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Cysteine scanning mutagenesis of putative transmembrane helices IX and X in the lactose permease of *Escherichia coli*

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Abstract

Using a functional lactose permease mutant devoid of Cys residues (C-less permease), each amino-acid residue in putative transmembrane helices IX and X and the short intervening loop was systematically replaced with Cys (from Asn-290 to Lys-335). Thirty-four of 46 mutants accumulate lactose to high levels (70–100% or more of C-less), and an additional 7 mutants exhibit lower but highly significant lactose accumulation. As expected (see Kaback, H.R., 1992, *Int. Rev. Cytol. 137A*, 97–125), Cys substitution for Arg-302, His-322, or Glu-325 results in inactive permease molecules. Although Cys replacement for Lys-319 or Phe-334 also inactivates lactose accumulation, Lys-319 is not essential for active lactose transport (Sahin-Tóth, M., Dunten, R.L., Gonzalez, A., & Kaback, H.R., 1992, *Proc. Natl. Acad. Sci. USA 89*, 10547–10551), and replacement of Phe-334 with leucine yields permease with considerable activity. All single-Cys mutants except Gly-296 → Cys are present in the membrane in amounts comparable to C-less permease, as judged by immunological techniques. In contrast, mutant Gly-296 → Cys is hardly detectable when expressed at a relatively low rate from the *lac* promoter/operator but present in the membrane in stable form when expressed at a high rate from the T7 promoter. Finally, studies with *N*-ethylmaleimide (NEM) show that only a few mutants are inactivated significantly. Remarkably, the rate of inactivation of Val-315 → Cys permease is enhanced at least 10-fold in the presence of β-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) or an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$). The results demonstrate that only three residues in this region of the permease—Arg-302, His-322, and Glu-325—are essential for active lactose transport. Furthermore, the enhanced reactivity of the Val-315 → Cys mutant toward NEM in the presence of TDG or $\Delta\bar{\mu}_{H^+}$ probably reflects a conformational alteration induced by either substrate binding or $\Delta\bar{\mu}_{H^+}$.

Keywords: active transport; Cys modification; Cys replacements; lactose permease mutants; scanning mutagenesis

β-Galactoside transport in *Escherichia coli* is dependent upon the lactose permease, a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of sugar and H^+ with a 1:1 stoichiometry (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 responsible for β-galactoside transport, probably as a monomer (see Kaback [1989, 1992] and Kaback et al. [1990] for reviews). Circular dichroic studies and hydropathy analysis of the amino-acid sequence (Foster et al., 1983) led to the proposal that the permease has a short hydrophilic N-terminus, 12 hydrophobic domains in α-helical configuration that traverse the membrane in a zig-zag fashion connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail (Fig. 1). Evidence supporting the general features of the secondary-structure model and demonstrating that both the N- and C-termini are on the cytoplasmic face of the membrane has been obtained from laser Raman spectroscopy (Vogel et al., 1985), from limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986; Page & Rosenbusch, 1988), immunological studies with monoclonal (Carrasco et al., 1982, 1984b; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibod-

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Abbreviations: C-less permease, functional lactose permease devoid of Cys residues; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; $\Delta\bar{\mu}_{H^+}$, the H^+ electrochemical gradient across the membrane; NEM, *N*-ethylmaleimide; lac, lactose; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; IPTG, *i*-propyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; SDS, sodium dodecyl sulfate.

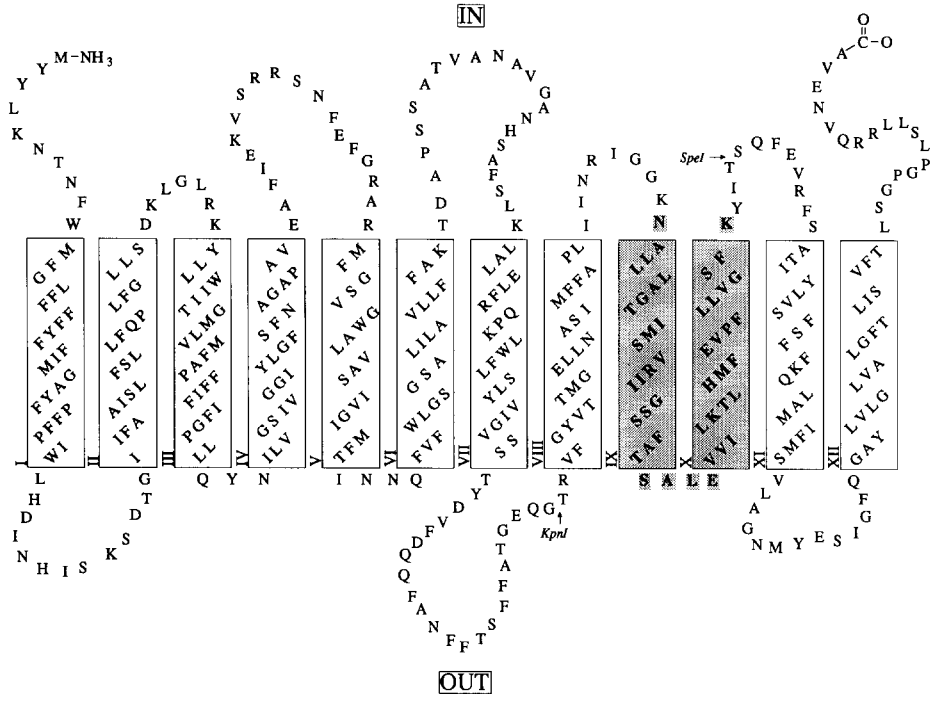

Fig. 1. Secondary-structure model of *Escherichia coli* lac permease (Foster et al., 1983). The single letter amino-acid code is used, and putative transmembrane helices are shown in boxes. The shaded area highlights the region of lac permease subjected to Cys scanning mutagenesis. Also indicated are the restriction endonuclease sites used for construction of the mutants.

ies (Seckler et al., 1983, 1986; Carrasco et al., 1984a; Seckler & Wright, 1984; Danho et al., 1985), and chemical modification (Page & Rosenbusch, 1988). Moreover, exclusive evidence for the presence of 12 transmembrane domains has been obtained from analyses of a large number of lac permease-alkaline phosphatase (*lacY-phoA*) fusions (Calamia & Manoil, 1990).

Oligonucleotide-directed, site-specific mutagenesis of lac permease has been useful for identifying functionally important residues (see Kaback, 1989, 1992; Kaback et al., 1990). Site-directed replacement of Arg-302 (Menick et al., 1987) in putative helix IX, His-322 (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a,b, 1990a; Brooker, 1990, 1991) or Glu-325 (Carrasco et al., 1986, 1989) in helix X indicate that these residues play a particularly important role in lac/H^+ symport and/or substrate recognition. Moreover, differences in the properties of the mutants with respect to various translocation reactions catalyzed by the permease have led to the suggestion that Arg-302, His-322, and Glu-325 may function as components of an H^+ translocation pathway, although it is also possible that the residues form part of a coordination site for H_3O^+ (see Kaback et al. [1990] for a discussion). Lys-319 in helix X is also important for activity (Persson et al., 1992), but recent experiments (Lee et al., 1992; Sahin-Tóth et al., 1992) demonstrate that Lys-319 interacts functionally with Asp-240 and that neither residue is mandatory for active lac transport. In addition, Ile-303 (Eelkema et al., 1991) and Ser-306 (Collins et al., 1989; King & Wilson, 1989b, 1990b) in helix IX have been suggested to be important for sugar recognition.

The observations that putative helices IX and X contain residues essential for both H^+ translocation and substrate recognition indicate that this region of the permease plays an important role in the mechanism of action of the permease, a contention that is also consistent with insertional mutagenesis studies (McKenna et al., 1992b). Therefore, this region of lac permease was subjected to systematic "scanning" mutagenesis using a functional mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991). Residues from Asn-290 to Lys-335 were individually replaced with Cys, and activity, expression, and sensitivity to inhibition by NEM were studied. Cys was chosen as a "scanner" residue because: (1) the side-chain is of intermediate bulk; (2) Cys is relatively hydrophobic (Kyte & Doolittle, 1982); (3) Cys reacts specifically with a variety of sulfhydryl reagents; and (4) Cys mutagenesis has been used to study other membrane proteins (e.g., bacteriorhodopsin [Flitsch & Khorana, 1989], the tar protein [Lynch & Koshland, 1991; Pakula & Simon, 1992], maltoporin [Francis et al., 1991], and the nicotinic acetylcholine receptor [Akabas et al., 1992]).

The great majority of the Cys-replacement mutants in lac permease retain highly significant lac transport activity, and evidence is presented demonstrating that only three residues, Arg-302, His-322, and Glu-325, play an important role in the transport mechanism. Furthermore, studies with NEM show that only a few of the Cys-replacement mutants are inactivated significantly, and one mutant — V315C — exhibits markedly enhanced rates of inactivation in the presence of ligand or an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$).

Results

Construction and verification of mutations

Individual Cys-replacement mutants were constructed in C-less permease from residue Asn-290 to Lys-335, a region containing putative transmembrane helices IX and X and a short intervening hydrophilic loop (Fig. 1). Each Cys mutant was constructed by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13mp19. The replicative forms of verified M13 clones were restricted with *Kpn* I and *Spe* I (Fig. 1) and the fragments ligated into plasmid pT7-5/cassette *lacY* encoding C-less permease (van Iwaarden et al., 1991). After propagation in *E. coli* HB101, recombinant plasmid DNA was isolated, and the *Kpn* I–*Spe* I fragments were subjected to double-stranded DNA sequencing as described in Materials and methods. Except for the base changes summarized in Table 1 the sequences were identical to that of C-less cassette *lacY*. Mutant C333, C-less permease with a native Cys residue restored, was constructed by ligating the *Kpn* I–*Spe* I fragment of pT7-5/cassette *lacY* into pT7-5/cassette *lacY* encoding C-less permease.

Colony morphology

Escherichia coli HB101 (*lacZ*$^+$*Y*$^-$) is a "cryptic" strain that expresses active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of external lac to cytosolic β-galactosidase, and subsequent metabolism of the monosaccharides causes acidification and the appearance of red colonies on MacConkey-agar indicator plates containing lac. Cells that are impermeable to lac appear as white colonies, and permease mutants with low activity grow as red colonies with a white halo. It is important that indicator plates report "downhill" lac translocation only and give no indication as to whether or not cells catalyze active transport (i.e., accumulation against a concentration gradient). HB101 expressing mutants containing individual Cys replacements for Gly-296, Arg-302, Lys-319, Glu-325, Pro-327, Gly-332, Phe-334, or Lys-335 in C-less permease grow as red colonies with white halos. The remainder of the mutants grow as red colonies indistinguishable from C-less. Therefore, judging from indicator plates, all the mutants retain at least some ability to translocate lac.

Active lactose transport

As a more quantitative measure of function, [1-^{14}C]lac transport was assayed in *E. coli* T184 expressing each Cys-replacement mutant. When rates of lac uptake are measured at 1 min and expressed as a percentage of C-less permease, it is apparent that the great majority of the 46 mutants transport lac at significant rates (Fig. 2A). Twenty-five mutants transport lac at rates of 80–100% or more of C-less, and an additional 12 mutants exhibit rates that are between 30 and 70% of C-less. In contrast, 9 mutants (G296C, R302C, K319C, H322C, E325C, P327C, G332C, F334C, and K335C)[1] exhibit markedly decreased

Table 1. *DNA sequence analysis of cysteine replacement mutants in the lac permease*

Mutant	Mutagenic oligonucleotide[a]	Codon change
N290C	CAGCAGGGC**ACA**CTTCCCACC	AAT → TGT
A291C	CAGCAGCAG**GCA**ATTCTTCCC	GCC → TGC
L292C	AGCCAGCAG**ACA**GGCATTCTT	CTG → TGT
L293C	GCCAGCCAG**ACA**CAGGGCATT	CTG → TGT
L294C	AGTGCCAGC**ACA**CAGCAGGGC	CTG → TGT
A295C	AATAGTGCC**ACA**CAGCAGCAG	GCT → TGT
G296C	CATAATAGT**GCA**AGCCAGCAG	GGC → TGC
T297C	AGACATAAT**ACA**GCCAGCCAG	ACT → TGT
I298C	TACAGACAT**ACA**AGTGCCAGC	ATT → TGT
M299C	ACGTACAGA**ACA**AATAGTGCC	ATG → TGT
S300C	AATACGTAC**ACA**CATAATAGT	TCT → TGT
V301C	AATAATACG**ACA**AGACATAAT	GTA → TGT
R302C	GCCAATAAT**ACA**TACAGACAT	CGT → TGT
I303C	TGAGCCAAT**ACA**ACGTACAGA	ATT → TGT
I304C	CGATGAGCC**ACA**AATACGTAC	ATT → TGT
G305C	GAACGATGA**GCA**AATAATTCG	GGC → TGC
S306C	GGTGGCGAACGA**ACA**GCCAATAATACG	TCA → TGT
S307C	TGAGGTGGCGAA**ACA**TGAGCCAATAAT	TCG → TGT
F308C	TGAGGTGGC**GCA**CGATGAGCC	TTC → TGC
A309C	CAGCGCTGAGGT**GCA**GAACGATGAGCC	GCC → TGC
T310C	TTCCAGCGCTGA**GCA**GGCGAACGATGA	ACC → TGC
S311C	TTCCAGCGC**ACA**GGTGGCGAA	TCA → TGT
A312C	CACTTCCAG**ACA**TGAGGTGGC	GCG → TGT
L313C	AACCACTTC**ACA**CGCTGAGGT	CTG → TGT
E314C	CAGAATAACCAC**ACA**CAGCGCTGAGGT	GAA → TGT
V315C	TTTCAGAATAAC**GCA**TTCCAGCGCTGA	GTA → TGC
V316C	CGTTTTCAGAAT**ACA**CACTTCCAGCGC	GTT → TGT
I317C	CAGCGTTTTCAG**ACA**AACCACTTCCAG	ATT → TGT
L318C	ATGCAGCGTTTT**GCA**AATAACCACTTC	CTG → TGC
K319C	CATATGCAGCGT**ACA**CAGAATAACCAC	AAA → TGT
T320C	AAACATATGCAG**ACA**TTTCAGAATAAC	ACG → TGT
L321C	TTCAAACATATG**GCA**CGTTTTCAGAAT	CTG → TGC
H322C	TACTTCAAACAT**ACA**CAGCGTTTTCAG	CAT → TGT
M323C	CGGTACTTCAAA**GCA**ATGCAGCGTTTT	ATG → TGC
F324C	CGGTACTTC**ACA**CATATGCAG	TTT → TGT
E325C	CAGGAACGGTAC**ACA**AAAACATATGCAG	GAA → TGT
V326C	CAGCAGGAACGGG**GCA**TTCAAACATATGC	GTA → TGC
P327C	CACCAGCAGGAAG**GCA**TACTTCAAACAT	CCG → TGC
F328C	GCCCACCAGCAGG**CA**CGGTACTTCAAA	TTC → TGC
L329C	GGAGCCCACCAGG**CA**GAACGGTACTTC	CTG → TGC
L330C	AAAGGAGCCCAC**GCA**CAGGAACGGTAC	CTG → TGC
V331C	TTTAAAGGAGCC**GCA**CAGCAGGAACGG	GTG → TGC
G332C	ATATTTAAAGGA**GCA**CACCAGCAGGAA	GGC → TGC
F334C	AGTAATATATTT**ACA**GGAGCCCACCAG	TTT → TGT
K335C	ACTAGTAATATA**GCA**AAAAGGAGCCCAC	AAA → TGC

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

[1] 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.

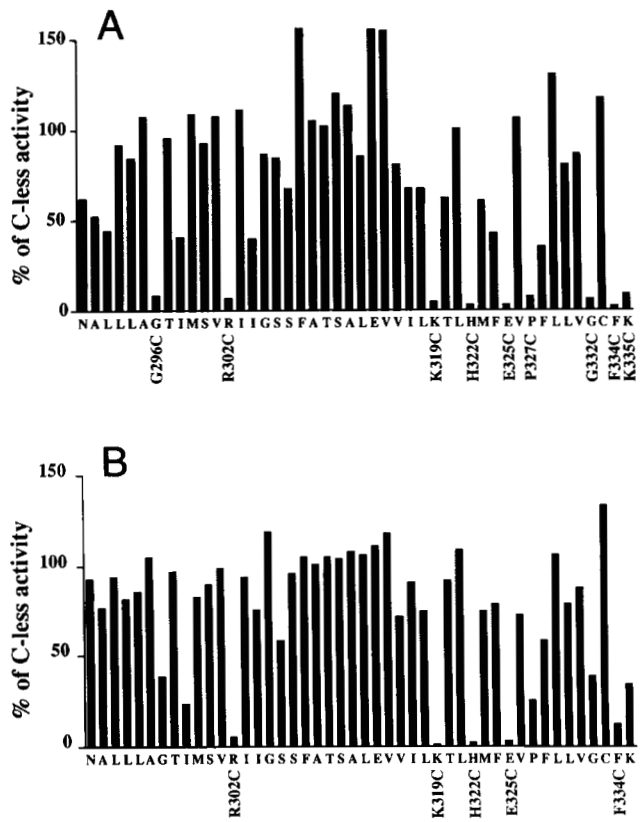

Fig. 2. Lactose transport by *E. coli* T184 expressing C-less permease or individual Cys-replacement mutants. Cells were grown overnight at 30 °C as described in Materials and methods. Aliquots of cell suspensions (50 μL containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lac (10 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched by addition of 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl and rapidly filtered through Whatman GF/F filters. The single letter amino-acid code is used along the horizontal axis to denote the original residues in increasing order from Asn-290 to Lys-335. Mutants with diminished activity are indicated. **A:** Rates of lac transport measured at 1 min. The rate of C-less permease was 31.6 nmol lac/min/mg protein. Results are expressed as a percentage of C-less activity. Although not shown (see Fig. 3), the rate of transport by T184 cells harboring pT7-5 (vector with no *lacY* gene) was 0.87 nmol/min/mg protein (i.e., 2.7% of C-less). **B:** Steady-state levels of accumulation. The steady-state level of lac accumulation observed for C-less permease was 142.5 nmol lac/mg protein. Results are expressed as a percentage of this value. Although not shown (see Fig. 3), T184 harboring pT7-5 (vector with no *lacY* gene) took up 8.1 nmol of lac/mg protein in 1 h (i.e., 5.7% of C-less).

rates of transport, <10% of C-less permease. Steady-state levels of lac accumulation for most of the mutants also approximate C-less permease (Fig. 2B). Thirty-four mutants accumulate lac to steady-state levels of 70–100% or more of C-less permease, 7 mutants accumulate lac to lower but significant steady-state levels of accumulation, and 5 mutants appear to be unable to catalyze significant lac accumulation (R302C, K319C, H322C, E325C, and F334C).

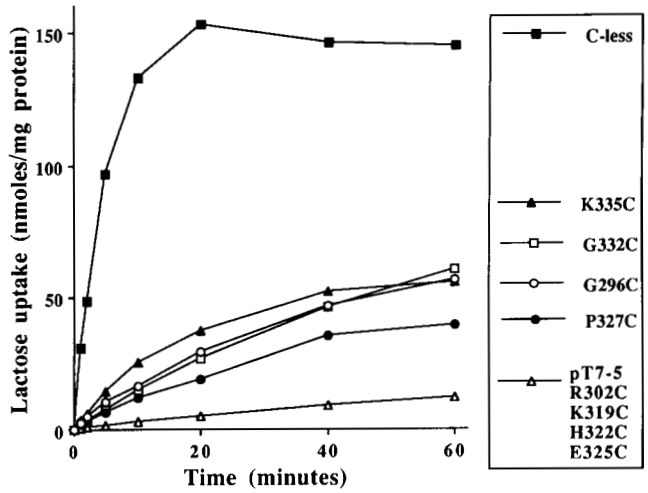

Fig. 3. Time courses of lac transport by single Cys mutants with low activity. *Escherichia coli* T184 transformed with plasmid pT7-5/C-less *lacY*, pT7-5 (vector with no *lacY* gene), or pT7-5 encoding given Cys-replacement mutants were grown and assayed as described in Figure 2. ■, C-less; △, pT7-5, R302C, K319C, H322C, E325C; ○, G296C; ●, P327C; □, G332C; ▲, K335C.

The data presented in Figures 3 and 4 represent time courses of lac accumulation for the 9 mutants with the lowest rates of transport. As shown (Fig. 3), mutants G296C, P327C, G332C, and K335C accumulate at low rates, but reach significant levels of accumulation in 1 h (i.e., 25–35% of C-less permease, which represents at least a 10-fold concentration gradient). Furthermore, lac accumulation by these mutants does not reach steady-state by 1 h and is still increasing. Also, previous experiments (Lolkema et al., 1988; Consler et al., 1991) demonstrate that P327A permease catalyzes lac transport in a manner indistinguishable from wild-type permease. On the other hand, mutants R302C, K319C, H322C, E325C, and F334C are unable to catalyze accumulation to a significant extent. Importantly, Lys-319 has been demonstrated to interact functionally with Asp-240 and is not essential for lac transport (Lee et al., 1992; Sahin-Tóth et al., 1992). Moreover, the data presented in Figure 4 show clearly that replacement of Phe-334 with Tyr, Trp, or Leu yields permease molecules that catalyze accumulation to increasingly significant steady states (20%, 30%, or 70% of C-less permease, respectively, at 1 h). Therefore, only 3 of the 46 residues in this region of lac permease—Arg-302, His-322, and Glu-325—are required for active lac transport.

Expression of Cys-replacement mutants

In order to test whether or not the alterations in transport activity observed are due to differences in the amount of permease in the membrane, immunoblots were carried out on membrane preparations from *E. coli* T184 transformed with plasmids encoding the different mutants.

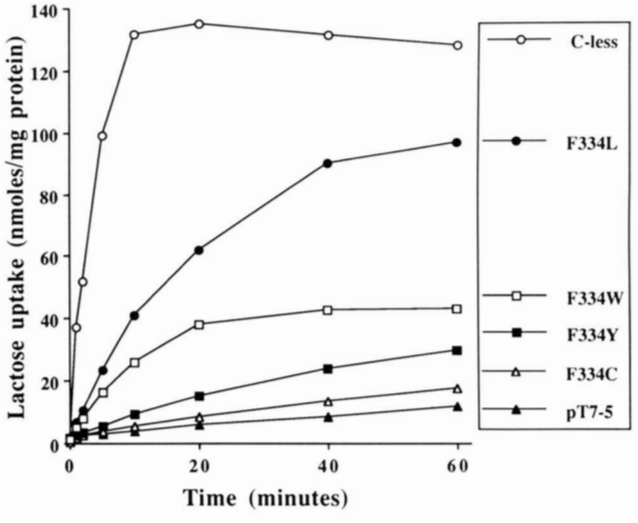

Fig. 4. Effect of various amino-acid replacements for Phe-334. Transport of lac in *E. coli* T184 expressing C-less permease, no permease (pT7-5 with no *lacY* gene), or permease mutants with given replacements for Phe-334 was assayed as described in Figure 2. ○, C-less; ▲, pT7-5; △, F334C; ●, F334L; □, F334W; ■, F334Y.

With the exception of G296C, which catalyzes significant lac accumulation (Fig. 3), each of the other mutants is present in the membrane in an amount comparable to C-less permease (Fig. 5). Strikingly, G296C is hardly detected in the membrane when the gene is expressed at a relatively low rate from the *lac* promoter/operator. However, when the mutant is expressed at a high rate from the T7 promoter after heat shock, it is present in the membrane in significant amounts (Fig. 6).

One explanation for the unique behavior of mutant G296C is that its lifetime in the membrane is diminished due to an enhanced rate of proteolysis (Roepe et al., 1989; McKenna et al., 1991, 1992a). Under these circumstances, little or no permease is observed when synthesis takes place at relatively low rates via the *lac* promoter/operator, but the mutant protein is detected upon synthesis at a high rate from the T7 promoter because the rate of synthesis exceeds the rate of degradation. However, [^{35}S]methionine pulse-chase experiments demonstrate that the intensity of the radioactive band corresponding to G296C permease remains essentially constant for 10 h after addition of unlabeled methionine, as observed for C-less permease (Fig. 7). Thus, as described for certain other mutants in lac permease (Dunten et al., 1993), mutant G296C appears to be defective in a step between translation and insertion into the membrane.

Effect of NEM on lactose transport activity of Cys-replacement mutants

The effect of NEM, a membrane-permeable sulfhydryl reagent, on lac transport by *E. coli* T184 expressing each active Cys mutant was tested (Fig. 8). As shown, the rate of transport by most of the single-Cys mutants in putative helices IX and X is relatively unaffected by the alkylating agent. Significant but incomplete inhibition is observed for mutants N290C, V315C, M323C, L329C, or L330C, whereas lac transport by mutant V326C or V331C is essentially completely inactivated by NEM. The time required for NEM (1 mM, final concentration) to inactivate V326C or V331C permease by 50% (i.e., $t_{1/2}$) is about 10 s and about 5 s, respectively (data not shown), rates that are approximately 10 times faster than those reported for wild-type permease (Viitanen et al., 1985).

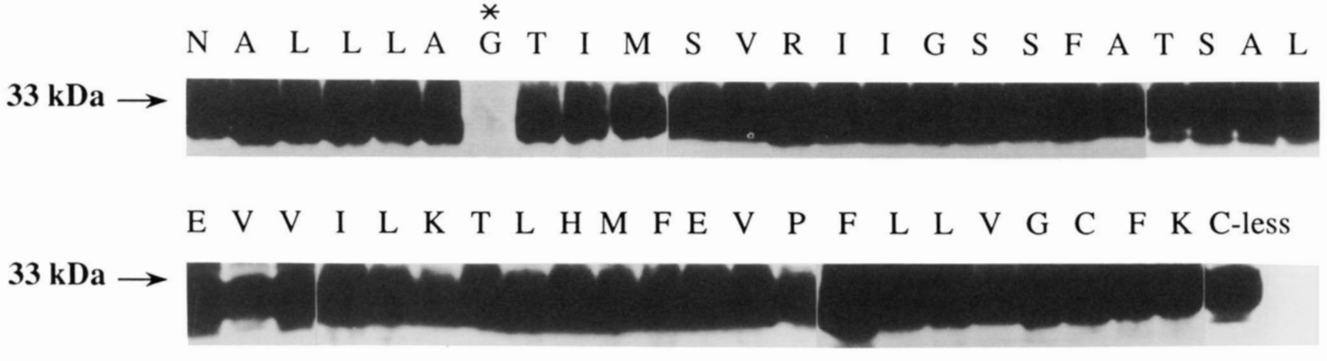

Fig. 5. Western blots of membranes containing C-less lac permease or Cys-replacement mutants. *Escherichia coli* T184 transformed with pT7-5/C-less *lacY* or pT7-5/C-less *lacY* encoding given Cys mutations were grown and induced with IPTG as described in Materials and methods. Membranes were prepared, and samples containing approximately 200 μg of protein per sample were subjected to SDS–polyacrylamide gel electrophoresis, electroblotted, and the nitrocellulose paper was incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase-conjugated protein A, followed by a short incubation with fluorescent substrate (Amersham), the nitrocellulose paper was exposed to film for 5 min. The single letter amino-acid code is used to denote the amino-acid residues replaced with Cys in increasing order from Asn-290 to Lys-335. The asterisk marks mutant G296C. Although not shown, membranes prepared from cells harboring pT7-5 with no *lacY* gene exhibit no immunoreactive material (see McKenna et al., 1992b).

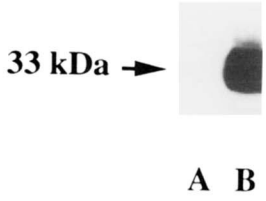

Fig. 6. Immunoblot of membranes from mutant G296C permease overexpressed from the T7 promoter. *Escherichia coli* T184 were cotransformed with pGP1-2 encoding T7 RNA polymerase under the control of a heat-shock promoter and pT7-5 encoding mutant G296C. Cells were grown at 30 °C and heat shocked for 60 min at 42 °C. Membranes were prepared from the heat-shocked cultures and subjected to electrophoresis and Western-blotting as described in Materials and methods and in Figure 5. Lane A, 100 μg of G296C membrane protein expressed from the *lac* promoter/operator by IPTG induction; lane B, 50 μg of G296C membrane protein expressed from the T7 promoter.

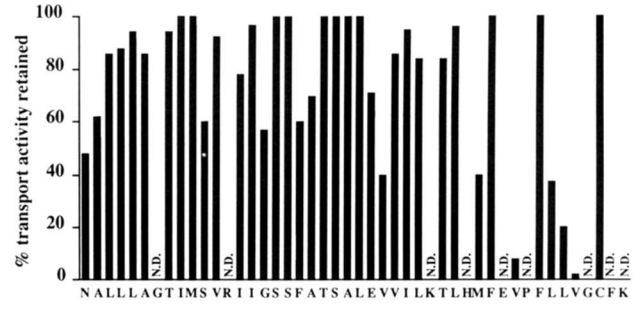

Fig. 8. Effect of NEM on lac transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 2 mM NEM at room temperature for 10 min and assayed for initial rates of lac uptake in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (Konings et al., 1971). The single letter amino-acid code denotes the amino-acid residues replaced with Cys in increasing order from Asn-290 to Lys-335. Mutants with low activity were not tested (N.D.). The results are presented as a percentage of the untreated controls.

When right-side-out membrane vesicles containing mutant V315C are incubated in the presence of 1 mM NEM, a $t_{1/2}$ of about 30 min is observed in the absence of substrate or an appropriate electron donor (Fig. 9). Strikingly, in the presence of TDG, $t_{1/2}$ is decreased to 2–3 min. That is, the rate of NEM inactivation increases approximately 10-fold in the presence of TDG. A similar effect is observed when a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) is generated across the vesicle membrane. Thus, in the presence of ascorbate and phenazine methosulfate, the $t_{1/2}$ for NEM inactivation is also decreased to 2–3 min, and the effect is abolished by the protonophore CCCP. Conversely, the effect of TDG is completely unaffected

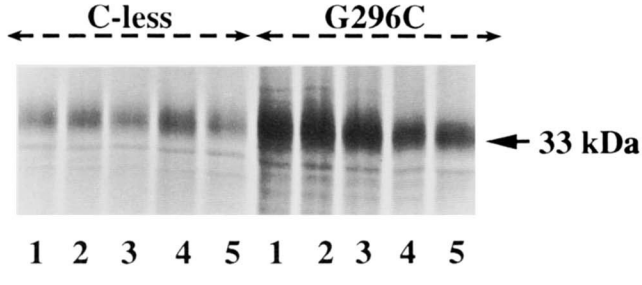

Fig. 7. [^{35}S]Methionine labeling and pulse-chase with C-less permease and mutant G296C expressed from the T7 promoter. Specific labeling of lac permease with [^{35}S]methionine was carried out in the presence of rifampicin as described in Materials and methods. *Escherichia coli* T184 cells were cotransformed with pGP1-2 encoding T7 RNA polymerase under the control of a heat-shock promoter and pT7-5 encoding C-less permease or mutant G296C. Cells were grown at 30 °C and heat shocked for 60 min at 42 °C. After incubation with [^{35}S]methionine at 30 °C for 10 min, an aliquot was removed as the 0-min time point (lane 1). A 200-fold excess of cold methionine was then added, and aliquots were removed for membrane preparation at 5 min (lane 2), 30 min (lane 3), 2 h (lane 4), and 10 h (lane 5). Membranes (approximately 25 μg of membrane protein for C-less and 50 μg for G296C) were subjected to polyacrylamide gel electrophoresis, and the dried gel was exposed to film for 20 h.

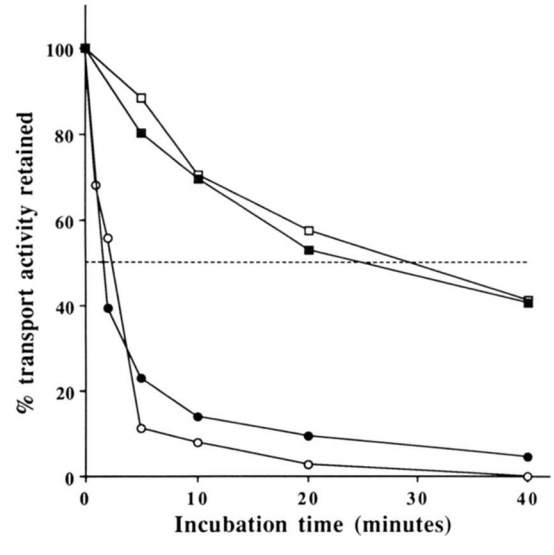

Fig. 9. Effect of TDG or $\Delta\bar{\mu}_{H^+}$ on the rate of NEM inactivation of lac transport by mutant V315C. Right-side-out membrane vesicles (2 mg/mL of protein in 100 mM KP_i, pH 7.5/10 mM $MgSO_4$) prepared from *E. coli* T184 transformed with pT7-5/C-less *lacY* encoding V315C permease were incubated with 1 mM NEM for the indicated times in the absence (□) or presence (○) of 10 mM TDG. Reactions were stopped by addition of 10 mM dithiothreitol (final concentration), and the vesicles were washed extensively with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ to remove TDG. The initial rate of lac transport was then assayed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate under oxygen (Konings et al., 1971). To test the effect of $\Delta\bar{\mu}_{H^+}$, incubation with 1 mM NEM was carried out in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate under oxygen in the absence (●) or presence (■) of 20 μM CCCP. Reactions were terminated by addition of 10 mM dithiothreitol, and the vesicles were washed with 5% bovine serum albumin/10 mM dithiothreitol/100 mM KP_i (pH 7.5)/10 mM $MgSO_4$. Samples were then assayed for lac transport, and the initial rate of transport was estimated from the linear portion of of the time course (i.e., from 0 to 30 s). The broken line represents 50% inhibition.

by CCCP (not shown). Finally, although data are not presented, when TDG and ascorbate/phenazine methosulfate are added simultaneously, no further increase in the rate of inactivation is observed. It is also relevant that the galactoside analog has no effect on the rate of NEM inactivation with mutants N290C, M323C, L329C, V326C, L330C, or V331C in intact cells.

Discussion

A systematic scanning mutagenesis strategy has been employed to identify functionally important amino-acid residues in putative transmembrane helices IX and X and the intervening hydrophilic segment in lac permease. Previous evidence (see Kaback [1992] for a review) indicates that certain residues fundamental for lac accumulation against a concentration gradient and/or substrate binding are clustered in this region of the permease. Therefore, the primary objective of the study was to determine whether or not other residues in this region play an important role in the mechanism of action of the permease. The results provide a strong indication that 3 residues identified previously—Arg-302[2] (Menick et al., 1987), His-322 (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988), and Glu-325 (Carrasco et al., 1986, 1989)—are the only residues in this region of the permease that are important for active lac transport. The remainder of the residues are either completely unimportant for activity or, like Lys-319 (Lee et al., 1992; Sahin-Tóth et al., 1992), are not mandatory but appear to be necessary for full activity. Thus, individual Cys replacement for about half of the 46 residues does not cause a significant change in lac transport activity, and another quarter of the mutants exhibit decreased rates of transport but retain the ability to accumulate the disaccharide to high levels. Based on these findings, it seems reasonable to conclude that the alterations in transport activity observed as a result of site-directed Cys replacements reflect the consequences of replacement of specific amino-acid residues and are not due to secondary long-range perturbations in the permease. Similarly, the great majority of single amino-acid substitutions do not cause significant changes in synthesis, folding, or insertion of the permease. Although studies with 45 Cys-replacement mutants support this notion, mutant G296C is a clear exception to the rule.

Comparison of the primary amino acid sequence of *E. coli* lac permease with the lac permease of *Klebsiella pneumoniae* (McMorrow et al., 1988), the raffinose permease of *E. coli* (Aslanidis et al., 1989), and a recently described sucrose permease from *E. coli* (Bockmann et al., 1992; Lengeler et al., 1992) reveals that Arg-302, Lys-319, His-322, and Glu-325 are conserved in all four proteins. It is particularly interesting that these residues are charged and thought to be located within transmembrane domains. In addition to the findings presented here, Sahin-Tóth et al. (1992) have found that Cys replacement of each of the eight charged residues in putative transmembrane helices results in lac permease molecules that are unable to catalyze lac accumulation. Either of two possibilities can provide a reasonable explanation for inactivation by the replacements: (1) the charged residue itself is required for the catalytic mechanism; or (2) the residue is not essential mechanistically but interacts with an oppositely charged residue elsewhere in the molecule (King et al., 1991; Lee et al., 1992; Sahin-Tóth et al., 1992; Dunten et al., 1993). Under the latter condition, replacement with a neutral residue disrupts the charge pair, leaving an unpaired charge within the low dielectric of the membrane, which leads to inactivation, and neutralization of the other member of the charge pair with a neutral residue restores activity by eliminating the unpaired charge. For example, replacement of Lys-319 with Cys inactivates the permease, but replacement of both Lys-319 and Asp-240 with Cys yields permease with significant activity (Sahin-Tóth et al., 1992). In addition to demonstrating that there is a functional interaction between the two residues, the findings imply that neither Lys-319 nor Asp-240 is essential for transport, although the charge pair is needed for full activity. In an even more clearcut fashion, replacement of Lys-358 or Asp-237 with neutral residues inactivates the permease, while neutral replacement of both residues or reversal of the charged residues has little effect on activity. Thus, neither residue nor the charge pair itself is necessary for activity (King et al., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993). In contrast, significant lac accumulation is not observed when mutants R302C, H322C, or E325C are combined with each of other Cys-replacements for oppositely charged residues (Sahin-Tóth et al., 1992). The implication is that Arg-302, His-322, and Glu-325 play a direct role in the transport mechanism; however, there is the caveat that the charge-pair neutralization approach is dependent upon permease activity and will not reveal paired residues if the interaction is essential for activity.

In an initial screen for the accessibility or reactivity of the Cys residues at each position from Asn-290 to Lys-335, the effect of NEM on lac transport in each of the active mutants was studied. As demonstrated, most of the mutants are unaffected by treatment with the alkylating agent. The simple explanation is that most of the Cys residues are inaccessible to NEM and do not react. Although this is a possibility, it seems unlikely because: (1) NEM is relatively permeant; and (2) mutants V326C and V331C are rapidly and completely inactivated by NEM, and partial inactivation is observed for mutants N290C, M323C, L329C, and L330C, although these residues are presumably in the middle of the membrane or disposed toward the inner surface (Fig. 1). In any case, each of the mutants that exhibit sensitivity to NEM was examined for TDG

[2] In addition to Leu, Arg-302 has also been replaced with Lys, His, or Ala with similar results (L. Patel & H.R. Kaback, unpubl.).

protection against alkylation (Fox & Kennedy, 1965), and surprisingly, lac transport in cells expressing V315C permease exhibited enhanced rates of NEM inactivation in the presence of the ligand (data not shown). For this reason, right-side-out membrane vesicles containing V315C permease were prepared in order to examine the phenomenon under more controlled conditions. In the absence of either TDG or an appropriate electron donor, lac transport by vesicles containing V315C permease is relatively insensitive to NEM, exhibiting a $t_{1/2}$ for inactivation of about 30 min. Remarkably, in the presence of either the galactoside analog or ascorbate/phenazine methosulfate, a redox pair that generates a large $\Delta\bar{\mu}_{H^+}$, the rate of inactivation increases by at least an order of magnitude. Moreover, although the effect of ascorbate/phenazine methosulfate is abolished when $\Delta\bar{\mu}_{H^+}$ is dissipated by CCCP, the effect of TDG is unchanged in the presence of the protonophore. The experiments provide an indication that this region of lac permease may undergo an important conformational change during turnover. Presumably, the conformational change causes an increase in the accessibility or reactivity of the Cys residue at position 315. With regard to accessibility, V315C permease can be studied readily by site-directed spin labeling or fluorescent labeling (see van Iwaarden et al., 1991). As for reactivity, the Cys residue at position 315 is preceded by a Glu residue, which raises the possibility that its reactivity may reflect deprotonation of Glu-314. On the other hand, replacement of Glu-314 with Cys has no effect on transport activity, indicating that the protonation state of the carboxylate at this position is unimportant for activity. In any event, the question can be resolved by replacing Glu-314 with Ala in the V315C mutant. Regardless of the ultimate explanation, the results lead to the hypothesis that a similar conformational change may be induced by ligand binding or $\Delta\bar{\mu}_{H^+}$. Thus, although Val-315 is unimportant for lac permease function (i.e., V315C exhibits good transport activity), replacement of the residue with Cys may provide a means of probing dynamic changes in the protein.

Materials and methods

Materials

[1-^{14}C]Lactose and [^{35}S]methionine were purchased from Amersham (Arlington Heights, Illinois). 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., 1984a) was prepared by BabCo (Richmond, California). NEM and TDG were from Sigma Chemical Company (St. Louis, Missouri). All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and plasmids

Escherichia coli 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) was used as carrier for the plasmids described and for detection of lac permease activity on MacConkey plates (Difco Laboratories) containing 25 mM lac. *Escherichia 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 by induction with IPTG. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (van Iwaarden et al., 1991) containing the *lac* promoter/operator was used for all *lacY* gene manipulations. For overexpression via the T7 promoter (Tabor & Richardson, 1985), *E. coli* T184 were cotransformed with plasmid pGP1-2 encoding T7 RNA polymerase.

Oligonucleotide-directed site-specific mutagenesis

The casette *lacY* gene encoding C-less permease was inserted into the replicative form of phage M13 or into plasmid pT7-5 and used as template for mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers. Cys replacements were created by the method of Kunkel (1985) as described by Consler et al. (1991), using the primers listed in Table 1. Mutants F334L, F334W, and F334Y were constructed by a two-stage polymerase chain reaction (overlap extension [Ho et al., 1989]) with two complementary mutagenic primers, the sequences of which were identical to those listed for F334C (Table 1), except that codon CTT was used for leucine, TAT for tyrosine, and TGG for tryptophan.

DNA sequencing

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

Colony morphology

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

Active transport

Active transport was measured in *E. coli* T184 (Z^-Y^-) transformed with each plasmid described. Fully grown overnight cultures of cells were diluted 200-fold and grown aerobically for 12 h at 30 °C in the presence of 10 μg/mL

streptomycin, 100 μg/mL ampicillin, and 0.2 mM IPTG. Cells were harvested by centrifugation, washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$, and assayed by the rapid filtration method as described (Consler et al., 1991).

Preparation of membranes

Right-side-out membrane vesicles were prepared by lysozyme-EDTA treatment and osmotic lysis as described (Kaback, 1971; Short et al., 1975) from 2.4-L cultures of *E. coli* T184 harboring given plasmids.

Alternatively, crude membrane fractions from *E. coli* T184 harboring plasmids with given mutations were prepared as follows: cells washed with 50 mM Tris-HCl (pH 8.0)/100 mM NaCl/1 mM EDTA were resuspended in ice-cold osmotic shock buffer (25 mM Tris-HCl [pH 8.0]/45% sucrose/1 mM EDTA), incubated on ice for 20 min, harvested by centrifugation, resuspended in cold distilled water, and allowed to stand 10 min on ice before adding 0.1 mg lysozyme. After incubation for 20 min, the cell suspensions were briefly sonicated. Unlysed cells were removed by low-speed centrifugation, and membranes were harvested by ultracentrifugation in a Beckman Optima TL™ ultracentrifuge.

Immunological analyses

Membrane fractions were subjected to SDS-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).

[^{35}S]Methionine labeling

[^{35}S]Methionine labeling of lac permease and pulse-chase experiments were carried out in vivo using the T7 polymerase system as described (McKenna et al., 1991).

Protein determinations

Protein was assayed in the presence of SDS by a modified Lowry method (Peterson, 1977).

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