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Cysteine scanning mutagenesis of the N-terminal 32 amino acid residues 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 the hydrophilic N-terminus and the first putative transmembrane helix was systematically replaced with Cys (from Tyr-2 to Trp-33). Twenty-three of 32 mutants exhibit high lactose accumulation (70–100% or more of C-less), and an additional 8 mutants accumulate to lower but highly significant levels. Surprisingly, Cys replacement for Gly-24 or Tyr-26 yields fully active permease molecules, and permease with Cys in place of Pro-28 also exhibits significant transport activity, although previous mutagenesis studies on these residues suggested that they may be required for lactose transport. As expected, Cys replacement for Pro-31 completely inactivates, in agreement with previous findings indicating that "helix-breaking" propensity at this position is necessary for full activity (Consler TG, Tsolas O, Kaback HR, 1991, *Biochemistry 30*:1291–1297). Twenty-nine mutants are present in the membrane in amounts comparable to C-less permease, whereas membrane levels of mutants Tyr-3 → Cys and Phe-12 → Cys are slightly reduced, as judged by immunological techniques. Dramatically, mutant Phe-9 → Cys is hardly detectable when expressed from the *lac* promoter/operator at a relatively low rate, but is present in the membrane in a stable form when expressed at a high rate from the T7 promoter. Finally, studies with *N*-ethylmaleimide show that 6 Cys-replacement mutants that cluster at the C-terminal end of putative helix I are inactivated significantly. The results demonstrate that although no residue per se in this region is essential for activity, the structural integrity of the periplasmic half of the first transmembrane helix is important for active lactose transport.

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

Lactose permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides 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 solely responsible for β-galactoside transport, probably in monomeric form (reviewed by Kaback, 1989, 1992, and Kaback et al., 1990). Based on circular dichroic studies and hydropathy analysis of the primary amino acid sequence (Foster et al., 1983), a secondary structure was proposed in which the permease has a short hydrophilic N-terminus, 12 α-helical hydrophobic domains that traverse the membrane in zigzag fashion connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail. Evidence supporting the general features of the 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), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), immunological studies (Carrasco et al., 1982, 1984a, 1984b; Seckler et al., 1983, 1986; Herzlinger et al., 1984, 1985; Seckler & Wright, 1984; Danho et al., 1985), and chemical modification (Page & Rosenbusch, 1988). Unequivocal support for the 12-helix motif has been obtained by analyzing an extensive series of lac permease–alkaline phosphatase (*lacY–phoA*) fusions (Calamia & Manoil, 1990).

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Abbreviations: C-less permease, functional lactose permease devoid of Cys residues; NEM, *N*-ethylmaleimide; lac, lactose; IPTG, *i*-propyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate. Site-directed mutants are designated as follows: the 1-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.

In vitro mutagenesis has been useful in identifying functionally important residues in the permease. Random and site-specific mutations provided the first indications that, in addition to essential residues described in the C-terminal transmembrane helices (see Kaback, 1992, for a review), the first putative transmembrane helix may also contain residues that are important for transport. Thus, Overath et al. (1987) isolated and sequenced permease mutants defective in lactose transport and found Arg or Glu in place of Gly-24 or Ser in place of Pro-28. In addition, oligonucleotide-directed site-specific mutagenesis of Tyr residues (Roepe & Kaback, 1989) and Pro residues (Consler et al., 1991) indicates that Tyr-26 and Pro-28 may be important for transport activity, whereas Tyr-2, Tyr-3, and Pro-31 are not essential. Similarly, Trp-10 and Trp-33 are dispensable because permease devoid of Trp residues is almost fully active (Menezes et al., 1990). Recently, Bibi et al. (1992) analyzed a series of N-terminal deletion mutants and found that the first 22 amino acid residues are not obligatory for active lactose transport or membrane insertion, whereas the region between residues 23 and 38 probably contains elements essential for both. Thus, permease with amino acids 2–9 deleted (Δ8) exhibits high transport activity, whereas deletion of residues 2–11 (Δ10) or 2–23 (Δ22) results in loss of transport, and the truncated permeases are absent from the membrane when expressed from the *lac* promoter/operator. However, Δ10 and Δ22 permeases are detected in the membrane and exhibit significant transport activity when expressed from the T7 promoter at a high rate. Deletion of residues 2–39 (Δ38) abolishes transport activity and results in the complete absence of the protein from the membrane. Importantly, when the *ompA* leader sequence is fused to Δ38 permease, the construct is inserted into the membrane at normal levels but does not catalyze lactose transport, indicating that the region from Met-23 to His-39 is important for transport activity.

The observations that mutations and deletions in putative helix I abolish transport activity indicate that this region may play an important role in the mechanism of action of the permease. Therefore, this region of lac permease was subjected to systematic "scanning" mutagenesis using a functional mutant devoid of Cys residues (van Iwaarden et al., 1991). Residues from Tyr-2 to Trp-33 were individually replaced with Cys, and lactose transport activity, levels of expression of the permease, and sensitivity to inhibition by *N*-ethylmaleimide were studied. Cys is particularly suitable for scanning hydrophobic membrane proteins because the side chain is of intermediate bulk and is relatively hydrophobic (Kyte & Doolittle, 1982). Furthermore, the unique reactivity of Cys toward a variety of sulfhydryl reagents can be utilized in further studies of the Cys-replacement mutants (Jung et al., 1993). Cys mutagenesis has been used to study other membrane proteins (for references see Sahin-Tóth & Kaback, 1993) and has been extremely useful in delineating functionally important residues in helices IX and X of lac permease. In this region, only 3 residues—Arg-302, His-322, and Glu-325—are important for transport (Sahin-Tóth & Kaback, 1993). Mutations in the other residues in these 2 putative helical domains lead to permease molecules with significant ability to concentrate lactose.

With the exception of P31C[2], which is inactive, 31 Cys-replacement mutants in the N-terminal portion of the permease and in the first transmembrane helix exhibit significant lactose transport activity. Furthermore, only a few Cys-replacement mutants at the C-terminal (periplasmic) end of helix I are inactivated significantly by alkylation with NEM.

Results

Construction and verification of mutations

Individual Cys-replacement mutants were constructed by oligonucleotide-directed site-specific mutagenesis in C-less permease from residue Tyr-2 to Trp-33, a region containing the short hydrophilic N-terminus and the first putative transmembrane helix (Fig. 1). Mutants in the N terminus (from Y2C to W10C) were created by PCR using pT7-5/*lacY*/C-less as template, and mutants in helix I (from M11C to W33C) were constructed in bacteriophage M13mp19, as described in Materials and methods. The PCR products or the replicative forms of verified M13 clones were restricted with *Bam* HI and *Acc* I or *Pst* I (see

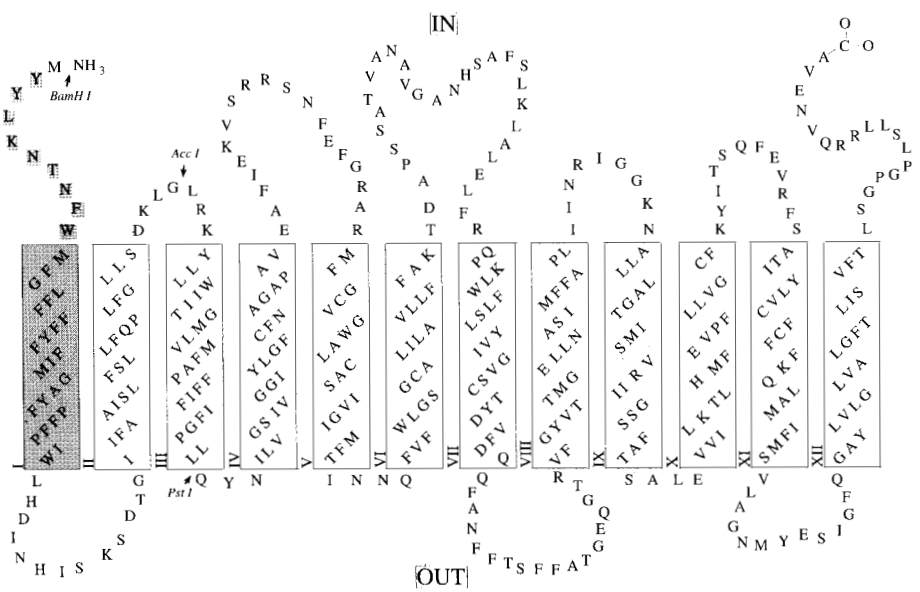

Fig. 1. Secondary-structure model of *E. coli* lac permease (Foster et al., 1983). The single letter amino acid code is used, and putative transmembrane helices are shown in boxes. The topology of helix VII was modified according to results obtained from the analyses of a series of *lacY-phoA* fusions (M.L. Ujwal & H.R. Kaback, unpubl. obs.). 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.

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 *Bam* HI-*Acc* I or *Bam* HI-*Pst* I fragments were subjected to 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* (EMBO-X56095).

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 monosaccharides 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 mutant P31C appears as red colonies with a white halo, whereas the rest of the individual Cys-replacements grow as red colonies indistinguishable from C-less permease. Therefore, judging from indicator plates, 31 mutants retain the ability to translocate lactose, whereas mutant P31C is apparently defective.

Active lactose transport

As a more quantitative measure of function, the ability of each Cys-replacement mutant to accumulate lactose against a concentration gradient was assayed in *E. coli* T184 (*Z*$^-$*Y*$^-$). When rates of lactose transport are measured at 1 min and expressed as a percentage of C-less permease activity, it is apparent that most of the 32 mutants transport lac at very significant rates (Fig. 2A). Twenty-two mutants transport lac at rates of 70–100% or more of C-less, and an additional 8 mutants exhibit rates that are 25–70% of C-less. Interestingly, mutant F21C catalyzes lactose accumulation at a significantly higher rate (166%)

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

Mutant	Mutagenic oligonucleotide[a]		Codon change
Y2C	AGGAAAGGATCCATG**TGT**TATTTGAAAAACAC		TAC → TGT
Y3C	GGAAAGGATCCATGTAC**TGT**TTGAAAAACAC		TAT → TGT
L4C	GGAAAGGATCCATGTACTAT**TGT**AAAAACACAAAC		TTG → TGT
K5C	GGAAAGGATCCATGTACTATTTG**TGT**AACACAAACTTTTGG		AAA → TGT
N6C	*Sense*	TATTTGAAA**TGC**ACAAACTTT	AAC → TGC
	Antisense	AAAGTTTG**TGC**ATTTCAAATA	
T7C	*Sense*	TTGAAAAAC**TGT**AACTTTTGG	ACA → TGT
	Antisense	CCAAAAGTT**ACA**GTTTTTCAA	
N8C	*Sense*	AAAAACACA**TGC**TTTTGGATG	AAC → TGC
	Antisense	CATCCAAAA**GCA**TGTGTTTTT	
F9C	*Sense*	AACACAAAC**TGT**TGGATGTTC	TTT → TGT
	Antisense	GAACATCC**AACA**GTTTGTGTT	
W10C	*Sense*	ACAAACTTTT**GC**ATGTTCGGT	TGG → TGC
	Antisense	ACCGAACAT**GCA**AAAGTTTGT	
M11C	TAAACCGAAG**CA**CCAAAAGTT		ATG → TGC
F12C	GAATAAACC**GCA**CATCCAAAA		TTC → TGC
G13C	AAAGAATAA**ACA**GAACATCCA		GGT → TGT
L14C	GAAAAAGAA**GCA**ACCGAACAT		TTA → TGC
F15C	AAAGAAAAA**GCA**TAAACCGAA		TTC → TGC
F16C	GTAAAAGAA**ACA**GAATAAACC		TTT → TGT
F17C	AAAGTAAAA**GCA**AAAGAATAA		TTC → TGC
F18C	AAAAAAGTA**ACA**GAAAAAGAA		TTT → TGT
Y19C	GATAAAAAA**GCA**AAAGAAAAA		TAC → TGC
F20C	CATGATAAAA**ACA**GTAAAAGAA		TTT → TGT
F21C	TCCCATGAT**ACA**AAAGTAAAA		TTT → TGT
I22C	GTAGGCTCCCAT**ACA**AAAAAAGTAAAA		ATC → TGT
M23C	GAAGTAGGCTCC**ACA**GATAAAAAAGTA		ATG → TGT
G24C	CGGGAAGTAGGC**ACA**CATGATAAAAAA		GGA → TGT
A25C	AAACGGGAAGTA**GCA**TCCCATGATAAA		GCC → TGC
Y26C	GAAAAACGGGAA**GCA**GGCTCCCATGAT		TAC → TGC
F27C	CGGGAAAAACGG**GCA**GTAGGCTCCCAT		TTC → TGC
P28C	AATCGGGAAAAAA**CA**GAAGTAGGCTCC		CCG → TGT
F29C	CCAAATCGGGAAA**CA**CGGGAAGTAGGC		TTT → TGT
F30C	TAGCCAAATCGG**GCA**AAACGGGAAGTA		TTC → TGC
P31C	ATGTAGCCAAAT**ACA**GAAAAACGGGAA		CCG → TGT
I32C	ATCATGTAGCCA**ACA**CGGGAAAAACGG		ATT → TGT
W33C	GATATCATGTAG**ACA**AATCGGGAAAAA		TGG → TGT

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

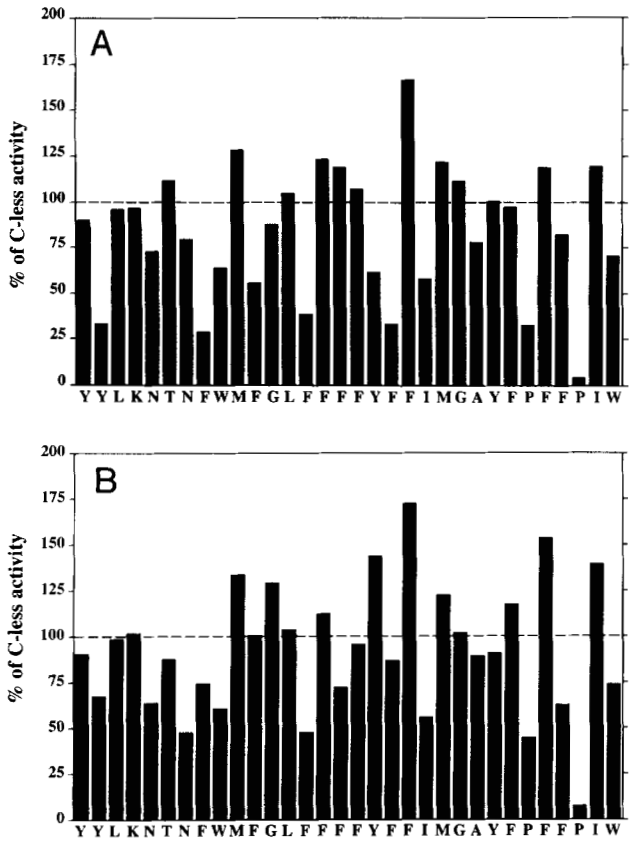

Fig. 2. Lactose transport by *E. coli* T184 expressing C-less permease or individual Cys-replacement mutants. Cells were grown at 37 °C as described in Materials and methods. Aliquots of cell suspensions (50 μL containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (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 Tyr-2 to Trp-33. **A:** Rates of lactose transport measured at 1 min. The rate of C-less permease was 24 nmol lactose/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 0.96 nmol/min/mg protein (i.e., 4% of C-less). **B:** Steady-state levels of accumulation. The steady-state level of lactose accumulation observed for C-less permease was 118 nmol lactose/mg protein. Results are expressed as a percentage of this value. Although not shown, T184 harboring pT7-5 (vector with no *lacY* gene) took up 8.4 nmol of lactose/mg protein in 1 h (i.e., 7% of C-less).

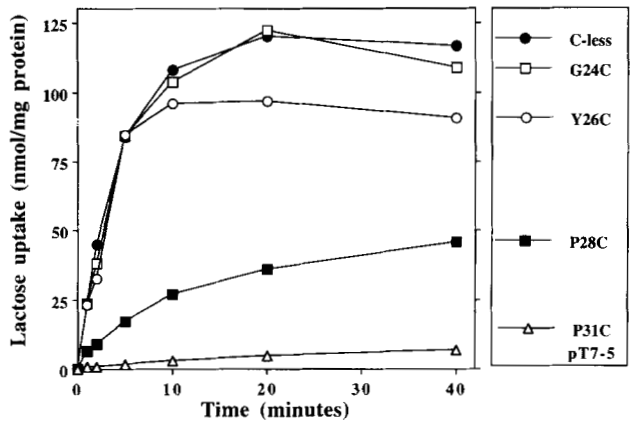

Fig. 3. Time courses of lactose transport by single-Cys mutants G24C, Y26C, P28C, and P31C. *E. coli* T184 transformed with plasmid pT7-5/C-less *lacY*, pT7-5 (vector with no *lacY* gene) or pT7-5 encoding given Cys-replacement mutants were grown and assayed as described in Figure 2. ●, C-less; □, G24C; ○, Y26C; ■, P28C; △, P31C and pT7-5.

than C-less. In contrast, mutant P31C exhibits markedly decreased transport rates, indistinguishable from cells harboring pT7-5 vector with no *lacY* insert. Steady-state levels of lactose accumulation for the great majority of the mutants also approximate or exceed that of C-less permease (Fig. 2B). Twenty-three mutants accumulate to steady-state levels of 70–100% or more of C-less permease, and 8 mutants accumulate to lower but significant steady-state levels (30–70% of C-less). Curiously, several mutants, most notably F21C, which reaches 172% of C-less steady state, accumulate to significantly higher levels than C-less. In contrast, mutant P31C is unable to accumulate lactose above background levels.

Time courses of lactose accumulation for mutants G24C, Y26C, and P28C are presented in Figure 3. Previous experiments (Overath et al., 1987; Roepe & Kaback, 1989; Consler et al., 1991) indicate that these residues may be important for lactose transport. Clearly, however, permease with Cys replacements for Gly-24 or Tyr-26 exhibits activity comparable to C-less permease, and P28C permease transports at approximately 30% of the C-less rate to about 40% of the control steady state. On the other hand, the time course of lactose accumulation by P31C permease is indistinguishable from that of cells expressing no permease (i.e., harboring pT7-5 with no *lacY* gene). It should be emphasized, however, that previous experiments (Consler et al., 1991) demonstrate that Gly replacement for Pro-31 yields fully active permease, demonstrating that the residue per se is also not essential for active transport.

Expression of Cys-replacement mutants

In order to test the effect of the Cys-replacements on expression and/or insertion, immunoblots were carried out on membrane preparations from *E. coli* T184 harboring plasmids encoding each mutant. The majority of the mutants are expressed at levels comparable to C-less permease (Fig. 4), with the exception of mutants Y3C and F12C, which exhibit reduced but significant levels of permease. Dramatically, mutant F9C, which catalyzes lactose accumulation to a relatively high steady state at a slow rate (Fig. 2B), is hardly detectable when expressed at a low rate from the *lac* promoter/operator. However, when the mutant is expressed at a high rate from the T7 promoter, it is detected in the membrane in significant amounts (Fig. 5). There are at least 3 possible explanations for the decreased amounts of F9C permease in the membrane. (1) Efficiency of transcription and/or translation is compromised due to changes in the 5′ end of the gene, and diminished amounts of protein are synthesized. In this case, increasing the rate of synthesis by expression via the T7 promoter would result in higher protein levels in the membrane. Although data are not shown, in vitro synthesis of C-less and F9C permeases using a bacterial coupled transcription/translation system (see Materials and methods) yields comparable amounts of translation product, indicating

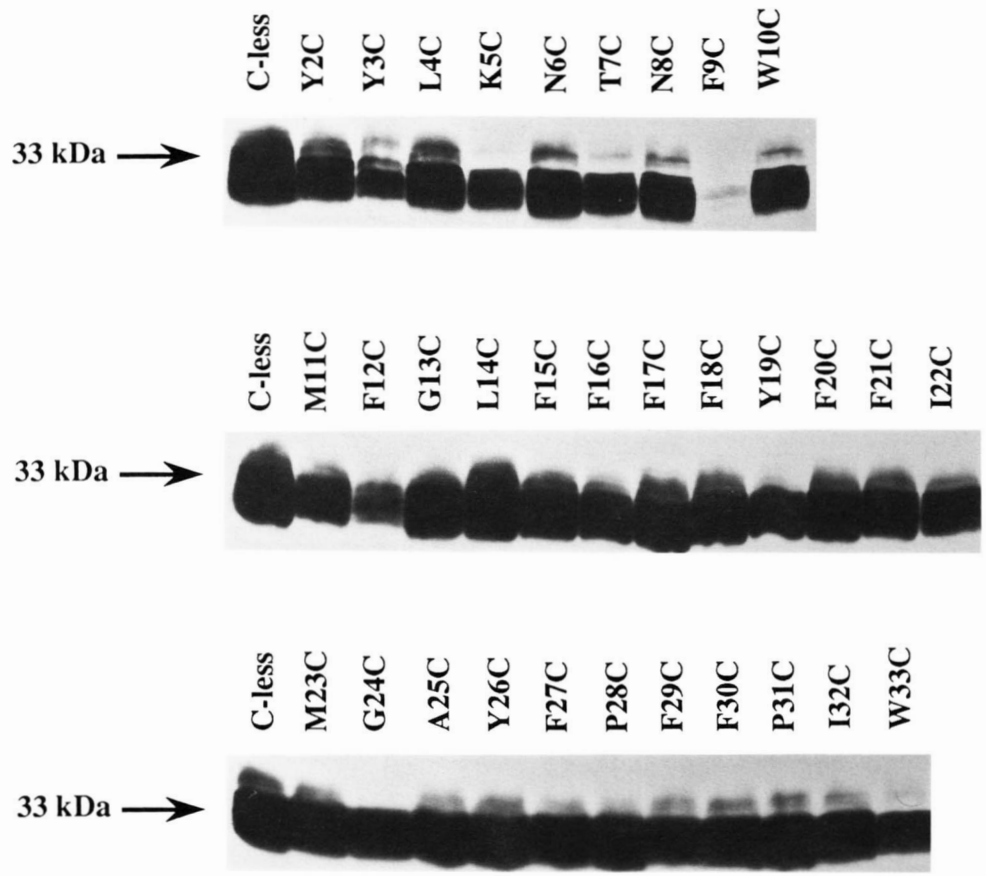

Fig. 4. Western blots of membranes containing C-less lac permease or Cys-replacement mutants. *E. coli* T184 transformed with pT7-5/C-less *lacY* or pT7-5/C-less *lacY* encoding given Cys mutations were grown and induced with IPTG as described in Materials and methods. Membranes were prepared, and samples containing approximately 100 μg of protein per sample were subjected to SDS-PAGE and 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 luminescent substrate (Amersham), the nitrocellulose paper was exposed to film for 1 min. Although not shown, membranes prepared from cells harboring pT7-5 with no *lacY* gene exhibit no immunoreactive material (see McKenna et al., 1992b).

that the proteins are synthesized at similar rates. (2) The lifetime of mutant F9C in the membrane may be decreased due to an enhanced rate of proteolysis (Roepe et al., 1989; McKenna et al., 1991, 1992a). Under these circumstances little or no protein is detected when synthesis takes place at a relatively low rate 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 F9C permease remains constant up to 16 h after addition of unlabeled methionine, as observed for C-less permease (Fig. 6). (3) Therefore, as described for certain other permease mutants – D237C/K358C (Dunten et al., 1993a) and G296C (Sahin-Tóth & Kaback, 1993) – mutant F9C is apparently defective in a step between translation and insertion into the membrane.

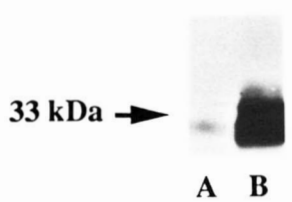

Fig. 5. Immunoblot of membranes from F9C permease overexpressed from the T7 promoter. *E. coli* T184 were co-transformed with pGP1-2 encoding T7 RNA polymerase under the control of a heat-shock promoter and pT7-5 encoding mutant F9C. Cells were grown at 30 °C and heat-shocked for 60 min at 42 °C. Membranes were prepared from the heat-shocked cultures and subjected to electrophoresis and Western blotting as described in Materials and methods and in Figure 4. Lane A, 100 μg of F9C membrane protein expressed from the *lac* promoter/operator by IPTG induction; lane B, 50 μg of F9C membrane protein expressed from the T7 promoter.

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

The effect of NEM, a membrane-permeable sulfhydryl reagent, on lactose transport by *E. coli* T184 expressing each active Cys-replacement mutant was tested (Fig. 7). As shown, the rate of transport by most of the single-Cys mutants in the hydrophilic N-terminus and putative helix I is relatively unaffected by the alkylating agent. Significant but incomplete inhibition is observed for a cluster of mutants at the C-terminal end of helix I, from F27C to W33C.

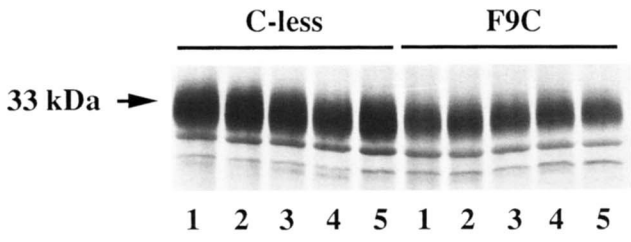

Fig. 6. [^{35}S]methionine labeling and pulse-chase with C-less permease and mutant F9C expressed from the T7 promoter. Specific labeling of lac permease with [^{35}S]methionine was carried out in the presence of rifampicin as described in Materials and methods. *E. coli* T184 cells were co-transformed with pGP1-2 encoding T7 RNA polymerase under the control of a heat-shock promoter and pT7-5 encoding C-less permease or mutant F9C. 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 16 h (lane 5). Membranes (approximately 50 μg of membrane protein) were subjected to PAGE, and the dried gel was exposed to film for 12 h.

Discussion

A systematic Cys-scanning mutagenesis strategy used previously to study putative transmembrane helices IX and X (Sahin-Tóth & Kaback, 1993) has been employed to identify functionally important amino acid residues in the N-terminus and the first putative transmembrane helix of lac permease. Previous evidence from single amino acid replacements and analysis of a series of deletion mutants indicated that certain residues in this region may be essential for active lactose transport. Therefore, the aim of the present study was to determine whether or not residues in this region play an important role in the mechanism of action of the permease. The results clearly demonstrate that no residue per se in the N-terminus or in the first putative trans membrane helix is mandatory for lactose transport. Thus, transport activity in approximately 70% of the mutants is either unaffected or improved relative to C-less permease, and another quarter of the mutants exhibit lower but significant activity. Although 1 mutant, P31C, is inactive, previous studies (Consler et al., 1991) demonstrate that permease with Gly at this position is fully active. Therefore, Pro-31 cannot be obligatory for active lactose transport. The present findings are also in contradiction with previous observations indicating that mutations (Overath et al., 1987; Roepe & Kaback, 1989; Consler et al., 1991) as well as deletions (Bibi et al., 1992) in this area often lead to seriously impaired function. Apparently the essential elements in putative transmembrane helix I are more related to the general physicochemical properties (e.g., bulk, hydrophobicity) of the amino acid residues than to more specific properties like shape or ability to hydrogen bond. In this respect, previous studies (Consler et al., 1991) on Pro residues in the permease show that Ser, Gly, Ala, or Leu replacements for Pro-28 lead to diminished lactose transport activity, although P28S permease transports the substrate analogue β-galactopyranosyl 1-thio-β, D-galactopyranoside reasonably well. Similarly, Pro-31 tolerates Gly replacement well, perhaps because flexibility is important at this position. Thus, Ala replacement results in significantly reduced activity, and introduction of Leu, similar to Cys, completely inactivates the permease (Consler et al., 1991). In the same context, it is surprising that replacement of Tyr-26 with Phe abolishes transport (Roepe & Kaback, 1989), whereas Y26C permease is fully active. Based on these observations, it is reasonable to speculate that only the hydroxyl group on Tyr-26 is important for activity and that it can be mimicked by the sulfhydryl moiety of Cys. It is noteworthy, however, that an unmodified sulfhydryl group is apparently not required for activity, as NEM treatment of Y26C does not significantly affect lactose transport (Fig. 7).

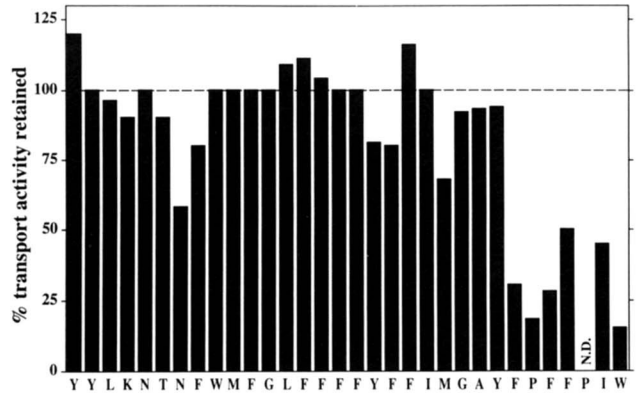

Fig. 7. Effect of NEM on lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 1 mM NEM (final concentration) at room temperature for 30 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lactose 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 Tyr-2 to Trp-33. The very low-activity mutant P31C was not determined (N.D.). The results are presented as a percentage of the untreated controls.

Progressive deletions of the N-terminus and the first transmembrane helix result in a progressive decrease in the concentration of the permease in the membrane (Bibi et al., 1992) when the mutants are expressed at a relatively low rate from the *lac* promoter/operator. Expression at a high rate via the T7 promoter of mutants with residues 2–9, 2–11, or 2–23 deleted results in stable insertion at normal levels, indicating that the N-terminus and the N-terminal half of the first transmembrane helix contain information that is not absolutely required for membrane insertion or folding. Rather, residues 2–23 probably serve to increase the kinetic efficiency of the insertion process. The great majority of single Cys-replacements in this region do not alter expression, but mutants Y3C and F12C are expressed at reduced levels and mutant F9C is virtually absent from the membrane when expressed from the *lac* promoter/operator. Importantly, the properties of F9C permease are essentially identical to those described for the N-terminal deletion mutants (Bibi et al., 1992). Thus, overexpression from the T7 promoter results in normal amounts of permease in the membrane and the protein is inserted in a functional and stable form, indicating that these mutants are defective in a step between translation and insertion.

Finally, in order to test the accessibility and/or reactivity of the Cys-replacements at each position from Tyr-2 to Trp-33, the effect of NEM on lactose transport in each active mutant was studied. As demonstrated for Cys replacements in helices IX and

X, the transport activity of the majority of the Cys mutants is unaffected by treatment with the alkylating agent. Although it is possible that most of the Cys residues are inaccessible to NEM and do not react, this seems unlikely because NEM is relatively permeant, and a number of Cys mutants in helices X and XI located in the middle of the membrane or disposed toward the inner surface are readily inhibited (Dunten et al., 1993b; Sahin-Tóth & Kaback, 1993). Interestingly, the Cys-replacement mutants that are significantly inactivated are all located at the C-terminus of the first transmembrane helix, from F27C to W33C. Apparently the addition of a relatively bulky hydrophobic ring to Cys residues in this region interferes with the transport mechanism, confirming previous observations that this region is important for lactose transport. The continuous distribution of NEM-sensitive Cys-replacement mutants at the C-terminus of helix I is markedly different from observations in helices VIII (Hinkle et al., 1989), X (Sahin-Tóth & Kaback, 1993), and XI (Dunten et al., 1993b), where important residues appear to be localized predominantly on 1 face of the helices.

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 was from Sigma Chemical Company (St. Louis, Missouri). All other materials were reagent grade and were obtained from commercial sources.

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*, 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 lactose. *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 by induction with IPTG. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (van Iwaarden et al., 1991) and containing the *lac* promoter/operator was used for all *lacY* gene manipulations. For overexpression via the T7 promoter (Tabor & Richardson, 1985), *E. coli* T184 was co-transformed 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 M13mp19 or into plasmid pT7-5 and used as template for mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers, the sequences of which are given Table 1. Mutants from Y2C to K5C were constructed by the polymerase chain reaction as described by McKenna et al. (1992b), and a 2-stage PCR method (overlap extension; Ho et al., 1989) with 2 complementary mutagenic primers was used to create mutants from N6C to W10C. Cys replacements for residues from Met-11 to Trp-33 were created by the method of Kunkel (1985) as described by Consler et al. (1991).

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 (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 C-less permease with given mutations, and the cells were plated on MacConkey indicator plates containing 25 mM lactose.

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 10-fold and grown aerobically for 2 h at 37 °C in the presence of 10 μg/mL streptomycin and 100 μg/mL ampicillin. To induce expression of lac permease, 0.5 mM IPTG (final concentration) was added and cells were grown for 2 h more. 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 by Consler et al. (1991).

Preparation of membranes

Crude membrane fractions from *E. coli* T184 harboring plasmids with given mutations were prepared by osmotic shock and sonication as described by Sahin-Tóth and Kaback (1993).

Immunological analyses

Membrane fractions were subjected to SDS-PAGE as described by 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 by McKenna et al. (1991).

In vitro translations

In vitro translations of lac permease mutants were carried out with a prokaryotic coupled transcription/translation system (*E. coli* S30 Extract System; Promega) in the presence of [^{35}S]methionine, according to the manufacturer's instructions. Approximately 2 μg purified plasmid was used for each translation reaction. The translation products were precipitated with

acetone, resuspended in sample buffer, and subjected to SDS-PAGE. The gel was dried and exposed to film for 15 h.

Protein determinations

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

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