Cysteine 148 in the Lactose Permease of *Escherichia coli* Is a Component of a Substrate Binding Site. 1. Site-Directed Mutagenesis Studies

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ABSTRACT: Cys148 in the lactose permease of *Escherichia coli* has been replaced with hydrophobic (Ala, Val, Ile, Phe), hydrophilic (Ser, Thr), or charged (Asp, Lys) residues, and the properties of the replacement mutants have been analyzed. Although Cys148 is not essential for transport, the size and polarity of the side chain at this position modifies transport activity and substrate specificity. Thus, small hydrophobic side chains (Ala, Val) generally increase the apparent affinity of the permease for substrate, while hydrophilic side chains (Ser, Thr, Asp) decrease apparent affinity and bulky or positively charged side chains (Phe, Lys) virtually abolish activity. In addition, hydrophilic substitutions (Ser, Thr, Asp) alter the specificity of the permease toward monosaccharides relative to disaccharides. On the basis of these and other observations, it is concluded that Cys148 is located in a sugar binding site of lac permease and probably interacts hydrophobically with the galactosyl moiety. The postulate receives more direct support from site-directed fluorescence labeling studies presented in the following paper in this issue [Wu, J., & Kaback, H. R. (1994) *Biochemistry* (following paper in this issue)].

The lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that 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 into proteoliposomes, and shown to be solely responsible for β-galactoside transport in monomeric form [reviewed in Kaback (1989, 1992), Kaback et al. (1993), and Poolman and Konings (1993); see Sahin-Tóth et al. (1994) in addition]. Based on circular dichroism and hydropathy analysis of the primary amino-acid sequence, a secondary-structure model was proposed in which the protein consists of a short hydrophilic N-terminus, 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a 17 amino acid residue hydrophilic C-terminal tail (Foster et al., 1983). Evidence supporting the general features of the model and demonstrating that both the N and C termini (hydrophilic domains 1 and 13, respectively), as well as hydrophilic domains 5 and 7, are on the cytoplasmic face of the membrane was obtained from other spectroscopic techniques, limited proteolysis, immunological studies, and chemical modification [see Kaback (1992)]. Moreover, the 12-helix model has received unequivocal support from the analysis of an extensive series of lac permease–alkaline phosphatase (*lacY–phoA*) fusions (Calamia & Manoil, 1990). Most recently, the use of site-directed fluorescence labeling has led to a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

Fox and Kennedy (Fox & Kennedy, 1965; Kennedy et al., 1974) demonstrated initially that lac permease is irreversibly inactivated by *N*-ethylmaleimide (NEM) 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 (Figure 1). In addition, the permease is reversibly inactivated by other sulfhydryl reagents like *p*-chloromercuribenzenesulfonate or by sulfhydryl oxidants such as diamide (Kaback & Patel, 1978) or plumbagin (Konings & Robillard, 1982), and TDG blocks inactivation by these reagents as well.

In view of the importance attributed to sulfhydryl groups in lac permease [see 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., 1986). Surprisingly, although Cys148 is required for substrate protection against alkylation by NEM, it is not essential 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 with wild-type steady-state levels of lactose accumulation, 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, none of the eight cysteinyl residues in the permease

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[1] Abbreviations: lac, lactose; NEM, *N*-ethylmaleimide; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; TMG, methyl 1-thio-β,D-galactopyranoside; IPTG, isopropyl 1-thio-β,D-galactopyranoside; Na-DodSO_4, sodium dodecyl sulfate; PMS, phenazine methosulfate.

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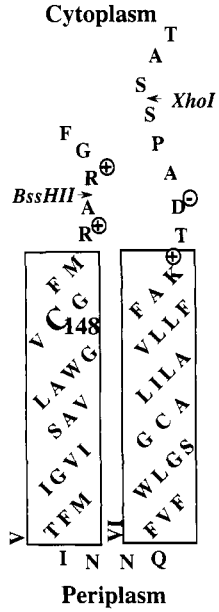

FIGURE 1: Secondary-structure model of putative transmembrane domains V and VI and adjacent hydrophilic loops of lactose permease (Foster et al., 1983). Cys148 is highlighted. The location of relevant restriction endonuclease sites in the corresponding DNA sequence are also indicated.

is essential for activity. Finally, experiments in which each of the Cys mutants was purified and reconstituted (van Iwaarden et al., 1991a) indicate that sulfhydryl–disulfide interconversion probably does not play a role in regulation of permease activity (Konings & Robillard, 1982; Robillard & Konings, 1982).

More recent studies (van Iwaarden et al., 1991b) provide definitive support for the contention that cysteinyl residues in lac permease do not play an essential role in the mechanism of lac permease. When Cys154 is replaced with Val and each of the other cysteinyl residues in lac permease is replaced with Ser, "C-less" permease catalyzes active lactose transport at about 30% of the initial rate and to about 60% of the steady-state level of accumulation of wild-type permease. Moreover, active lactose transport in right-side-out vesicles containing C-less permease is not inactivated by NEM, in dramatic contrast to vesicles containing wild-type permease.

The observation that Cys148 is not an essential residue raises the important question of whether substrate protection of this residue reflects location in or near a binding site (i.e., a steric effect) or decreased reactivity secondary to a long-range conformational change. This consideration is particularly relevant in view of recent observations demonstrating that permease mutants containing a single Cys residue at position 315 (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a), 269, or 322 (Jung et al., 1994b) exhibit a marked increase in reactivity in the presence of TDG. In order to begin to address the question, we have replaced Cys148 with a variety of amino acid residues so that the effect of size, polarity, and charge can be assessed. The results indicate that Cys148 is located in a substrate binding site and probably interacts hydrophobically with the galactosyl moiety of the substrate. In the following paper (Wu & Kaback, 1994), more direct evidence for this contention is presented.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lactose, [U-^{14}C]galactose, and [α-^{35}S]-ATP were purchased from Amersham, Arlington Heights, IL. [^{14}C]Methyl 1-thio-β,D-galactopyranoside (TMG) was from Du Pont NEN, Boston, MA. [^{3}H]TDG was synthesized by Yu-Yen Liu under the supervision of Arnold Leibman in the Isotope Synthesis Group of Hoffmann-La Roche, Nutley, NJ. [^{3}H]Melibiose was a generous gift from Gérard Leblanc, Laboratoire J. Maetz, Villefranche sur mer, France. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C-terminus of lac permease (Carrasco et al., 1984) was prepared by BabCo (Richmond, CA). All restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were from New England Biolabs, Beverly, MA. All other materials were reagent grade and obtained from commercial sources.

Table 1: Mutagenic Oligonucleotides Used To Change Codon TGT Encoding Cys148 in the Cassette *lacY* Gene

mutant	mutagenic oligonucleotides used to change codon TGT[a]
C148A	GAATTTGGTCGCGCGCGGATGTTTGGC**GCT**GTTGGCTGG
C148D	GAATTTGGTCGCGCGCGGATGTTTGGC**GAT**GTTGGCTGG
C148F	GAATTTGGTCGCGCGCGGATGTTTGGC**TTT**GTTGGCTGG
C148I	GAATTTGGTCGCGCGCGGATGTTTGGC**ATT**GTTGGCTGG
C148K	GAATTTGGTCGCGCGCGGATGTTTGGC**AAG**GTTGGCTGG
C148S	GAATTTGGTCGCGCGCGGATGTTTGGC**TCT**GTTGGCTGG
C148T	GAATTTGGTCGCGCGCGGATGTTTGGC**ACT**GTTGGCTGG
C148V	GAATTTGGTCGCGCGCGGATGTTTGGC**GTT**GTTGGCTGG

[a] Sequences of mutagenic primers are presented in the 5′ → 3′ order with altered codons in boldface type.

Bacterial Strains and Plasmids. *E. coli* HB101 [*hsdS20 (r^-B, m^-B), recA13, ara-14, proA2, lacY1, galK2, rpsL20-(Sm^r), xyl-5, mtl-1, supE44, l^-/F$^-$*] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described. *E. coli* T184 [*lacI$^+$O$^+$Z$^-$Y$^-$(A), rpsL, met$^-$, thr$^-$, recA, hsdM, hsdR/F′, lacI^{q}O$^+$Z^{D118}(Y$^+$A$^+$)*] (Teather et al., 1980) harboring plasmid pT7-5/*lacY* with given mutations was used for expression from the *lac* promoter. A cassette *lacY* gene (EMBL-X56095) containing the *lac* promoter/operator was used for all *lacY* gene manipulations.

Oligonucleotide-Directed Site-Specific Mutagenesis. Replacement of Cys148 in lac permease was performed by oligonucleotide-directed, site-specific mutagenesis of the cassette *lacY* gene in the plasmid pT7-5. The polymerase chain reaction (PCR) overlap extension method (Ho et al., 1989) was employed to create each mutation. The sequences of the synthetic mutagenic primers used are presented in Table 1. PCR products were purified in agarose gels, gene-cleaned (Bio 101), and digested with *Bss*HII and *Xho*I restriction endonucleases. The *Bss*HII–*Xho*I fragments were isolated from low melting point agarose gels and ligated to similarly treated pT7-5/cassette *lacY* vector.

DNA Sequencing. Double-stranded plasmid DNA prepared by Magic Minipreps was sequenced by using the dideoxynucleotide termination method (Sanger et al., 1977) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Colony Morphology. For preliminary qualitative assessment of permease activity, *E. coli* HB101 (*Z$^+$Y$^-$*) was transformed with plasmid pT7-5/cassette *lacY* encoding lac permease with given mutations, and the cells were plated on MacConkey indicator plates containing 25 mM lactose.

Active Transport in Intact Cells. Active transport was measured in *E. coli* T184 (*Z$^-$Y$^-$*) transformed with each plasmid described. The cells were grown aerobically in Luria broth containing 10 μg/mL streptomycin and 100 μg/mL ampicillin at 37 °C. Over-night cultures were diluted 10-fold

and allowed to grow for 2 h before induction with isopropyl 1-thio-β,D-galactopyranoside (IPTG). After further growth for 2 h, cells were harvested by centrifugation, washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$, and adjusted to an optical density of 10 at 420 nm in the same buffer. Transport was assayed at given concentrations of sugars in the presence of 20 mM potassium ascorbate, 0.1 mM phenazine methosulfate (PMS), and oxygen by the rapid filtration method as described (Consler et al., 1990).

Immunological Analysis. For Western blot analysis, T184 cells grown as described for *Active Transport* were adjusted to an OD_{420} of 100 (ca. 7 mg of protein/mL). A 100-μL aliquot of the suspension was sonicated, unlysed cells were removed by low-speed centrifugation, and membranes were harvested by ultracentrifugation at 250000g_{max} for 1 h at 4 °C and solubilized in an equal volume of 2× concentrated sample buffer (Laemmli, 1970). Equal volumes of each sample were subjected to sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis as described (Newman et al., 1981). Proteins were electroblotted and immunoblots were probed with site-directed polyclonal antibody against the C-terminus of lac permease (Herzlinger et al., 1985).

Preparation of Right-Side-Out Membrane Vesicles. Right-side-out membrane vesicles were prepared by lysozyme–EDTA treatment and osmotic lysis as described (Kaback, 1971; Short et al., 1975) from 3.0-L cultures of *E. coli* T184 harboring given plasmids.

Protein Determinations. Protein was assayed by a modified Lowry method (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Construction and Verification of Mutants. Cys148 in wild-type permease was replaced with Ala, Asp, Ile, Lys, Phe, Ser, Thr, or Val using synthetic oligonucleotide primers (Table 1) and PCR overlap extension (Ho et al., 1989). The PCR products were cloned into the cassette *lacY* gene by using the *Bss*HII and *Xho*I restriction endonuclease sites (see Figure 1). All mutations were verified by double-stranded DNA sequencing, and, except for the desired base changes summarized in Table 1, the sequences were identical to those of the cassette *lacY* gene encoding wild-type permease.

Colony Morphology. E. coli HB101 (*lacZ*$^+$*Y*$^-$) expresses active β-galactosidase but carries a defective *lacY* gene. Cells transformed with plasmids encoding functional lac permease allow access of external lactose to cytosolic β-galactosidase, and subsequent metabolism of the sugar causes acidification and the appearance of red colonies on MacConkey indicator plates containing lactose. Cells devoid of permease activity appear as white colonies, and permease mutants with low activity form red colonies with white halos of varying size. HB101 expressing mutants C148A[2] or C148S appear as red colonies indistinguishable from wild-type permease; cells expressing C148D, C148I, C148F, C148T, or C148V permease yield red colonies with white halos, and cells expressing mutant C148K grow as white colonies. Therefore, judging from indicator plates, lac permease with Ala or Ser in place of C148 retains the ability to translocate lactose, whereas replacement with Asp, Ile, Phe, Thr, Val, or Lys alters downhill lactose transport to varying degrees.

Active Lactose Transport. Active transport of lactose by the mutants was assayed more quantitatively by using *E. coli*

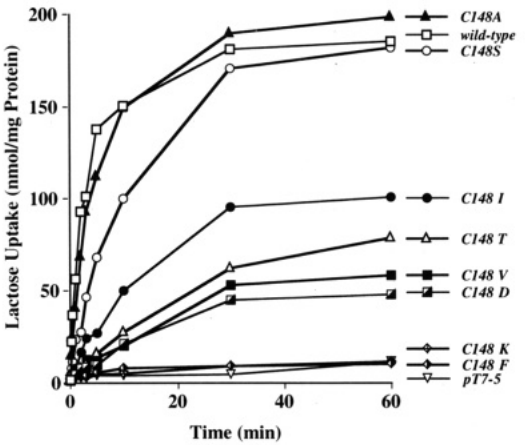

FIGURE 2: Time course of lactose transport by Cys148 replacement mutants. *E. coli* T184 harboring plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding wild-type permease, or pT7-5 encoding mutant permease with given amino acid substitutions for Cys148 were grown at 37 °C, and 50-μL aliquots of cell suspensions (containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed for sugar uptake at 25 °C as described under Experimental Procedures. The concentration of [^{14}C]lactose (2.5 Ci/mol) was 0.4 mM.

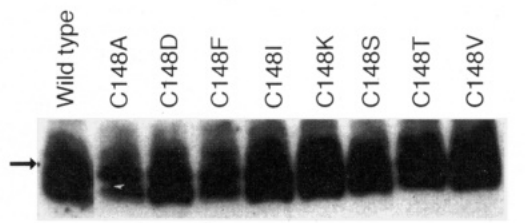

FIGURE 3: Western blot of membranes containing wild-type lac permease or Cys148 replacement mutants. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids as described under Experimental Procedures, and 80 μg of membrane protein was subjected to 12% $NaDodSO_4$–polyacrylamide gel electrophoresis and electroplotting. The blot was incubated with antibody directed against the C-terminal dodecapeptide of lac permease, followed by horseradish peroxidase-linked protein A, and finally luminescent substrate before exposure to film. The arrow indicates the positions of the marker protein carbonic anhydrase (32.5 kDa).

T184 (*Z*$^-$*Y*$^-$) which lacks β-galactosidase and cannot metabolize lactose (Figure 2). Mutants containing Ser or Ala in place of Cys148 transport lactose at about 50% and 90%, respectively, of the initial rate of wild-type permease to steady-state levels of accumulation that are comparable to wild-type. Replacement of Cys148 with amino acid residues carrying bulkier side chains or a charge results in much lower initial rates and reduced steady states. Mutants C148I, C148T, C148V, or C148D transport lactose at only 10–25% of the initial rate of wild-type to 25–50% of the steady-state level of accumulation. Time courses of lactose transport with cells expressing C148F and C148K are indistinguishable from cells carrying plasmid pT7-5 without the *lacY* gene.

Expression of Mutant Permeases. The relative concentration of each mutant in membranes from *E. coli* T184 was approximated by Western blot analysis with anti-C-terminal antibody (Figure 3). All eight mutants are present in amounts comparable to wild-type permease. Therefore, the lactose transport activities reported for the mutants cannot be attributed to defects in insertion of the permease into the membrane or to proteolytic degradation subsequent to insertion.

Transport of Substrate Analogs. In order to examine whether size, polarity, or charge at position 148 alters substrate specificity, transport of different substrate analogs was analyzed in each mutant (Figures 4 and 5). Transport of the disaccharides TDG (Figure 4A) and melibiose (Figure 4B)

[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 at this position.

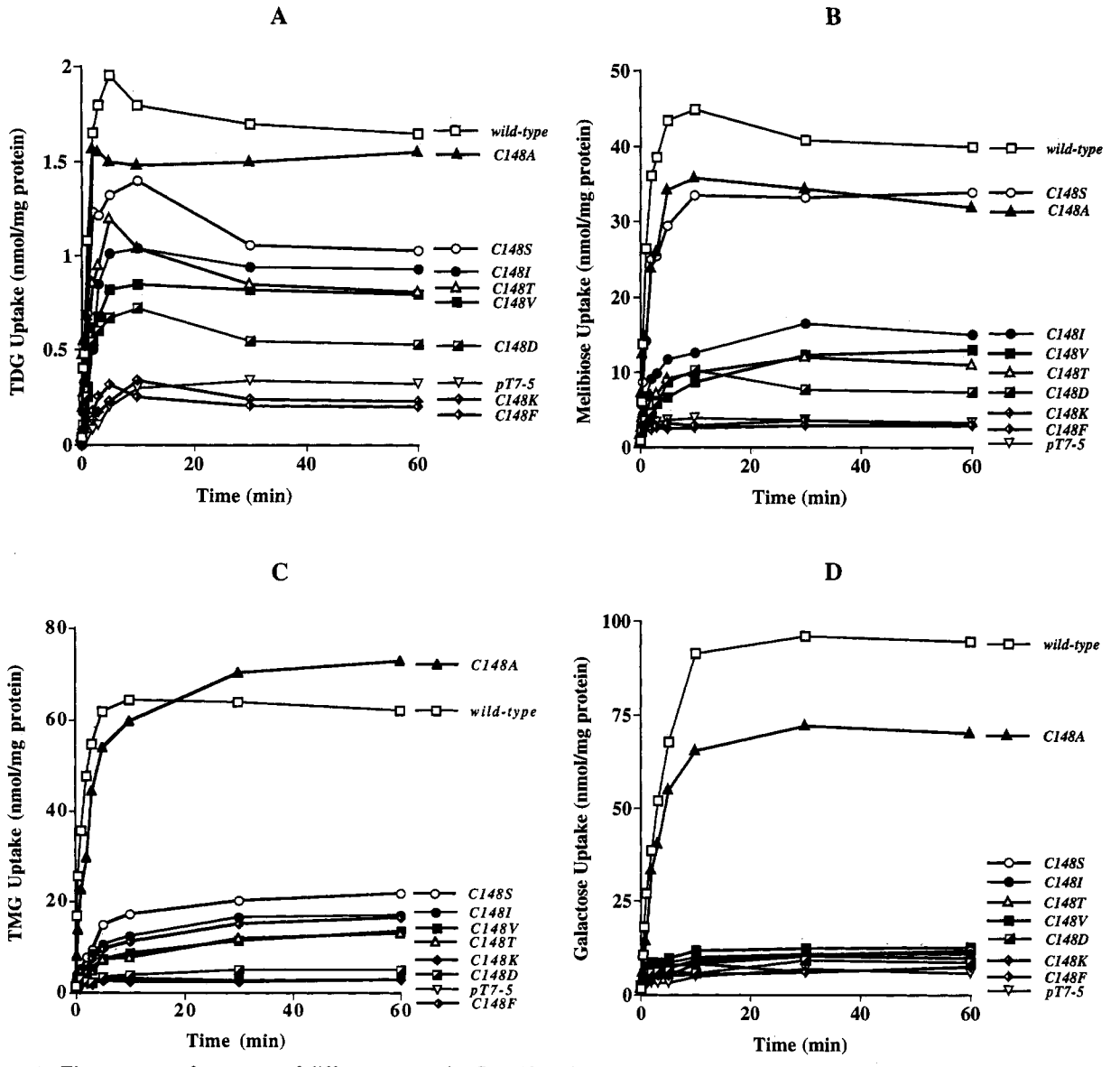

FIGURE 4: Time courses of transport of different sugars by Cys148 replacement mutants. *E. coli* T184 harboring plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding wild-type permease, or pT7-5 encoding mutant permease with given amino acid substitutions for Cys148 were grown and assayed for sugar uptake at 25 °C as described in the legend to Figure 2. (A) TDG transport; final concentration of [^{3}H]TDG (100 Ci/mol) was 10 μM. (B) Melibiose transport; final concentration of [^{3}H]melibiose (40 Ci/mol) was 0.4 mM. (C) TMG transport; final concentration of [^{14}C]TMG (2.5 Ci/mol) was 0.4 mM. (D) Galactose transport; final concentration of [^{14}C]galactose (5 Ci/mol) was 1 mM.

by the Cys148 mutants exhibits the same pattern of initial rates and steady-state levels of accumulation as observed for lactose. Thus, mutants C148S and C148A accumulate TDG and melibiose to the same extent as wild-type permease at about 50% and 90%, respectively, of the initial rate. Amino acid residues with larger side chains or a charge cause a more marked decrease in transport activity, effecting initial rates (Figure 5A) as well as steady-state levels of accumulation (Figure 5B). Moreover, as shown for lactose, TDG and melibiose transport are abolished when Cys148 is replaced with Phe or Lys.

Studies with TMG and galactose as transport substrates reveal patterns of initial rates and steady-state levels of accumulation that differ from those obtained with the disaccharides (Figures 4C,D, and 5). C148A permease has the highest activity with 60–80% of the initial rate of wild-type and steady-state levels of accumulation for both sugars that are comparable to the wild-type. However, replacement of Cys148 with Ser alters transport of TMG and galactose more dramatically, causing decreases in initial rates and steady states to 5–20% of wild-type. C148V, C148I, and C148T permeases exhibit more dramatic effects on galactose and TMG transport than observed for the disaccharides, particularly with respect to steady-state levels of accumulation. Mutants C148D, C148F, and C148K are completely inactive with respect to active TMG and galactose transport.

Kinetic Analyses. To obtain more information on the effect of the amino acid replacements for Cys148, kinetics of lactose and TMG transport were determined. Rates of transport were measured over the initial linear time course of transport, and the data were analyzed by Eadie–Hofstee plots to obtain apparent K_m and V_{max} values (Table 2). Mutants with Ala, Val, Ser, or Thr in place of Cys148 exhibit relatively small changes in apparent K_m for lactose. Replacement with Ala or Val decreases the apparent K_m for lactose by about a factor of 2 relative to wild-type, while replacement with Ser or Thr causes a slight but significant (five independent determinations) increase in apparent K_m. More dramatic changes in apparent K_m are observed with TMG. Apparent K_m values for C148A and C148V permease are reduced by a factor of 3–4 relative to wild-type, while the apparent K_ms for C148S and C148T are increased by a factor of 2–3. Finally, the apparent K_m of mutant C148A for galactose is decreased by a factor of 6–7 relative to wild-type.

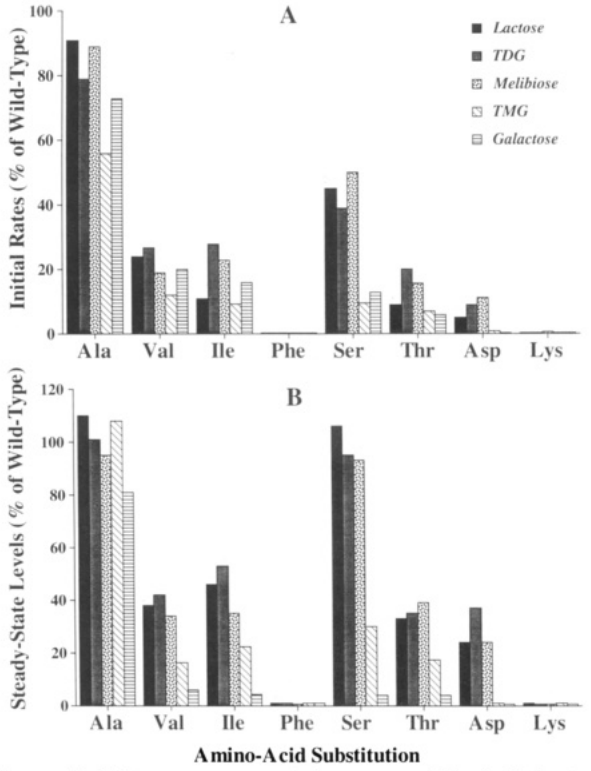

FIGURE 5: Histogram representation summarizing initial rates of transport and steady-state levels of accumulation in *E. coli* T184 cells expressing C148 replacement mutants. Cells were grown and transport was measured as described in the legends to Figures 2 and 3. (A) Initial rates of transport. Rates were taken from the linear part of the transport curve (usually 5 –10 s). The data represent the average of 3–5 measurements. Results are expressed as a percentage of wild-type permease activity after correcting for nonspecific transport by T184 cells harboring pT7-5 (vector without *lacY* gene). (B) Steady-state levels of accumulation. Results are expressed as percentage of the wild-type value and are corrected for the value obtained with T184 harboring pT7-5. Values were determined after 1 h of transport.

Table 2: K_m and V_{max} Values for Transport of Lactose, TMG, and Galactose by Wild-Type Permease and Cys148 Replacement Mutants[a]

	lactose		TMG		galactose	
construct	K_m (mM)	V_{max} [nmol/(min·mg of protein)]	K_m (mM)	V_{max} [nmol/(min·mg of protein)]	K_m (mM)	V_{max} [nmol/(min·mg of protein)]
wild-type	0.23	124	0.75	112	2.4	152
C148A	0.09	62	0.23	57	0.37	35
C148V	0.14	5	0.17	6	nd[b]	nd
C148S	0.34	51	2.13	33	nd	nd
C148T	0.38	8	1.43	18	nd	nd

[a] Initial rates of transport were measured at substrate concentrations from 0.02 to 4 mM. The data were plotted according to Eady–Hofstee. The values presented present the average of three to five independent experiments. [b] nd, not determined.

The effects of replacements for Cys148 on V_{max} for lactose or TMG differ from the effects on the apparent K_ms. Although C148V permease exhibits lower apparent K_ms for both substrates, V_{max} values are reduced by about a factor of about 20, while mutant C148T exhibits marked decreases in V_{max} for lactose and TMG despite relatively small increases in apparent K_m for both substrates. On the other hand, V_{max} values ranging from 30% to 50% of wild-type are obtained with C148A and C148S permeases with lactose or TMG as substrates, and C148A permease transports galactose with a V_{max} that is about 25% of wild-type.

DISCUSSION

This paper focuses on Cys148 in lac permease, a residue that is protected from reaction with thiol reagents by ligands of the permease (Fox & Kennedy, 1965; Kennedy et al., 1974; Beyreuther, 1981) but inessential for activity (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986). These properties can be explained in either of two ways: (i) Cys148 may be a component of a substrate binding site in the permease that is sterically blocked in the presence of ligand, as postulated originally by Fox and Kennedy (1965), but does not interact in an essential manner with ligand; or (ii) Cys148 may be far removed from a substrate binding site, and protection could result from long-range conformational effects such as those observed with V315C (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a), H322C, or E269C permease (Jung et al., 1994b). The results presented in this paper, particularly when considered together with the findings presented in the following paper (Wu & Kaback, 1994), are clearly consistent with the first alternative.

The observations presented here confirm and extend earlier studies (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986) demonstrating that Cys148 is not essential for lactose/H^+ symport. Thus, lactose transport by C148S or particularly C148A permease is comparable to wild-type demonstrating that a thiol at position 148 does not play an essential role in the mechanism of the permease. The lactose transport activity of the other mutants fall into two groups: C148I, C148T, C148V, and C148D permeases which exhibit reduced but clearly significant activities, and C148F or C148K permeases which exhibit essentially no activity. From this pattern of activities, it seems reasonable to conclude that although the side chain at position 148 is not essential, alterations in hydropathy, size, and/or charge at this position can modify the activity of the permease. With respect to lactose, the physiological substrate of the permease, it appears that a small, relatively hydrophobic side chain is optimal, but the differences between Ala, Ser, and Gly are not dramatic. Thus, maximum activity is observed with Ala at position 148, while permease with either Ser [Figures 3 and 4; see Sarkar et al. (1986) in addition] or Gly (Trumble et al., 1984; Viitanen et al., 1985) accumulate lactose to a normal steady state at about 50% or 25%, respectively, the rate of wild-type. The activities of the other mutants are generally consistent with this conclusion. Permeases with Ile, Val, or Thr, which are larger than Ala or Ser, at position 148 exhibit grossly similar activity, and permease with a large hydrophobic Phe residue at position 148 is completely inactive. Finally, the effect of charge at position 148 is interesting. With mutant C148D, low but significant lactose transport activity is observed, while C148K permease is inactive indicating that the permease will tolerate a negative but not a positive charge at position 148 to a limited extent. Furthermore, when lactose transport by C148D permease is assayed at pH 5.5 rather than 7.5, the rate of lactose transport is stimulated about 5-fold (data not shown), demonstrating that protonation of the carboxylate improves activity and indicating that this position in the permease is accessible to solvent.

The same general pattern is observed with the disaccharides TDG and melibiose. Thus C148A and C148S exhibit similar initial rates and steady-state levels of accumulation as the wild-type relative to lactose, C148V, C148I, C148T, and C148D exhibit lower but significant activities, and C148F and C148K are inactive. Remarkably, much more marked differences are observed when transport of TMG or galactose is measured. Most dramatically, C148A permease transports the two monosaccharides in a manner comparable to wild-

type, while C148S permease transports these sugars much less effectively than wild-type. With the exceptions of C148F and C148K which are completely inactive, a similar decrease in transport of the monosaccharides relative to the disaccharides is observed with the other mutants, particularly with respect to steady-state levels of accumulation.

The observed changes in K_m values for lactose, TMG, and galactose reveal that replacement of Cys148 with small hydrophobic amino acid residues (Ala, Val) leads to an increase in apparent substrate affinity (i.e., a decrease in K_m), whereas hydrophilic substitutions (Ser, Thr) cause the opposite effect. Thus, hydrophobic interactions with the galactosyl moiety probably contribute to substrate binding. Although these interactions are of minor importance with respect to disaccharides, removal of the "nongalactosyl" moiety of the substrate apparently diminishes certain binding interactions and thereby reveals the functional importance of hydrophobic interactions of the galactosyl moiety with position 148. Indeed, Cys is relatively hydrophobic (Kyte & Doolittle, 1982) and much poorer at hydrogen-bonding than Ser (Richardson & Richardson, 1989). Taken together with the results presented here, it appears very unlikely that Cys148 interacts with galactosides via hydrogen bonding. Moreover, hydrophobic sugar–protein interactions for lactose have been reported for other proteins. For example, on the basis of the three-dimensional structure of the complex formed between lactose and a lectin from *Erythrina corallodendron*, Shaanan et al. (1991) demonstrated that the galactose moiety is held by hydrophobic interactions with Tyr, Phe, and two Ala residues, as well as seven hydrogen bonds.

In conclusion, therefore, the results presented in this paper are consistent with the original postulate of Fox and Kennedy (1965) that a Cys residue in lac permease is located in a substrate binding site. This residue, identified as Cys148 by Beyreuther et al. (1981), is not essential for transport and interacts relatively weakly and hydrophobically with the galactosyl moiety of the substrate. In the following paper (Wu & Kaback, 1994), more direct evidence indicating that Cys148 is a component of a substrate binding site is presented.

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