sun et al., 1996) to the periplasmic loop between helices VII and VIII. 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 in the N-terminal half of the permease, as judged by site-directed and Cys-scanning mutagenesis [see Kaback et al. (1994) and Kaback (1996)].

Bibi and Kaback (1990) restricted the *lacY* gene into two approximately equal-size fragments which were subcloned individually or together under separate *lacZ* operator/promoters [see Wrubel et al. (1990, 1994) and also Zen et al. (1994)]. Under these conditions, lac permease is expressed in two portions: (i) the N-terminus, the first six putative transmembrane helices and most of putative loop 7; and (ii) the last six putative transmembrane helices and the C-terminus. Cells expressing both fragments catalyze active lactose transport, while cells expressing either half of the permease independently exhibit no activity whatsoever. Methionine labeling and immunoblotting experiments demonstrate that intact permease is completely absent from the membrane of cells expressing *lacY* fragments either individually or together. Thus, transport activity must result from an association between independently synthesized pieces of lac permease. When the gene fragments are expressed individually, the N-terminal portion of the permease is observed sporadically and the C-terminal portion is not observed. When the gene fragments are expressed together, polypeptides identified as the N- and C-terminal moieties of the permease are found in the membrane. Thus, the N- or C-terminal halves of lac permease are proteolyzed when synthesized independently, and association between the two complementing polypeptides leads to a more stable, catalytically-active complex.

Recently (Wu et al., 1995), a polypeptide fragment containing the C-terminal half of the permease (C6) with an additional five amino acid residues at the N-terminus was expressed independently in a relatively stable form, thereby allowing studies on this portion of the permease devoid of the other half of the molecule. In this report, it is demonstrated that C6 interacts with two different monoclonal antibodies (mAbs) that bind to conformationally dependent epitopes on either side of the membrane, that C6 catalyzes downhill lactose transport, and that the solubilized, purified fragment retains ability to bind ligand with relatively high affinity. It is concluded that C6 expressed independently of N6 probably retains much of the structure of the analogous portion of native lac permease and at least some of the structural requirements for substrate binding and translocation.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Plasmid pC6 was constructed in two steps. First, the *Bam*HI-*Hin*dIII *lacY* DNA fragment encoding intact lac permease was ligated into pSU2718 (Martinez & Cruz, 1988) that had been digested with both *Bam*HI and *Hin*dIII. The DNA fragment encoding the N-terminal half of the permease was then removed by digesting the construct with *Sac*I and *Xho*I, followed by intramolecular ligation after filling in both ends with the Klenow fragment of DNA polymerase. Plasmid pC6-(V331C) was constructed by replacement of the *Bxt*XI-*Hin*dIII fragment of pC6 with the corresponding *lacY* DNA fragment encoding Cys-less C6 with a single single mutation at codon 331 that changes the native Val into a Cys residue [C6(V331C)]. To facilitate purification of C6(V331C), a DNA fragment encoding the biotin acceptor domain from the oxaloacetate decarboxylase of *Klebsiella pneumoniae* was placed at the 5′ end of the *Bxt*XI-*Hin*dIII fragment (Consler et al., 1993) to make plasmid pC6(V331C)/BD.

Downhill Lactose Transport. E. coli HB101 harboring a given plasmid were grown at 37 °C and induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 2 h. Cells were harvested and suspended in 50 mM KP_i (pH 7.4)/10 mM $MgSO_4$, and carbonyl cyanide-*m*-chlorophenylhydrazone was added to a final concentration of 20 μM. Where indicated, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) or sucrose was added to final concentration of 20 mM, and the cell suspensions were incubated at room temperature for 10 min prior to addition of 1 mM [1-^{14}C]-lactose (10 mCi/mmol). Samples were taken at given time and rapidly filtered as described (Trumble et al., 1984).

Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared by osmotic lysis of spheroplasts as described (Kaback, 1971; Short et al., 1975).

4B1 Binding. Aliquots of RSO vesicles [0.5 mL containing 0.1 mg of protein/mL in 100 mM KP_i (pH 7.0) containing 5% bovine serum albumin] were mixed with 5 μL of affinity-purified 4B1 (5 mg/mL), incubated at room temperature for 1 h, centrifuged, washed once, and resuspended in 0.4 mL of the same solution. A 2 μL amount of [^{125}I]-labeled protein A (30 mCi/mg; 100 mCi/mL) was added, and incubation was continued for 45 min. The vesicles 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.

4B11 Binding. RSO vesicles were sonified and subjected to low-speed centrifugation to remove debris. Membranes were then harvested by centrifugation at 100 000g_{max} for 30 min. Binding of mAb 4B11 (Carrasco et al., 1982) to membranes was determined as described for mAb 4B1 binding except that the sonified membranes with bound 4B11 and [^{125}I]-labeled protein A were harvested at 100 000g_{max} for 30 min in order to assay bound mAb.

Expression and Purification of C6. E. coli T184 (Y^-Z^-) transformed with pC6(V331C)/BD were grown at 37 °C in LB broth (12 L) with streptomycin (10 μg/mL) and chloramphenicol (20 μg/mL), 0.5 mM IPTG was added at an OD_{600} of 0.8, and growth was continued for 4 h. Cells were harvested, and crude membrane fractions were prepared (Viitanen et al., 1986) and solubilized with 2.0% dodecyl β-D-maltopyranoside (DM) by incubation at 30 °C for 30 min with continuous stirring. Solubilized biotinylated lac permease was purified by affinity chromatography on immobilized monovalent avidin (Consler et al., 1993; Wu & Kaback, 1994). The purity of each preparation was ascertained by electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide gel (Laemmli, 1970) followed by silver staining. Protein was determined as described (Bradford, 1976) with bovine serum albumin as standard.

Labeling of Purified Single-Cys C6 Permease with 2-(4′-Maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) and Fluorescence Measurements. MIANS (Molecular Probes, Inc.) was dissolved in methanol, and the concentration was determined by measuring absorbance at 322 nm and using a

extinction coefficient of 17 000 (Haugland, 1994). Fluorescence was measured at 30 °C in an SLM 8000C spectrofluorimeter (SLM-Amico Instruments Inc., Urbana, IL). Emission spectra were recorded using an excitation wavelength of 330 nm and 4 nm slits for both excitation and emission.

To determine the rate of MIANS reaction with purified C6(V331C) polypeptide, 25 μg of protein/mL was preincubated with given ligands in an assay mixture containing 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM for 5 min at 30 °C. Reactions were initiated by addition of MIANS to a final concentration of 4 μM from a 1 mM stock solution, and fluorescence was monitored continuously at an emission wavelength of 418 nm. Where indicated, TDG or lactose was added to given final concentrations.

For complete modification of Cys residues, reactions were carried out at 4 °C for 60 min with a 10-fold molar excess of MIANS over protein. Unreacted MIANS was quenched with DTT and removed by dialysis against three changes of 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM. To study the effect of ligand on the fluorescence of MIANS-labeled permease, labeled protein was preincubated with a given ligand for 5 min at 30 °C followed by measurement of emission spectra. Bound TDG was calculated from the fluorescence decrease measured at given TDG concentrations. K_d and n were calculated by Scatchard analysis:

$$B/L \cdot E = (n/K_d) - (B/K_d \cdot E)$$

where B is the amount of bound ligand, L is the free ligand concentration, E is the concentration of MIANS-labeled C6 (V331C) permease, n is the number of ligand-binding sites, and K_d is the dissociation constant.

RESULTS

Cloning and Expression of C6. The 5′ end of a *lacY* fragment extending from codon 193 to 417 was fused in-frame to the 3′ end of a *lacZ* DNA fragment encoding the five N-terminal amino acid residues in β-galactosidase in plasmid pSU2718 (Martinez & Cruz, 1988), resulting in the addition of Met-Thr-Met-Ile-Thr to the N-terminus of C6 (Figure 1). As opposed to the C6 fragments described by Bibi and Kaback (1990) or Zen et al. (1994) which have no N-terminal extension and are proteolyzed completely when expressed independently, the C6 fragment described here is expressed and inserted into the membrane in a relatively stable form (Figure 2). Thus, the level of expression is approximately 30% of that observed with wild-type permease. Furthermore, as observed previously [data not shown; see Bibi and Kaback (1990) and Zen et al. (1994)], co-expression of N6 enhances the level of expression of the C6 construct described here. Although the calculated size is about 25 kDa, like full-length permease, C6 migrates with a smaller M_r (ca. 20 kDa).

Binding of mAbs. To test whether C6 is inserted into the membrane in a conformation resembling that of the analogous portion of full-length lac permease, binding of two different mAbs that interact with conformationally dependent epitopes on either side of the membrane were studied. mAb 4B1 binds to one face of a short helical segment of the periplasmic loop between helices VII and VIII and uncouples H^+ and lactose translocation (Carrasco et al., 1984; Sun et al., 1996), while the epitope for mAb 4B11 is comprised of residues in the last two cytoplasmic loops of the permease

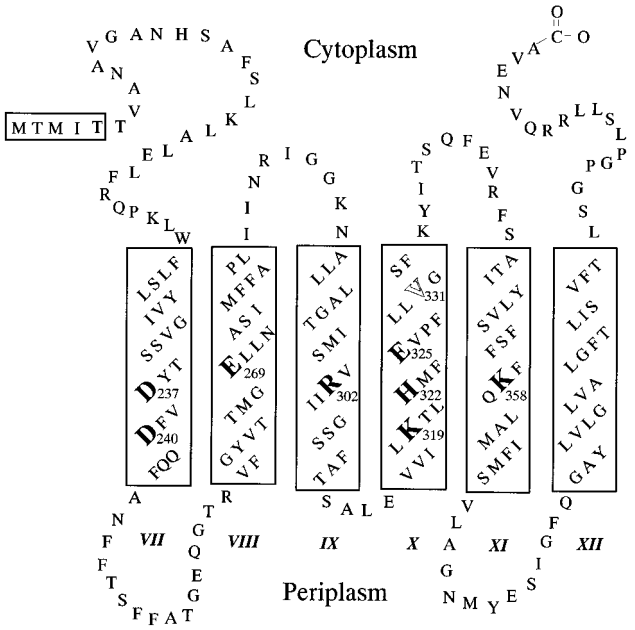

FIGURE 1: Secondary structure model of C6. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. Residues essential for active transport (Glu269, Arg302, His322, and Glu325) and the interacting charge-paired residues in helix VII (Asp237 and Asp240) and helices X (Lys319) and XI (Lys358), respectively, are enlarged and emboldened. Val331 (helix X) is replaced with Cys as the sole Cys residue in C6(V331C) permease. The first five residues (Met-Thr-Met-Ile-Thr) are from the expression vector pSU2818 (Martinez & Cruz, 1988).

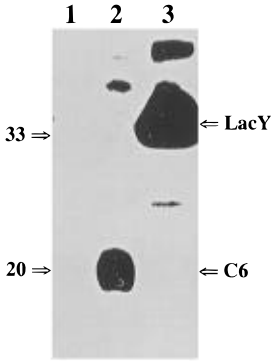

FIGURE 2: Expression of C6. Membranes prepared from *E. coli* T184 transformed with pT7−5 (lane 1), pC6 (lane 2), or pT7-5/lacY (C-less) (lane 3) were electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide gel, followed by immunoblotting using site-directed antibody against the C-terminus of lac permease (Carrasco et al., 1984) as described (Frillingos et al., 1994).

between helices VIII and IX and between helices X and XI (J. Sun and H. R. Kaback unpublished observations). RSO vesicles containing C6 permease bind mAb 4B1 in a manner comparable to that of RSO vesicles with full-length permease (Figure 3A). Furthermore, although spheroplasts or RSO vesicles containing either full-length permease or C6 do not bind mAb 4B11 significantly (J. Sun and H. R. Kaback, unpublished observations), sonified membrane vesicles containing C6 bind 4B11 about as well as sonified membranes with full-length permease (Figure 3B).

Downhill Lactose Translocation. As demonstrated previously with other C6 fragments (Bibi & Kaback, 1990; Zen et al., 1994), lactose accumulation against a concentration gradient is not observed in cells independently expressing the polypeptide described here; however, co-expression with the N-terminal half results in lactose accumulation against a

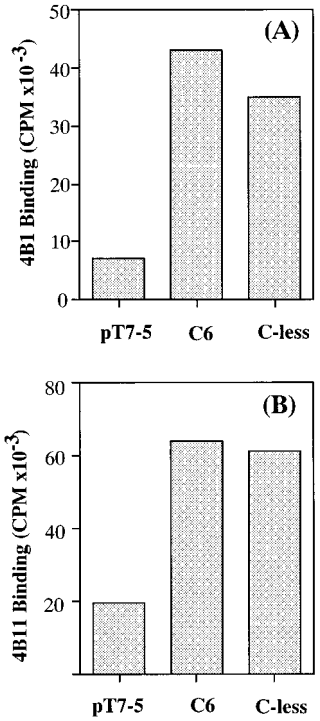

FIGURE 3: Binding of monoclonal antibodies to C6. RSO membrane vesicles (A) or sonified membrane vesicles (B) prepared from cells of *E. coli* T184 harboring plasmid pT7-5 with no *lacY* insert (negative control), pT7-5/C-less cassette *lacY* (positive control) or pC6 were incubated with mAb 4B1 (A) or 4B11 (B) as described under Experimental Procedures. Bound antibodies were quantified with [^{125}I]-labeled protein A as described (Sun et al., 1996).

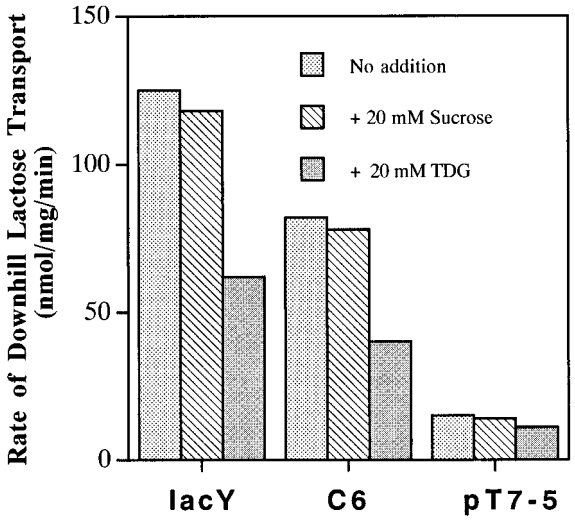

FIGURE 4: Downhill lactose transport by *E. coli* HB101 harboring plasmid pT7-5 with no *lacY* insert (negative control), pT7-5/C-less cassette *lacY* (positive control) or pC6. Cells were cultured in LB medium with given antibiotics and induced with IPTG for 2 h, and the rate of lactose influx was determined as described under Experimental Procedures. Where indicated, TDG or sucrose was added to final concentrations of 20 mM.

concentration gradient (not shown). Furthermore, as shown with N1C6 (Bibi et al., 1991), *E. coli* HB101 (Z^-Y^+) harboring pC6 catalyzes downhill lactose translocation at about 80% of the rate of full-length permease in a manner that is inhibited ca. 50% by the galactoside analog TDG but unaffected by sucrose (Figure 4). Thus, the sugar translocation pathway in the permease appears to be located in the C-terminal half of the permease, as suggested by Bibi et al. (1991).

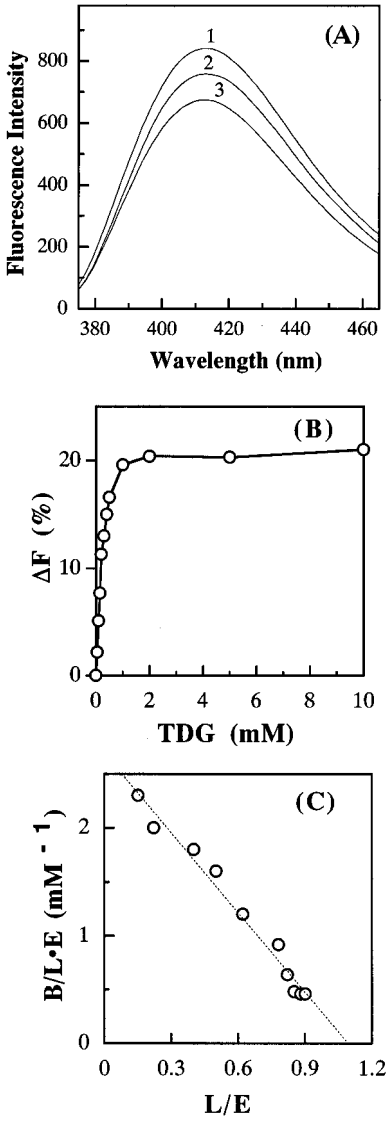

FIGURE 5: Effect of TDG on MIANS-labeled C6(V331C) fluorescence. (A) Purified C6(V331C) permease was labeled with MIANS, and the excess was removed by dialysis as described under Experimental Procedures. Fluorescence emission spectra of MIANS-labeled C6(V331C) permease (35 μg/mL) were recorded as described in Experimental Procedures. Curve 1, no addition, 50 mM sucrose or 50 mM galactose; curve 2, 20 mM lactose; curve 3, 10 mM TDG. (B) Titration of TDG quenching of MIANS-labeled C6(V331C) fluorescence. Fluorescence emission of MIANS-labeled C6(V331C) was measured at 418 nm in the absence and presence of TDG at given concentrations as described in A. (C) Scatchard plot of the data presented in B. Data were analyzed as described in Experimental Procedures assuming that 1 mol of TDG is bound per mol of permease at saturation [see Lolkema and Walz (1990)].

Site-Directed Fluorescence of C6(V331C). C6 with a single-Cys residue in place of Val331 [C6(V331C)] and a biotin acceptor domain at the C-terminus was constructed, since site-directed fluorescence studies with full-length V331C permease (Wu et al., 1994) demonstrate that this position participates in ligand-induced conformational changes. C6(V331C) with a biotin acceptor domain at the C-terminus was solubilized in DM, purified by avidin affinity chromatography, and labeled with MIANS. The emission spectrum of MIANS-labeled C6(V331C) exhibits a maximum at 418 nm in the absence of ligand or in the presence of galactose, a low-affinity substrate of the permease, or sucrose which is not a substrate (Figure 5A, curve 1). Addition of TDG

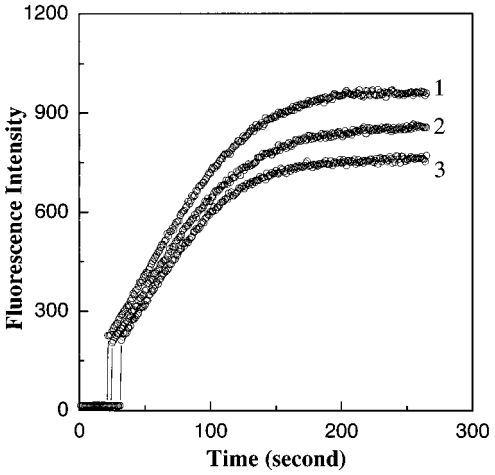

FIGURE 6: Effect of TDG on the reactivity of MIANS with C6-(V331C). Reactions were carried out with 25 μg of purified protein/mL in 0.5 mL of 50 mM KP_i (pH 7.4)/150 mM NaCl/0.02% DM. Where indicated, reactions were initiated by adding MIANS to a final concentration of 4 μM, and the fluorescence increase was recorded continuously at 418 nm (excitation 330 nm) as described under Experimental Procedures. Curve 1, no addition, 50 mM sucrose or 50 mM galactose; curve 2, 20 mM lactose; curve 3, 10 mM TDG.

or lactose, on the other hand, induces about 20% or 10% quenching, respectively, but unlike full-length V331C permease [see Figure 5A in Wu et al. (1994)], no change in the position of the emission maximum is observed (Figure 5A, curves 3 and 2, respectively). When the effect of TDG on the emission spectrum is quantitated, it is apparent that increasing concentrations of the analog up to about 0.8 mM quench markedly and progressively and that no further effect is observed above 1.0 mM (Figure 5B). Scatchard analysis reveals a single binding isotherm with a K_d of approximately 0.4 mM (Figure 5C). Analogous experiments with full-length V331C permease yield a K_d of 0.12 mM (Wu et al., 1994).

MIANS reacts rapidly with purified C6(V331C) permease for about 2 min, and labeling is completed in approximately 3 min (Figure 6, curve 1). The rate of MIANS labeling observed is similar to that observed with full-length V331C permease (Wu et al., 1994). As expected from the results presented above, TDG causes about a 20% decrease in the maximum level of fluorescence achieved (curve 3); however, as opposed to full-length V331C permease (Wu et al., 1994), the rate of labeling is not altered significantly. Lactose, the physiological substrate of the permease, also has no effect on labeling rate but causes about a 10% decrease in the maximum level of fluorescence (curve 2). In contrast, galactose or sucrose change neither the rate of labeling nor the maximum level of fluorescence (curve 1).

DISCUSSION

Site-directed mutagenesis with wild-type permease or Cys-scanning mutagenesis with a functional mutant devoid of Cys residues (C-less permease) has identified individual residues in lac permease that are essential for activity [reviewed in Kaback et al. (1994) and Kaback (1996)]. Over 380 of the 417 residues in C-less permease have been mutagenized, mostly by Cys-scanning mutagenesis. Remarkably, only four residues have been identified thus far as being clearly mandatory for active lactose transport, and they are located in the six C-terminal transmembrane domains of the permease. Thus, Glu269 (helix VIII) (Ujwal et al., 1994; Franco & Brooker, 1994), Arg302 (helix IX) (Menick et al., 1987; Matzke et al., 1992), His322 (helix X) (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988), and Glu325 (helix X) (Carrasco et al., 1986, 1989) are essential for active lactose transport and/or ligand binding. In addition, two pairs of interacting Asp and Lys residues which are not critical for activity are also located in the C-terminal half of the permease [see Kaback et al. (1994) and Kaback (1996)]. In contrast, although no essential residues have been found in the N-terminal half of the permease, Cys148 in helix V has been shown to interact hydrophobically and weakly with the galactosyl moiety of permease substrates (Jung et al., 1994; Wu & Kaback, 1994). Taken as a whole, the observations suggest that the C-terminal half of the permease may play a more direct role in the transport mechanism than the N-terminal half of the molecule, a hypothesis that led to experiments described here.

Although Bibi et al. (1991) found that N1C6 is relatively stable and exhibits only slightly less downhill lactose transport activity than that of wild-type permease, two different C6 constructs have been expressed independent of N6, and both fragments are completely inactive probably because they are highly unstable and rapidly proteolyzed (Bibi & Kaback, 1990; Zen et al., 1994). Therefore, it has not been possible to study the properties of the C-terminal half of the permease independent of the N-terminal half. However, for reasons that are obscure, by adding five amino acid residues (Met-Thr-Met-Ile-Thr) from the N-terminus of β-galactosidase to the N-terminus of C6, the polypeptide is sufficiently stabilized that it is expressed at about 30% of the wild-type level, thereby making possible the studies reported here.

Remarkably, C6 binds two mAbs that interact with conformationally dependent epitopes on either side of the membrane. Since C6 appears to bind both mAbs approximately as well as full-length permease in the appropriate preparations, it is likely that the fragment is inserted into the membrane in correct orientation and that the conformation of both epitopes in C6 is similar to that in the full-length permease. However, there is an apparent inconsistency regarding quantative aspects of the binding data. C6 is expressed to only about 30% of the level observed with full-length permease but appears to bind similar amounts of the mAbs. Since the mAb binding assays involve incubation with essentially stoichiometric amounts of mAb and permease, followed by washing, it is possible that a portion of the mAb dissociates from the full-length permease but not from C6. In other words, C6 may have increased affinity for mAbs 4B1 and 4B11 due to enhanced accessibility of the respective epitopes which results from removal of the N-terminal half of the permease. This interpretation is consistent with experiments (Sahin-Tóth et al., 1995) demonstrating that the periplasmic loop between helices VII and VIII which contains the eptiope for mAb 4B1 is relatively inaccessible to factor Xa protease. Thus, in order to achieve efficient factor Xa cleavage in this loop, it is necessary to insert three factor Xa sites in tandem, and it is highly likely that the effect is due to increased accessibility. In any event, it is apparent that C6 binds the two mAbs at least as well as full-length permease. Given the findings, it seems likely that independently expressed C6 forms a tightly packed structure

similar to that in full-length permease (Jung et al., 1993, 1995; He et al., 1995a,b; Wu et al., 1995) and that the N- and C-terminal halves of lac permease may form helical bundles that are relatively independent of each other structurally. The latter suggestion is consistent with recent observations (J. Sun and H. R. Kaback, unpublished observations) showing that biotinylation of a single-Cys residue in each of the three periplasmic loops in the N-terminal half of lac permease followed by the attachment of avidin has no significant effect on mAb 4B1 binding to the periplasmic loop between helices VII and VIII in the C-terminal half of lac permease. In contrast, avidin binding to each of the three C-terminal periplasmic loops blocks 4B1 binding [see Sun et al. (1996)]. In this respect, it is also interesting that disruption of the interaction between Asp237 (helix VII) and Lys358 (helix XI) causes the permease to be inserted into the membrane much less efficiently, thereby raising the possibility that the C-terminal half of lac permease may be inserted post-translationally (Dunten et al., 1993; Frillingos et al., 1995).

It has been suggested that lac permease contains two sugar binding sites (Lolkema & Walz, 1990; Lolkema et al., 1991), and more direct evidence for the hypothesis has been provided recently by site-directed fluorescence studies with purified single-Cys permease V331C in DM (Wu et al., 1994). Recent chemical labeling studies with right-side-out and inside-out membrane vesicles (Frillingos & Kaback, 1996) are consistent with a relatively high-affinity site on the periplasmically exposed surface of the permease and a low-affinity site on the cytoplasmically exposed surface. With full-length V331C permease, the fluorescence of the MIANS-labeled protein is quenched by TDG in a saturable manner (apparent K_d of ca. 0.12 mM) without a change in emission maximum. In contrast, over a higher range of TDG concentrations (1−10 mM), the reactivity of V331C permease with MIANS is enhanced and the emission maximum of MIANS-labeled V331C permease is blue-shifted by 3−7 nm. Clearly, the C6 fragment described here retains the properties associated with the relatively high-affinity binding site. Thus, TDG induces quenching of MIANS-labeled C6-(V331C) with an apparent K_d of 0.4 mM. In contradistinction, neither the blue shift in the emission spectrum nor the enhanced reactivity observed at high TDG concentrations in the full-length permease is observed in C6(V331C). Since it has been demonstrated (Jung et al., 1994; Wu & Kaback, 1994) that Cys148 in helix V interacts weakly and hydrophobically with the galactosyl moiety of permease substrates, it is tempting to attribute the small decrease in apparent affinity (i.e., increase in apparent K_d) observed with C6 to the absence of Cys148. If this is the case, then the structural components associated with high-affinity ligand binding are likely to be located exclusively in the C-terminal half of the permease. On the other hand, it is readily apparent that, although C6 mediates downhill lactose translocation, the half molecule is completely unable to catalyze lactose accumulation against a concentration gradient and exhibits none of the conformationally-related changes associated with low-affinity ligand binding. Therefore, interactions between the N- and C-terminal halves of the permease are required for active transport, as well as at least some of the conformational changes associated with ligand binding and translocation.

REFERENCES

Bibi, E., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4325−4329.

Bibi, E., Verner, G., Chang, C. Y., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7271−7275.

Bibi, E., Stearns, S. M., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3180−3184.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

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. (1984) *Biochemistry 23*, 3681−3687.

Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486−4488.

Carrasco, N., Puttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 2533−2539.

Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Privé, G. G., Verner, G. E., & Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6934−6938.

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

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

Franco, P. J., & Brooker, R. J. (1994) *J. Biol. Chem. 269*, 7379−86.

Frillingos, S., & Kaback, H. R. (1996) *Biochemistry* (in press).

Frillingos, S., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Biochemistry 33*, 8074−8081.

Frillingos, S., Sahin-Tóth, M., Lengeler, J. W., & Kaback, H. R. (1995) *Biochemistry 34*, 9368−9373.

Haugland, R. P. (1994) *Handbook of Fluorescent Probes and Research Chemicals*, 5th ed., Molecular Probes Inc., Eugene, OR.

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

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

Jung, H., Jung, K., & Kaback, H. R. (1994) *Biochemistry 33*, 12160−12165.

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. (1971) *Methods Enzymol. 22* 99−120.

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

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

Kaback, H. R. (1992) in *International Review of Cytology* (Friedlander, M., & Mueckler, M., Eds.) Vol. 137A, pp 97−125, Academic Press, New York.

Kaback, H. R. (1996) in *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., & Kaback, H. R., Eds.) Elsevier, Amsterdam (in press).

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.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lolkema, J. S., & Walz, D. (1990) *Biochemistry 29*, 11180−11188.

Lolkema, J. S., Carrasco, N., & Kaback, H. R. (1991) *Biochemistry 30*, 1284−1290.

Martinez, E. B. B., & Cruz, D. L. F. (1988) *Gene 68*, 159−162.

Matzke, E. A., Stephenson, L. J., & Brooker, R. J. (1992) *J. Biol. Chem. 267*, 19095−19100.

Menick, D. R., Carrasco, N., Antes, L. M., Patel, L., & Kaback, H. R. (1987) *Biochemistry 26*, 6638−6644.

Padan, E., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765−8.

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

Püttner, I. B., & Kaback, H. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1467−71.
Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483−4485.
Püttner, I. B., Sarkar, H. B., E., P., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry 28*, 2525−2533.
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.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291−4296.
Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−998.
Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun. 119*, 860−867.
Ujwal, M. L., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Mol. Membr. Biol. 1*, 9−16.
Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 429−52.
Wrubel, W., Stochaj, U., Sonnewald, U., Theres, C., & Ehring, R. (1990) *J. Bacteriol. 172*, 5374−5381.
Wrubel, W., Stochaj, U., & Ehring, R. (1994) *FEBS Lett. 349*, 433−438.
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., Perrin, D., Sigman, D., & Kaback, H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−9190.
Zen, K. H., McKenna, E., Bibi, E., Hardy, D., & Kaback, H. R. (1994) *Biochemistry 33*, 8198−8206.

BI960064F