Construction of a Functional Lactose Permease Devoid of Cysteine Residues

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ABSTRACT: By use of oligonucleotide-directed, site-specific mutagenesis, a lactose (*lac*) permease molecule was constructed in which all eight cysteinyl residues were simultaneously mutagenized (C-less permease). Cys154 was replaced with valine, and Cys117, -148, -176, -234, -333, -353, and -355 were replaced with serine. Remarkably, C-less permease catalyzes lactose accumulation in the presence of a transmembrane proton electrochemical gradient (interior negative and alkaline). Thus, in intact cells and right-side-out membrane vesicles containing comparable amounts of wild-type and Cys-less permease, the mutant protein catalyzes lactose transport at a maximum velocity and to a steady-state level of accumulation of about 35% and 55%, respectively, of wild-type with a similar apparent K_m (ca. 0.3 mM). As anticipated, moreover, active lactose transport via C-less permease is completely resistant to inactivation by *N*-ethylmaleimide. Finally, C-less permease also catalyzes efflux and equilibrium exchange at about 35% of wild-type activity. The results provide definitive evidence that sulfhydryl groups do not play an essential role in the mechanism of lactose/H^+ symport. Potential applications of the C-less mutant to studies of static and dynamic aspects of permease structure/function are discussed.

Lactose (*lac*) permease of *Escherichia coli* is a hydrophobic polytopic membrane protein that catalyzes symport i.e., co-transport) of a single β-galactoside with a single H^+ [for reviews, see Kaback (1989, 1990) and Kaback et al. (1990)]. The *lacY* gene, which encodes the permease, has been cloned and sequenced (Büchel et al., 1980), and the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be fully active (Newman et al., 1981; Foster et al., 1982; Viitanen et al., 1984, 1986; Wright et al., 1986) as a monomer (Dornmair et al., 1985; Costello et al., 1987). On the basis of circular dichroic studies and hydropathy analysis (Foster et al., 1983), a secondary structure was proposed in which the protein exhibits 12 transmembrane hydrophobic domains in α-helical conformation connected by hydrophilic "loops" (Figure 1). Evidence confirming some of the general features of the model and demonstrating that both the amino and carboxyl termini are on the cytoplasmic face of the membrane has been obtained from laser Raman (Vogel et al., 1985) and Fourier transform infrared (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished results) spectroscopy, from limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986; Page & Rosenbusch, 1988), from binding studies with monoclonal (Carrasco et al., 1982, 1984a; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibodies (Seckler et al., 1983, 1984, 1986; Carrasco et al., 1984b; Danho et al., 1985), and from chemical labeling (Page & Rosenbusch, 1988). Importantly, moreover, exclusive support for the topological predictions of the 12-helix motif has been obtained from analysis of a large number of *lac* permease–alkaline phosphatase (*lacY–phoA*) fusion proteins (Calamia & Manoil, 1990).

Fox and Kennedy (1965; Kennedy et al., 1974) demonstrated initially that *lac* permease is irreversibly inactivated by *N*-ethylmaleimide (NEM)[1] and that protection is afforded by substrates such as β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG). On the basis of these findings, it was postulated (Fox & Kennedy, 1965) that a cysteinyl residue is at or near the substrate binding site of *lac* permease, and Beyreuther et al. (1981) later showed that the substrate-protectable residue is Cys148. In addition, the permease is reversibly inactivated by other sulfhydryl reagents like *p*-(chloromercuri)benzenesulfonate or by sulfhydryl oxidants such as diamide (Kaback & Patel, 1978) or plumbagin (Konings & Robillard, 1982), and TDG blocks inactivations by these reagents as well.

In view of the importance attributed to sulfhydryl groups in *lac* permease [cf. Kaback and Barnes (1971), Konings and Robillard (1982), and Robillard and Konings (1982) in addition], particularly Cys148, site-directed mutagenesis was used initially to replace Cys148 with Gly (Trumble et al., 1984; Viitanen et al., 1985) or Ser (Neuhaus et al., 1985; Sarkar et al., 1986a). Surprisingly, although Cys148 is requried for substrate protection against alkylation by NEM, it is not important for lactose/H^+ symport. Subsequently, it was shown (Menick et al., 1985; Kaback, 1989) that replacement of Cys154 with Gly leads to complete loss of transport activity although the permease binds the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside normally. Moreover, replacement of Cys154 with Ser or Val yields permease with 10% or 30%, respectively, of the wild-type rate, indicating that although Cys154 is needed for full activity, it is not mandatory. Brooker and Wilson (1986) then replaced Cys176 or -234 with Ser, and Menick et al. (1987) replaced Cys117, -333, or -353 and -355 with Ser with little or no effect on activity. Therefore, out of a total of eight cysteinyl residues in the permease, only Cys154 appears to be important for transport, but even this

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[1] Abbreviations: NEM, *N*-ethylmaleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; C-less permease, permease devoid of cysteinyl residues; EPR, electron paramagnetic resonance; EMB, eosin/methylene blue; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RSO, right-side-out.

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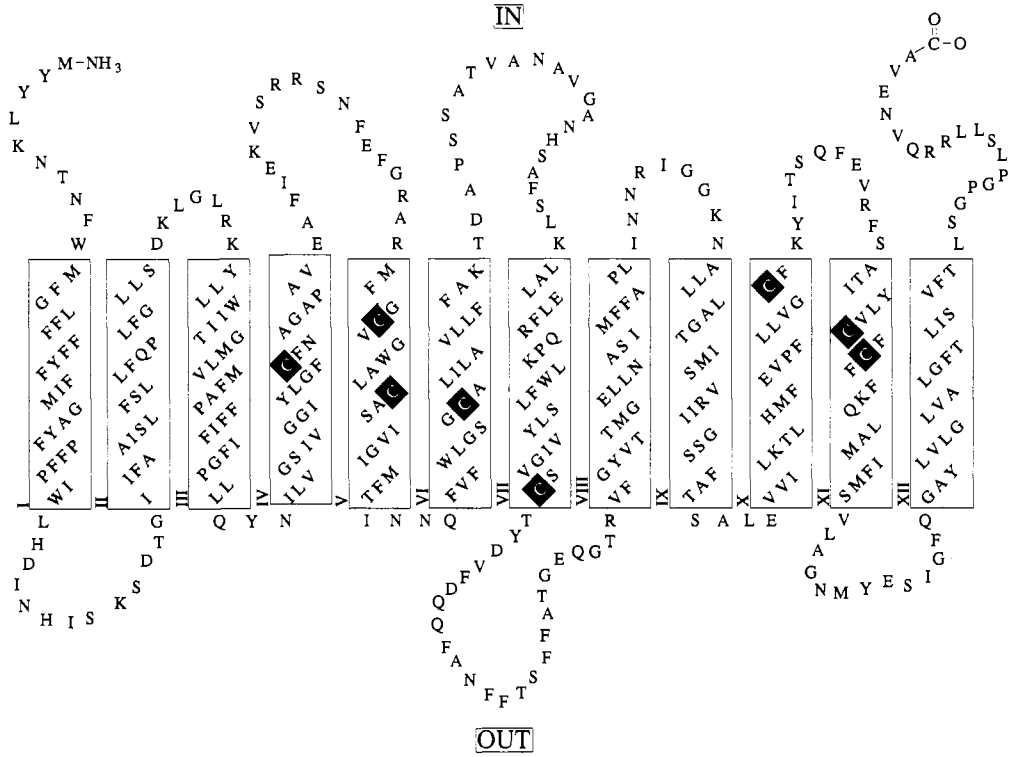

FIGURE 1: Secondary structure model of *lac* permease based on the hydropathy profile of the deduced amino acid sequence (Foster et al., 1983) with cysteinyl residues (C) highlighted. The single amino acid code is used.

residue is not essential. Finally, recent experiments (van Iwaarden et al., 1991) indicate that sulfhydryl–disulfide interconversion probably does not play a role in regulation of permease activity (Konings & Robillard, 1982; Robillard & Konings, 1982).

We now describe the properties of a *lac* permease molecule constructed by site-directed mutagenesis that is completely devoid of cysteinyl residues (C-less permease). In the construct, Cys154 was replaced with Val, and the other Cys residues were replaced with Ser. Transport studies with intact cells and right-side-out membrane vesicles demonstrate that the mutant permease catalyzes active lactose transport in a manner that is resistant to inactivation by NEM, as well as efflux and equilibrium exchange. In addition to confirming previous conclusions that cysteinyl residues are not essential for permease activity, the study provides the groundwork for future studies in which site-directed Cys replacements in C-less permease combined with chemical labeling and electron paramagnetic resonance (EPR) or fluorescence spectroscopy will be used to resolve static and dynamic aspects of permease structure and function.

Experimental Procedures

Materials

[1-^{14}C]Lactose (57 mCi/mmol) was obtained from Amersham (Buckinghamshire, U.K.). All other materials were reagent grade and obtained as described (Viitanen et al., 1986; Sarkar et al., 1986b).

Methods

Bacterial Strains and Plasmids. The following strains of *E. coli* K-12 were used: JM101, *supE,thi,*Δ(*lac-proAB*), [F′*traD36,proA*$^+$*B*$^+$,*lacI*^{q}*ZΔ15*] (Yanisch-Perron et al., 1985); BMH71-18 *mutL,*Δ(*lac-pro*),*supE,thi*/*proA*$^+$*B*$^+$,-*lacI*^{q}*ZΔM15*/*MutL*::*Tn10* (Kramer et al., 1984); T206 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*),*rpsL,met*$^-$,*thr*$^-$,*recA,hsdM,hsdR*/*F*′, *lacI*^{q}*O*$^+$*Z*^{U118}(*Y*$^+$*A*$^+$)] harboring plasmid pGM21 [*lac*Δ(*I*)*O*$^+$*P*$^+$Δ(*Z*)*Y*$^+$Δ(*A*),*tet*^{r}] (Teather et al., 1980); T184 (T206 cured of plasmid pGM21) (Teather et al., 1980); HB101, *hsdS20*(r^-_B, m^-_B),*recA13,ara-14,proA2,lacY1,galK2,rpsL20*-(*Sm*^{r}),*xyl-5,mtl-1,supE44,*λ$^-$/*F*$^-$ (Boyer & Roulland-Dussoix, 1969); CJ236, *dut-1,unf-1,thi-1,relA-1* harboring plasmid pCJ105 (*Cm*^{r}); and MV1190, Δ(*lac-proAB*),*thi,supE,*Δ(*srl-recA*)*306*::*Tn10*(*tet*^{r})[*F*′:*traD36,proAB,lacI*^{q}*ZΔM15*] (Bio-Rad Laboratories, Muta-Gene Mutagenesis Kit). Construction of the C-less mutant was facilitated by using a cassette *lacY* gene (EMBL-X56095) containing the *lac* promoter/operator [p/o(*lac*)] and unqiue restriction sites approximately every 100 bp within the coding sequence cloned into plasmid pT7-5 (Tabor & Richardson, 1985) (pT7-5/*cassette lacY*). Plasmid pBTacI was obtained from Boehringer-Mannheim.

Site-Directed Mutagenesis and Construction of C-less Permease. Oligonucleotide-directed, site-specific mutagenesis was performed essentially as described (Kunkel, 1985) with given modifications. Deoxyoligonucleotide primers containing given mismatches (Table I) with respect to the antisense wild-type *lacY* DNA sequence have been described previously (Menick et al., 1987) or were synthesized on an Applied Biosystems synthesizer. In each case, Cys codons (TGT or TGC) were replaced with Ser (AGT or TCC) or Val (GTT)

Table I: DNA Sequence Analyses of Cys Mutants in the Cassette *lacY* Gene

mutated cysteine codon	mutagenic primer[a]	codon change observed
117	3′-CCGAAA<u>TCA</u>AAATTGCGGCC-5′	TGT → AGT
148, 154	3′-CAAACCG<u>TCA</u>CAACCGACCC-GCGAC<u>CAA</u>CGGAGC-5′	TGT → AGT; TGT → GTT
176	3′-CCGAGACCG<u>TCA</u>CGTGAGTAG-5′	TGT → AGT
234	3′-CCGCAAAGG<u>AGG</u>TGGATGCTAC-5′	TGC → TCC
333	3′-GACCACCCG<u>AGG</u>AAATTTATA-5′	TCG → TCC
353, 355	see Menick et al. (1987)	TGT → AGT; TGC → AGC

[a] Altered codons are underlined.

codons as indicated. Construction of C-less permease, which contains Ser in place of Cys at positions 117, 148, 176, 234, 333, 353, and 355 and Val in place of Cys154, was carried out by using the six primers shown in Table I simultaneously. Single-stranded M13mp18 DNA containing the cassette *lacY* gene was transfected into *E. coli* CJ236 to obtain uracil-containing template (Kunkel, 1985). The six oligonucleotides were annealed to the template simultaneously, and heteroduplex DNA was synthesized during 90-min incubation at 37 °C. *E. Coli* MV1190 was then transfected with the resultant heteroduplex DNA to remove the uracil-containing wild-type strand. Mutations were verified by dideoxyoligonucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978). The replicative form of M13mp18 DNA containing specified mutations was isolated by alkaline lysis and digested with *Bam*HI and *Hin*dIII, and the fragment containing *lacY* was cloned into plasmid pT7-5 (Tabor & Richardson, 1985) digested with *Bam*HI and *Hin*dIII to yield plasmid pC_7S/C154V. Finally, the entire *lacY* gene was sequenced to ensure that no secondary mutations were present. Where indicated, the *Bam*HI/*Hin*dIII fragments from pT7-5/*cassette lacY* or pC_7S/C154V were cloned into plasmid pBTacI to yield pBTacI/*cassette lacY* or pC-less, respectively.

Qualitative Estimates of Permease Activity. E. coli HB101 (Z^+Y^-) was transformed with pT7-5/*cassette lacY* or pC_7S/C154V, and the cells were grown on eosin/methylene blue (EMB) plates containing 25 mM lactose as a qualtitative estimate of permease activity (Miller, 1972).

Growth of Bacteria. E. coli T184 was transformed with pBTacI-derived plasmids and grown at 37 °C in M9 minimal medium supplemented as described except that ampicillin (50 μg/mL) was substituted for tetracycline (Teather et al., 1980). At an OD_{420} of 0.2, the cells were induced with 0.13 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), and growth was continued overnight to an OD_{420} of about 1.5. The cells were then harvested by centrifugation, washed with 50 mM potassium phosphate (pH 7.5) containing 10 mM magnesium sulfate, and resuspended in the same solution.

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

Transport Measurements. Transport of [1-^{14}C]lactose (19 mCi/mmol; 1 Ci = 37 CBq) at a final concentration of 0.4 mM was assayed in intact cells (Consler et al., 1991). Transport in RSO vesicles was measured at various [1-^{14}C]-lactose concentrations and specific activities under oxygen and in the presence of ascorbate and phenazine methosulfate (PMS) (Konings et al., 1971; Kaback, 1974). Efflux and equilibrium exchange assays were carried out with RSO membrane vesicles as described (Kaczorowski & Kaback, 1979).

Immunological Analysis. Immunoblots were carried out with monoclonal antibody (Mab) 4A10R as described (Carrasco et al., 1982) except that horseradish peroxidase (HrP)-conjugated protein A (Amersham) was used to detect immune complexes.

Protein Determinations. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Results

Analysis of Mutations by DNA Sequencing. The cassette *lacY* gene was cloned initially from pT7-5/*cassette lacY* into the replicative form of M13mp18 DNA, and single-stranded phage DNA was isolated and used as template for site-directed mutagenesis. Subsequently, single-stranded phage DNA with

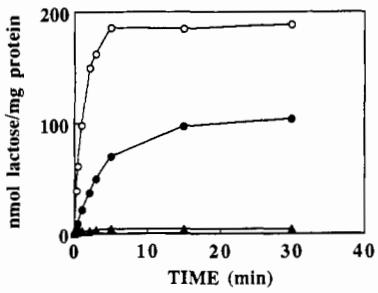

FIGURE 2: Active transport of lactose in *E. coli* T184 encoding wild-type or C-less *lac* permease. *E. coli* T184 was transformed with pBTacI containing no *lacY* insert (▲), wild-type *cassette lacY* (○), or C-less *lacY* (●). Aliquots (50 μL) of washed cells at an OD_{420} of 10 in 50 mM potassium phosphate (pH 7.5)/10 mM magnesium sulfate were assayed for [1-^{14}C]lactose (10 mCi/mmol) transport at a final concentration of 0.4 mM as described (Consler et al., 1991).

lacY encoding C-less permease was isolated and subjected to dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) with primers complementary to regions of *lacY* 50–100 bases downstream from each mutation. As summarized in Table I, the *lacY* gene encoding C-less permease contains given base changes at the sites predicted such that Cys codons (TGT or TGC) are replaced with Ser codons (AGT or TCC) at positions 117, 148, 176, 234, 333, 353, and 355 and by a Val codon (GTT) at position 154. Furthermore, sequencing of the entire *lacY* gene revealed no additional mutations, and the sequence was identical with that reported for the cassette *lacY* gene (EMBL-X56095) with the exception of the base changes described.

Colony Morphology of Cells Expressing C-less Permease. E. coli HB101 (Z^+Y^-) harboring plasmid pT7-5/*cassette lacY*, which expresses β-galactosidase from a chromosomal *lacZ* gene, grows as dark red colonies on EMB plates containing 25 mM lactose, indicating that lactose is transported and metabolized at a high rate. In contrast, HB101 harboring pT7-5 (the vector with no insert) grows as white colonies because lactose in the medium is inaccessible to β-galactosidase in the cytosol. *E. coli* HB101 transformed with pC_7S-C154V grows as dark red colonies, indicating that C-less permease is minimally able to catalyze downhill lactose translocation.

Lactose Transport in Intact Cells. E. coli T184 expressing C-less permease transports lactose at a rate that is about 30% of that observed in cells expressing wild-type *lac* permease from the cassette *lacY* gene, and the steady-state level of lactose accumlation in T184/pC-less is approximately 55% of that observed in T184 harboring pBTacI/*cassette lacY* (Figure 2). Importantly, moreover, immunoblot analyses carried out on membranes prepared from the cells used to assay transport demonstrate that comparable amounts of permease are present (Figure 3). Thus, it is apparent that replacement of all eight Cys residues in the same permease molecule does not markedly impair permease activity.

Lactose Transport in RSO Membrane Vesicles. In the absence of exogenous electron donors, RSO membrane vesicles containing wild-type or C-less permease transport lactose at negligible rates and to minimal steady-state levels of accumulation (Figure 4). In the presence of ascorbate and PMS, on the other hand, the initial rate of transport increases dramatically in vesicles containing wild-type *lac* permease, and a steady-state level of accumulation is achieved in about 3 min (Figure 4A). RSO vesicles containing C-less permease also transport lactose in the presence of ascorbate/PMS, and the initial rate and steady-state level of accumulation are about 35% and 55%, respectively, of wild-type vesicles (Figure 4B). Although data are not shown, when initial rates of transport

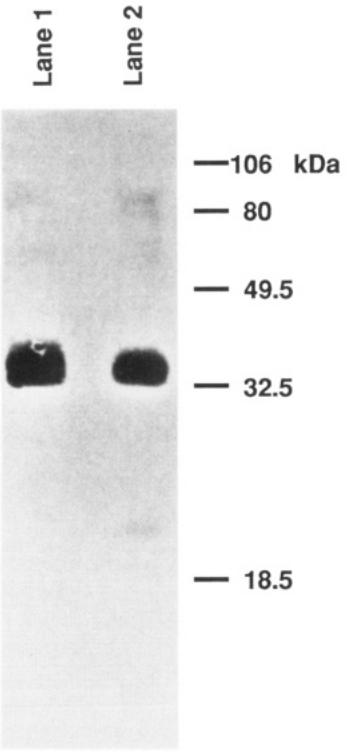

FIGURE 3: Immunoblot analyses of membranes containing wild-type or C-less permease. Membrane vesicles (35 μg of protein) from *E. coli* T184 harboring pBTacI/*cassette lacY* (lane 1) or pC-less (lane 2) were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and incubated with monoclonal antibody 4A10R (Herzlinger et al., 1985). The Amersham Western blotting detection system using HrP-conjugated protein A was used for detection. The permease migrates as a broad band with an apparent molecular mass of 33 kDa; the faint immunoreactive bands at 66 and 17 kDa correspond to a dimeric aggregate form and a proteolysis product of *lac* permease, respectively.

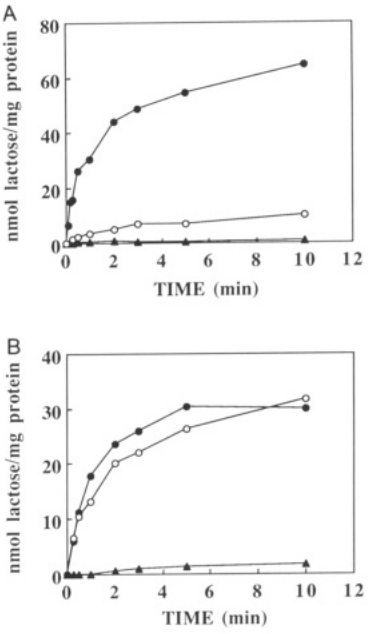

FIGURE 4: Active lactose transport in RSO vesicles containing wild-type (A) or C-less permease (B) in the presence or absence of NEM. Aliquots (50 μL) of membrane vesicles (ca. 100 μg of membrane protein) were assayed for [1-^{14}C]lactose (10 mCi/mmol) transport at a final concentration of 0.4 mM. The assays were carried out as described (Konings et al., 1971; Kaback, 1974), and at indicated times, transport was terminated by dilution and rapid filtration. (▲) No additions; (●) in the presence of 10 mM ascorbate and 0.1 mM PMS; (○) in the presence of 10 mM ascorbate, 0.1 mM PMS, and 0.5 mM NEM (after a 15-min preincubation).

are measured as a function of lactose concentration and the data are plotted according to Eadie and Hofstee, it is observed that C-less permease transports lactose with an apparent K_m comparable to wild type (approximately 0.3 mM) and at a maximum velocity that is 35% of wild type. Furthermore, while treatment with NEM inactivates lactose transport in RSO vesicles containing wild-type permease, the alkylating agent has a negligible effect on ascorbate/PMS-driven lactose transport in vesicles containing C-less permease (compare panels A and B of Figure 4).

Efflux and Equilibrium Exchange. RSO vesicles containing wild-type permease encoded by the *cassette lacY* gene were equilibrated overnight with 10 mM [1-^{14}C]lactose and subsequently diluted 200-fold into medium at pH 6.6 containing either no lactose (efflux) or 10 mM lactose (equilibrium exchange). Efflux (Figure 5A) and equilibrium exchange (Figure 5B) occur at rates similar to those reported previously (Kaczorowski & Kaback, 1979) (i.e., $t_{1/2}$ is ca. 25 s for efflux and less than 5 s for equilibrium exchange). Similar experiments performed with RSO vesicles containing C-less permease exhibit $t_{1/2}$ values for efflux and equilibrium exchange of approximately 75 and 15 s, respectively. Although the rates are diminished relative to wild-type permease, they are highly significant relative to the *p*-(chloromercuri)benzenesulfonate-inactivated control samples which reflect non-carrier-mediated passive processes.

Discussion

The results presented in this paper provide definitive support for previous experiments (Kaback, 1989; Trumble et al., 1984; Viitanen et al., 1985; Menick et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986a; Brooker & Wilson, 1986; van Iwaarden et al., 1991) indicating that cysteinyl residues in *lac* permease do not play a direct role in the mechanism of *lac* permease. Thus, as demonstrated here, when site-directed mutagenesis is used to replace each of the eight cysteinyl residues in *lac* permease simultaneously, the C-less permease catalyzes active lactose transport moderately well relative to wild-type permease. Moreover, active lactose transport in RSO vesicles containing C-less permease is not inactivated by NEM, in dramatic contrast to vesicles containing wild-type permease, and vesicles containing C-less permease also catalyze efflux and equilibrium exchange at significant rates. In addition, the results reinforce the conclusion (van Iwaarden et al., 1991) that sulfhydryl–disulfide interconversion is probably not important for regulation of permease activity (Konings & Robillard, 1982; Robillard & Konings, 1982).

Although use of site-directed mutagenesis is useful for delineating amino acid residues that are important for lactose/H^+ symport and/or substrate binding and recognition, it has become apparent that high-resolution structure is required to begin to determine the role of these residues in the mechanism. However, it is also clear that in order to solve the mechanism, dynamic information at high resolution will be required as well. In this respect, chemical labeling and spectroscopic approaches in which reactive cysteinyl residues are tagged with radioactive sulfhydryl reagents, spin-labels (Altenbach et al., 1989, 1990; Todd et al., 1989), or fluorescent probes (Dornmair et al., 1985) represent potentially powerful means for examining static and dynamic aspects of protein structure–function relationships at high resolution. A principle difficulty with the general approach, however, is the complexity resulting from the presence of multiple cysteinyl residues in most proteins, eight in the case of *lac* permease. Thus, in

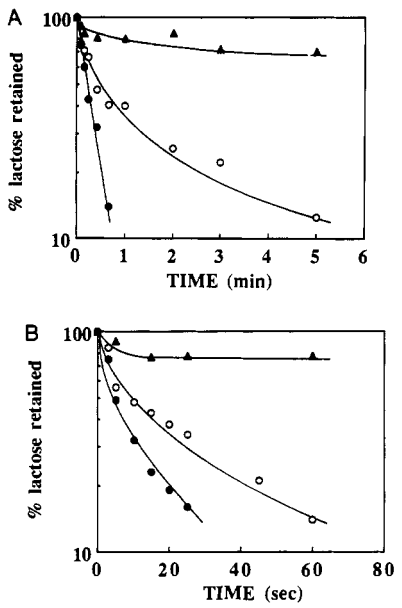

FIGURE 5: Lactose efflux (A) and equilibrium exchange (B) in RSO membrane vesicles. (A) Membrane vesicles (ca. 30 mg of protein/mL) were equilibrated at 4 °C overnight with 10 mM [1-^{14}C]lactose (3 mCi/mmol). Aliquots (2 μL) were then rapidly diluted into 400 μL of 100 mM potassium phosphate (pH 7.5). At the times indicated, the reactions were terminated by addition of 3.0 mL of 100 mM potassium phosphate (pH 5.5)/100 mM lithium chloride/20 mM mercuric chloride and filtered immediately as described (Kaczorowski & Kaback, 1979). (●) Wild-type vesicles; (○) C-less vesicles; (▲) wild-type vesicles pretreated with 1.4 mM *p*-(chloromercuri)-benzenesulfonate. Data are expressed as a percentage of lactose retained with zero time points for normalization. (B) Experiments were carried out as described in (A), except that the dilution medium contained 10 mM unlabeled lactose. (●) Wild-type vesicles; (○) C-less vesicles; (▲) wild-type vesicles pretreated with 1.4 mM *p*-(chloromercuri)benzenesulfonate. Data are expressed as a percentage of lactose retained with zero time points for normalization.

addition to the important conclusion that cysteinyl residues do not play a critical role in the mechanism of *lac* permease, the construction of a functional permease devoid of cysteinyl residues, in analogy to *lac* permease devoid of tryptophanyl residues (Menezes et al., 1990), provides the basis for an approach to the analysis of static and dynamic aspects of permease structure–function relationships. By using the *lacY* gene encoding C-less permease, for instance, it is now possible to design mutants in which an individual amino acid residue in putative hydrophilic or hydrophobic domains is replaced with a cysteinyl residue which can then be reacted specifically with either permeant or impermeant sulfhydryl reagent in RSO or inside-out membrane vesicles, followed by solubilization and immunoprecipitation. In addition, single Cys mutants can be tagged with appropriately reactive EPR or fluorescent probes after solubilization and purification and studied spectroscopically after reconstitution. Finally, it should be possible to study proximity relationships between transmembrane domains by placing single cysteinyl residues in pairs of helical domains predicted to lie close to each other within the membrane. In these contexts, it is encouraging that each amino acid residue in putative transmembrane helices I, IX, and X as well as putative loop 10 of C-less permease has already been replaced with Cys (cf. Figure 1), and the great majority of the mutants exhibit highly significant transport activity (M. Sahin-Toth, J. Schwieger, B. Persson, and H. R. Kaback, unpublished results).

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Registry No. Cys, 52-90-4; *lac* permease, 9068-45-5; lactose, 63-42-3.

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