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Role of Cysteine Residues in the *Lac* Permease of *Escherichia coli*

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ABSTRACT: Oligonucleotide-directed, site-specific mutagenesis has been utilized to replace cysteine residues 117, 333, or 353 and 355 with serine in the *lac* permease of *Escherichia coli*. Replacement of Cys-117 or Cys-333 has no significant effect on permease activity, while permease with serine residues in place of Cys-353 and Cys-355 has about 50% of wild-type permease activity. The results provide a clear demonstration that cysteine residues at positions 117, 333, 353, and 355 are not obligatory for lactose/H^+ symport. When considered in conjunction with previous findings, the results indicate that, of the eight cysteine residues in the *lac* permease, only Cys-154 is important for lactose transport. As discussed, the conclusion has important implications for the hypothesis that sulfhydryl–disulfide interconversion plays an important role in the symport mechanism.

The *lac* permease of *Escherichia coli* is an integral membrane protein encoded by the *lac Y* gene that catalyzes symport (i.e., cotransport) of β-galactosides with H^+ [cf. Kaback (1986) and Wright et al. (1986) for recent reviews]. The *lac Y* gene has been cloned and sequenced, and the permease has been purified to a single polypeptide species in a completely functional state, thereby demonstrating that the *lac Y* gene product is solely responsible for β-galactoside transport. Secondary structure models for the permease based on circular dichroic and laser Raman spectroscopy and on analyses of sequential hydropathic character suggest that the polypeptide is organized into 12–14 hydrophobic α-helical segments that traverse the membrane in a zig-zag manner, connected by more hydrophilic, charged segments. Preliminary evidence supporting certain general aspects of these models has been obtained from limited proteolysis studies and from binding studies with monoclonal and site-directed polyclonal antibodies.

During the past few years, oligonucleotide-directed, site-specific mutagenesis has been utilized to modify the structure and function of the *lac* permease (Sarkar et al., 1986a), and recent studies suggest that the technique has the potential to yield unique mechanistic information. Thus, a series of experiments utilizing site-directed mutagenesis (Padan et al., 1985; Püttner et al., 1986; Carrasco et al., 1986) has provided

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Table I: Bacterial Strains and Plasmids

strain	relevant genotype (chromosome/F′/plasmid)	ref
JM101	supE, thi, Δ(lac-proAB)/traD36, proAB lacI^{q}ZΔM15/−	*a*
JM105	thi, rspL endA, sbcB15, hspRT4, Δ(lac−proAB)/traD36 proAB lacI^{q}ZΔM15/−	*a*
MAA23	Δ(lac-pro)/lacI^q O$^+$ Z$^+$ Y$^-$/−	*b*
T184	Lac I$^+$ O$^+$ Z$^-$ Y$^-$/lac I^q O$^+$ Z^{U118} (Y$^+$)/−	*c*

[a] Yanisch-Perron et al. (1985). [b] Hobson et al. (1977). [c] Teather et al. (1980); Lac Z^{U118} is a polar (nonsense) mutation which results in *lac Z$^-$Y$^-$* phenotype.

evidence that His-322 and Glu-325, neighboring residues in putative helix 10, may be components of a charge-relay system that plays a critical role in the coupled translocation of lactose and H$^+$.

Given the importance attributed to sulfhydryl groups in the *lac* permease (Fox & Kennedy, 1965; Kaback & Barnes, 1971; Konings & Robillard, 1982), particularly Cys-148 (Beyreuther et al., 1981), site-directed mutagenesis was utilized initially to replace Cys-148 with glycine (Trumble et al., 1984; Viitanen et al., 1985) or serine (Neuhaus et al., 1985; Sarkar et al., 1986b). The results of these experiments demonstrate clearly that although Cys-148 is required for substrate protection against inactivation of the permease by *N*-ethylmaleimide, it is not mandatory for lactose/H$^+$ symport. Subsequently, Menick et al. (1985) showed that replacement of Cys-154 with either glycine or serine residues leads to a dramatic loss in transport activity, although the altered permease binds a high-affinity ligand normally. Most recently, Brooker and Wilson (1986) replaced cysteine residues at positions 176 or 234 with serine and found little effect on permease activity.

This paper describes site-directed mutagenesis of the remaining four cysteine residues in the *lac* permease, Cys-117, Cys-333, Cys-353, and Cys-355. When these residues are replaced with serine, the ability of the permease to catalyze lactose/H$^+$ symport is either uncompromised or mildly impaired relative to the wild-type permease. Taken as a whole, the results indicate that out of a total of eight cysteine residues in the permease, only Cys-154 is important for lactose/H$^+$ symport.

Experimental Procedures

Materials

T4 DNA ligase, bacterial alkaline phosphatase, *Eco*RI, *Hin*dIII, *Hin*cII, and dideoxynucleotide sequencing reagents were from BRL; *dam* methylase was from New England Biolabs; *S*-adenosylmethionine (AdoMet)[1] was from Boehringer/Mannheim; [γ-^{32}P]ATP, [α-^{32}P]dATP, [^{14}C]lactose, and T4 polynucleotide kinase were from Amersham; Klenow fragment of DNA polymerase I was from BRL, Boehringer/Mannheim, and New England Biolabs; nitrocellulose (BA85) was from S & S; Sea Plaque and Sea Kem agarose were from FMC; [^{14}C]methyl 1-thio-β-D-galactopyranoside (TMG) was purchased from New England Nuclear.

Methods

Bacterial Strains. Bacterial strains are listed in Table I.

Oligonucleotide Synthesis. The oligonucleotides described were synthesized on an Applied Biosystems Model 380B DNA synthesizer and were purified by polyacrylamide gel electrophoresis as described in the Applied Biosystems manual.

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1986a) using one of the following modifications to improve the frequency of mutations.

The first strategy used the "coupled priming" technique (Kramer et al., 1984; Brooker & Wilson, 1986). The *lac Y* gene was cloned initially from pGM21 into the *Eco*RI site of the replicative form (RF) of M13mp8. The genome of M13mp8 contains two amber mutations within critical M13 genes and can only be propagated in suppressor strains of *E. coli* such as JM101 (Table I). RF DNA from M13mp18, which contains no amber mutations, was isolated and restricted with *Eco*RI and *Hin*dIII to remove the polylinker region. The restricted M13mp18 fragment (~7200 bp) was isolated by agarose gel electrophoresis. The purified 7200 bp fragment of M13mp18 was added to the single-stranded (ss) M13mp8 DNA containing the wild-type *lac Y* gene (2.5 and 25 pmol/mL, respectively) and to the appropriate mutagenetic primer (Table II; 1.25 nmol/mL). The mixture was heated to 90 °C for 10 min and allowed to anneal at 55 °C for 30 min. The gaps in the resulting heteroduplex were filled in by incubation with the Klenow fragment of DNA polymerase I plus deoxynucleotide triphosphates, and ligation was achieved with T4 ligase. The resulting closed-circular, double-stranded DNA was used to transfect the nonsuppressor strain JM105. The M13mp8(+) DNA strand containing wild-type *lac Y* cannot be propagated because of the amber mutations in the phage genes. In contrast, the complementary M13mp18(−) DNA strand containing mutated *lac Y* can serve as a viable template for phage proliferation, and the resulting plaques should all contain the mutant *lac Y* gene. The ssDNA from putative mutants was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) by using appropriate primers complementary to regions of the *lac Y* gene 50–100 bases downstream from the mutation.

The second modification utilizes in vitro methylation, single-strand nicking, and denaturation of the heteroduplex prior to transfection in order to increase the frequency of mutant recovery (Sarkar et al., 1986a). The appropriate mutagenetic primer (Table II) was annealed to the ss M13mp19 DNA containing wild-type *lac Y*. Extension, ligation, and isolation of the heteroduplex DNA were carried out as described (Sarkar et al., 1986a). Closed-circular, heteroduplex DNA was methylated by using *dam* methylase and *S*-adenosylmethionine (AdoMet), nicked with *Hin*dIII in the presence of ethidium bromide and heat denatured before transfecting JM101. Phage harboring a given mutation were identified initially by colony-blot hybridization using the appropriate ^{32}P-labeled mutagenic primer (Carter et al., 1984). Phage from positive colonies were plaque purified, and the mutation was verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) utilizing a synthetic primer complementary to a region of *lac Y* 50–100 bases downstream from the site of mutation.

Double-stranded *lac Y* DNA from each mutant was restricted from M13mp18 or M13mp19 RF DNA and ligated into the *Eco*RI site of pACYC184. The resulting plasmids (Table II), pS117, pS333, and pS353–355, were used to transform *E. coli* MAA23 (*lac Z$^+$Y$^-$*) or T184 (*lac Z$^-$Y$^-$*).

Transport Assays. E. coli T184 and MAA23 containing appropriate plasmids were grown and induced with isopropyl 1-thio-β-D-galactopyranoside as described (Teather et al., 1980; Brooker & Wilson, 1986). Cells were harvested in mid-log

[1] Abbreviations: IPTG, D-galactopyranoside; TMG, methyl 1-thio-β-D-galactopyranoside; AdoMet, *S*-adenosylmethionine; ssDNA, single-stranded DNA; RF, replicative form; $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; bp, base pair(s).

Table II: DNA Sequence Analysis of Cysteine Mutants in *Lac Y*

plasmid	mutagenic primer	DNA sequence change	protein change
pGM21		*lac* Y^+	
pS117	5′-TAAAAC*TAAAGCCTAGAT-3′	codon 117, TGT → AGT	Cys-117 → Ser
pS154[a]	5′-GGCAC*TCAGCGCCCAGCC-3′	codon 154, TGT → AGT	Cys-154 → Ser
pS333	5′-AAAGC*TGCCCACCAGCAG-3′	codon 333, TGC → AGC	Cys-333 → Ser
pS353–355	5′-AAAGAAGC*TGAAAC*TGACCAGATA-3′	{codon 353, TGC → AGC codon 355, TGC → AGC}	{Cys-353 → Ser Cys-355 → Ser}

[a] Menick et al. (1985).

phase, washed in 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$, resuspended at an A_{420} of 10, and equilibrated to 25 °C. Transport was initiated by addition of a given radioactive β-galactoside at a specified concentration and specific activity. At the times indicated, transport was terminated by rapid dilution and filtration as described (Kaback, 1971, 1974).

Protein Determination. Protein was measured as described (Lowry et al., 1951) with bovine serum albumin as a standard.

Results

Table II summarizes the results of DNA sequence analyses of the mutated *lac Y* genes described. In each instance, a given *lac Y* gene contains the T to A change at the site predicted, thereby changing a specific cysteine residue in the permease to a serine residue.

Double-stranded *lac Y* DNA from each of the mutants was restricted from M13mp18 or M13mp19 RF DNA and ligated into the *Eco*RI site of pACYC184. The resulting plasmids, pS117, pS333, and pS353–355 (Table II), were used to transform *E. coli* MAA23 (*lac* Z^+Y^-) or T184 (*lac* Z^-Y^-). When the cryptic strain MAA23 is transformed with pS117, pS333, or pS353–355 and grown on eosinmethylene blue (EMB) lactose indicator plates, the cells form dark red colonies that are indistinguishable from MAA23 transformed with pGM21 which encodes wild-type *lac* permease. In contrast, MAA23 transformed with pS154 forms light red colonies on EMB lactose. Since S154 exhibits impaired lactose transport (Menick et al., 1985), the results provide a preliminary indication that *lac* permease encoded by pS117, pS333, or pS353–355 retains significant ability to catalyze lactose accumulation.

Transport of lactose in *E. coli* T184 transformed with pGM21 (T184/pGM21) or each of the mutant plasmids is shown in Figure 1. With S333, the initial velocity of transport and the steady-state level of accumulation are not significantly different from T184/pGM21. In transport experiments conducted with eight independently grown cultures, the initial rate of transport in S333 varied from 73% to 126% of that observed in T184/pGM21, while the steady-state level of accumulation varied from 62% to 100%. With S117, there is a small decrease in lactose transport which is marginally significant; the initial velocity is about 70% of the control, and the steady state approximates 50%. In transport studies with three independently grown cultures, initial rates and steady states varied from 70% to 118% and from 50% to 80%, respectively, of the controls. Finally, in the double-replacement mutant, S353–355, both the initial rate and the steady state are about 50% of the wild-type levels (four independent cultures; range ±5%). Importantly, the initial rate of lactose transport in each of these mutants is well above that observed in S154 which exhibits an initial rate of only 10% of T184/pGM21 [in addition, cf. Menick et al. (1985)].

When the cryptic strain MAA23 is transformed with pS117, pS333, or pS353–355, the rate of lactose uptake is very similar to that observed in MAA23 transformed with pGM21, while

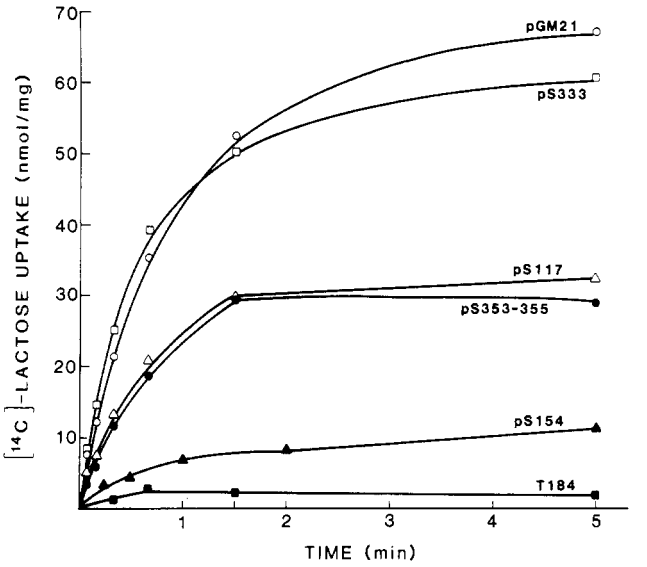

FIGURE 1: Lactose transport in *E. coli* T184 (■) and T184 transformed with pGM21 (○), pS117 (△), pS154 (▲), pS333 (□), or pS353–355 (●). Uptake was measured in 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$ at 25 °C with 0.38 mM [1-^{14}C]lactose (19.7 mCi/mmol).

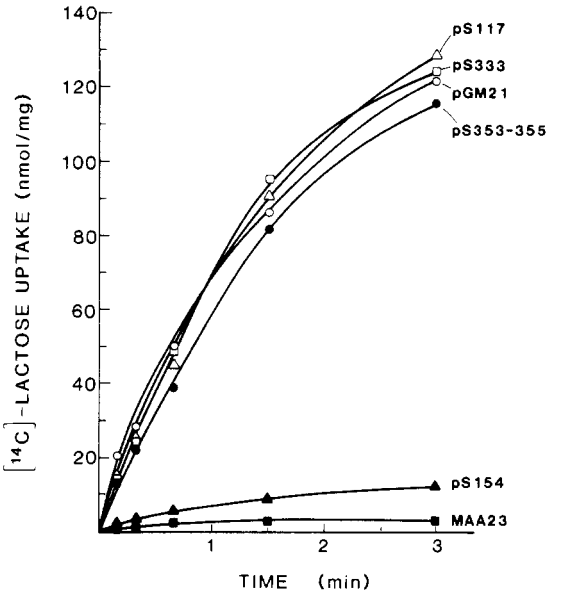

FIGURE 2: Lactose transport in *E. coli* MAA23 (■) and MAA23 transformed with pGM21 (○), pS117 (△), pS154 (▲), pS333 (□), or pS353–355 (●). Uptake was measured in 100 mM potassium phosphate (pH 7.5)/10 mM $MgSO_4$ at 25 °C with 0.38 mM [1-^{14}C]lactose (19.7 mCi/mmol).

the rate of uptake in MAA23/pS154 is only about 8% of that observed in MAA23/pGM21 (Figure 2). In this case, the host strain contains β-galactosidase, and the rate of hydrolysis is limited by the rate of entry. Thus, lactose uptake under these conditions does not occur against a concentration gradient, but is presumably "downhill" (Rickenberg et al., 1956). In any

Table III: Summary of Transport Activities in *Lac Y* Cysteine Mutants[a]

Cys residue	substitution	initial rate of transport (% wild type)	ref
117	Ser	70	this work
148	Gly	25	*b, c*
148	Ser	100	*d*
154	Gly	0	*e*
154	Ser	10	*e*
176	Ser	80	*f*
234	Ser	70	*f*
333	Ser	100	this work
353, 355	Ser	≥50	this work

[a] Apparent initial rate of lactose transport in *E. coli* T184 transformed with the appropriate plasmid. [b] Trumble et al. (1984). [c] Viitanen et al. (1985). [d] Sarkar et al. (1986b). [e] Menick et al. (1985). [f] Brooker & Wilson (1986).

case, it is readily apparent that the results are qualitatively similar to those observed with T184 and support the general conclusion that replacement of cysteines -117, -333, or -353 and -355 does not cause a dramatic effect on lactose transport.

Although data are not shown, transport of the nonhydrolyzable substrate methyl 1-thio-β-D-galactopyranoside (TMG) was also examined in both T184 and MAA23 transformed with each of the plasmids described. In both host strains, results similar to those presented in Figure 1 were obtained.

Discussion

The work presented here completes the systematic replacement of cysteine residues in the *lac* permease by site-directed mutagenesis. Thus, cysteines-117, -333, or -353 and -355 have been replaced with serine residues, and importantly, despite some quantitative differences, each of the altered permeases catalyzes lactose/H^+ symport at least 50% as well as the wild-type permease. When considered in conjunction with earlier studies describing site-directed mutagenesis of cysteines-148 (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986b), -154 (Menick et al., 1985), and -176 or -234 (Brooker & Wilson, 1986), it is readily apparent that out of a total of eight cysteine residues in the *lac* permease, only Cys-154 appears to be important for activity (Table III).

In view of the attention that has been given to the functional importance of sulfhydryl groups in the permease over the past 20 years, the conclusion is particularly interesting. In 1965, Fox and Kennedy first demonstrated that *lac* permease can be protected from inactivation by *N*-ethylmaleimide by certain substrates of the permease and demonstrated that the permease can be labeled specifically by this means [cf. Kennedy (1970) for a review]. For these reasons, it was postulated that there is an "essential" sulfhydryl group in the permease located at or near the galactoside binding site, and in 1981, Beyreuther et al. (1981) identified the residue as Cys-148. With the advent of oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983), however, it was demonstrated that Cys-148 is not mandatory for lactose/H^+ symport, although it is required for substrate protection against alkylation (Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986b).

In addition to the postulated essentiality of a sulfhydryl group at or near the binding site of the permease, other hypotheses implicating cysteine residues in *lac* permease function have been put forward. Specifically, it has been suggested that the permease might undergo sulfhydryl–disulfide interconversion during turnover either as a respiratory intermediate (Kaback & Barnes, 1971) or as a H^+ carrier in equilibrium with the proton electrochemical gradient (Konings & Robillard, 1982; Robillard & Konings, 1982). In this context, the results obtained from site-directed mutagenesis of the cysteine residues in the *lac* permease place important restrictions on any theory that invokes disulfide formation as part of the catalytic mechanism. Since Cys-154 alone appears to be important for activity (Figures 1 and 2; Table III; Menick et al., 1985), it follows that any postulated disulfide formation must occur between two permease monomers. Although evidence has been presented that is consistent with the notion that the permease may dimerize in the presence of a proton electrochemical gradient (Mieschendahl et al., 1981; Goldkorn et al., 1983), all efforts to document $\Delta\bar{\mu}_{H^+}$-induced dimerization with purified, reconstituted *lac* permease using diamide (Kaback & Patel, 1978), triphenylarsenine oxide, plumbagen (Konings & Robillard, 1982), copper *o*-phenanthroline (Kobashi & Horecker, 1967; Steck, 1972), or bis(dimaleimides) (Moore & Ward, 1956) have been unequivocally negative (L. Patel, J. A. Lee, and H. R. Kaback, unpublished results). Furthermore, it is particularly noteworthy that although Ser-154 permease is defective, it retains the ability to catalyze accumulation against a concentration gradient at about 10% of the rate of the wild-type molecule. On the basis of these considerations, it seems highly unlikely that sulfhydryl–disulfide interconversion plays a central role in the mechanism of action of the *lac* permease.

Finally, the observations of Cohn et al. (1981) demonstrating that the rate of inactivation of the permease by various maleimides is enhanced by the proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) and the findings of Viitanen et al. (1985) demonstrating that this property of the permease is retained when Cys-148 is replaced with glycine deserve discussion in the context of the present findings. Since Cys-154 is the only cysteine residue in the permease that is essential for activity, the observations of Cohn et al. (1981) and Viitanen et al. (1985) suggest that Cys-154 is the residue that exhibits enhanced reactivity to maleimides in the presence of $\Delta\bar{\mu}_{H^+}$. The behavior of the permease in this respect indicates that $\Delta\bar{\mu}_{H^+}$ increases the nucleophilic character of Cys-154 and suggests that this residue might be involved in H^+ translocation. The following considerations tend to exclude this notion, however. As discussed above, permease with serine in place of Cys-154 does catalyze lactose accumulation, albeit at 10% of the rate of the wild type. Since serine is similar to cysteine in that the hydroxyl function might replace the sulfhydryl to an extent, Cys-154 has been replaced with a valine residue (D. R. Menick and H. R. Kaback, unpublished results). Permease with Val-154 catalyzes transport in a manner similar to that observed with the Ser-154 mutant. Therefore, although Cys-154 appears to be the only cysteine residue in the *lac* permease whose replacement leads to dramatic loss of activity, the bulk of the evidence suggests that Cys-154 is not directly involved in catalysis of lactose/H^+ symport.

Registry No. Cysteine, 52-90-4; lactose permease, 9068-45-5.

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