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The role of transmembrane domain III in the lactose permease of *Escherichia coli*

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Abstract

Deletion of putative transmembrane helix III from the lactose permease of *Escherichia coli* results in complete loss of transport activity. Similarly, replacement of this region en bloc with 23 contiguous Ala, Leu, or Phe residues abolishes active lactose transport. The observations suggest that helix III may contain functionally important residues; therefore, this region was subjected to Cys-scanning mutagenesis. Using a functional mutant devoid of Cys residues (C-less permease) each residue from Tyr 75 to Leu 99 was individually replaced with Cys. Twenty-one of the 25 mutants accumulate lactose to >70% of the steady-state exhibited by C-less permease, and an additional 3 mutants transport to lower, but significant levels (40–60% of C-less). Cys replacement for Leu 76 results in low transport activity (18% of C-less). However, when placed in the wild-type background, mutant Leu 76 → Cys exhibits highly significant rates of transport (55% of wild type) and steady-state levels of lactose accumulation (65% of wild type). Immunoblots reveal that the mutants are inserted into the membrane at concentrations comparable to wild type. Studies with *N*-ethylmaleimide show that mutant Gly 96 → Cys is rapidly inactivated, whereas the other single-Cys mutants are not altered significantly by the alkylating agent. Moreover, the rate of inactivation of Gly 96 → Cys permease is enhanced at least 2-fold in the presence of β-galactopyranosyl 1-thio-β,D-galactopyranoside. The observations demonstrate that although no residue per se appears to be essential, structural properties of helix III are important for active lactose transport.

Keywords: active transport; bioenergetics; C-less permease; Cys replacements; lactose permease; scanning mutagenesis

The lactose permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of a single β-galactoside molecule with a single proton (i.e., β-galactoside/H^+ symport or cotransport). Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted in proteoliposomes, and shown to be solely responsible for β-galactoside transport (reviewed by Kaback, 1989, 1992). Furthermore, evidence has been presented (Dornmair et al., 1985; Costello et al., 1987; Sahin-Tóth et al., 1994a) indicating that the permease is functional as a monomer. Based on CD studies and hydropathy analysis (Foster et al., 1983), a secondary structure was proposed in which the permease has 12 putative transmembrane domains in α-helical conformation connected by hydrophilic loops (Fig. 1). Support for the general features of the model and evidence demonstrating that the C-terminus, as well as the second and third cytoplasmic loops, are on the cytoplasmic face of the membrane have been obtained from laser Raman spectroscopy (Vogel et al., 1985), immunological studies (Carrasco et al., 1982, 1984a, 1984b; Seckler et al., 1983, 1986; Seckler & Wright, 1984; Herzlinger et al., 1984, 1985; Danho et al., 1985), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and chemical labeling (Page & Rosenbusch, 1988). Finally, analysis of an extensive series of lac permease–alkaline phosphatase (*lacY-phoA*) fusions (Calamia & Manoil, 1990) has provided unequivocal evidence for the topological predictions of the 12-helix model.

Cys-scanning mutagenesis reveals that very few residues in lac permease are mandatory for activity, and the functionally important residues that are charged and in transmembrane domains are located predominantly in the C-terminal half of the

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Abbreviations: C-less permease, functional lactose permease devoid of Cys residues; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; lac, lactose; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; Tris, tris(hydroxymethyl)aminomethane; $\Delta\tilde{\mu}_{H^+}$, the H^+ electrochemical gradient across the membrane.

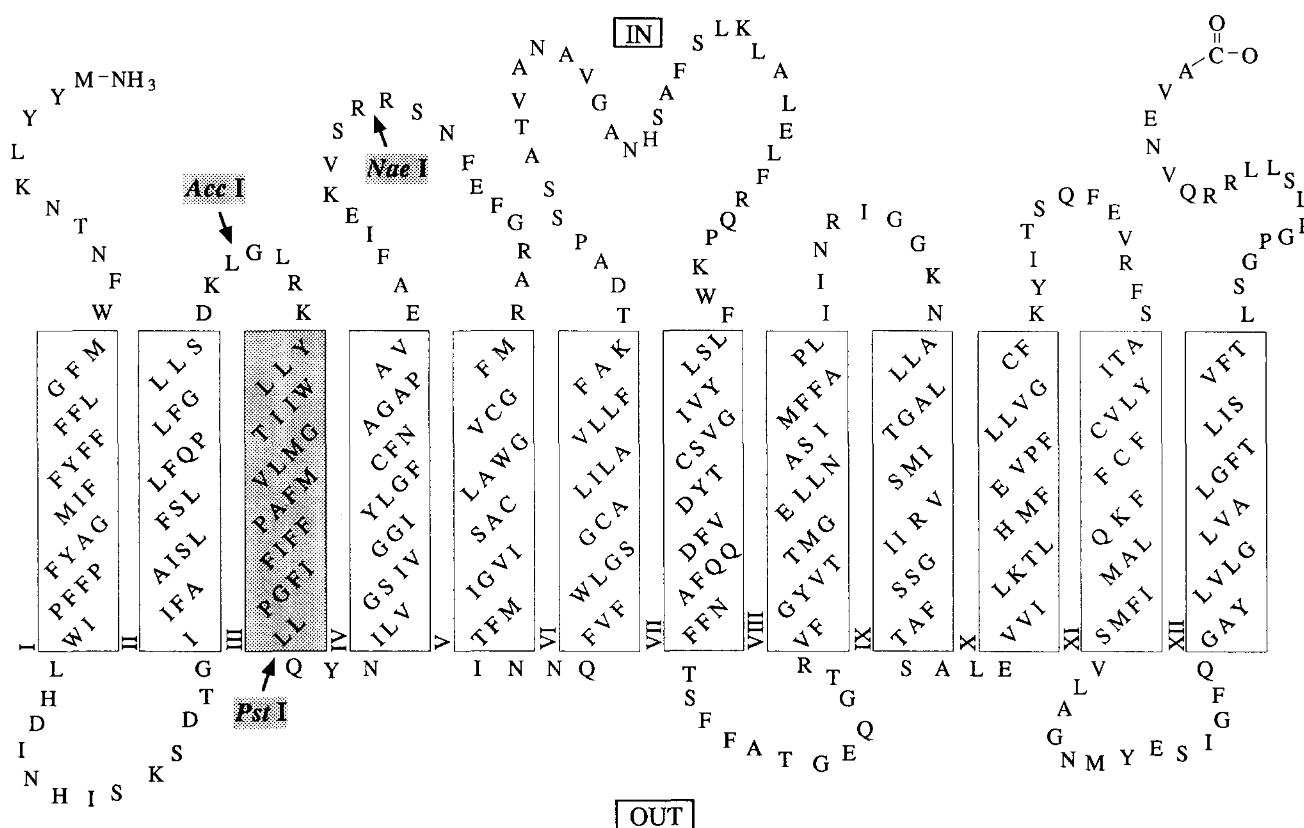

Fig. 1. Secondary-structure model of *E. coli* lac permease, based on hydropathy analysis (Foster et al., 1983). The 1-letter amino acid code is used and putative transmembrane helices are shown in boxes. The topology of helix VII is modified according to King et al. (1991) and the results obtained from expression of the permease in contiguous fragments (Zen et al., 1994). The shaded area highlights putative transmembrane domain III, which has been subjected to Cys-scanning mutagenesis. Also indicated are the restriction endonuclease sites used for construction of the mutants.

molecule (Sahin-Tóth et al., 1992, 1994b; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993). Thus, mutagenesis of Glu 269 in putative helix VIII (Hinkle et al., 1990; Franco & Brooker, 1994; Ujwal et al., 1994), Arg 302 in helix IX (Menick et al., 1987a; Matzke et al., 1992), His 322 (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988; King & Wilson, 1989a, 1989b; Brooker, 1990, 1991) or Glu 325 (Carrasco et al., 1986, 1989) in helix X indicates that these residues play key roles in lactose/H^+ symport and/or substrate recognition. Recently, site-directed pyrene excimer fluorescence was used to study proximity relationships between these residues (Jung et al., 1993). The results demonstrate that His 322 and Glu 325 are in an α-helical portion of the permease and that helices VIII (Glu 269) and IX (Arg 302) are in close proximity to helix X (His 322 and Glu 325, respectively). These and other findings showing that helix VII is close to helices X and XI form the basis of a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

In contrast to the relative abundance of information regarding the C-terminal half of the permease, little is known about the N-terminal half because mutagenesis studies have failed to identify essential residues in this half of the permease. Thus, systematic replacement of Cys (Trumble et al., 1984; Viitanen et al., 1985; Menick et al., 1987b; van Iwaarden et al., 1991), Pro (Consler et al., 1991), Trp (Menezes et al., 1990), or Gly (Jung et al., 1994d) residues indicates that none of these residues is essential for activity even though Cys 148 is in a substrate binding site (Jung et al., 1994b; Wu & Kaback, 1994). Similarly, none of the His (Püttner et al., 1986, 1989; Püttner & Kaback, 1988) or Tyr residues (Roepe & Kaback, 1989; Sahin-Tóth et al., 1994b) in the first 6 transmembrane domains is mandatory for lactose transport. Deletion-analysis (Bibi et al., 1992) and a recent Cys-scanning mutagenesis study (Sahin-Tóth et al., 1994b) demonstrate that helix I contains no essential residues; however, structural features of the periplasmic half of the domain appear to be important for active transport.

The third putative transmembrane domain is probably the most hydrophobic helix in the permease, containing very few polar amino acid residues. This raises the possibility that the role of helix III may be primarily "structural." To test this hypothesis, the entire transmembrane domain was deleted or replaced with runs of 23 identical "helix-forming" hydrophobic residues (Ala, Phe, or Leu). In both cases, transport activity is completely lost, suggesting that certain residues in this region may be important for the mechanism. For this reason, the entire region was subjected to Cys-scanning mutagenesis in a functional mutant devoid of Cys residues (van Iwaarden et al., 1991). The results demonstrate that the majority of the single-Cys mutants accumulate lactose to high levels, 3 mutants exhibit lower, but significant transport and replacement of Leu 76 results in low

activity. However, when the Leu 76 → Cys mutation is placed in the wild-type background, highly significant transport activity is observed. Furthermore, studies with *N*-ethylmaleimide indicate that alkylation of G96C[1] permease inactivates lactose transport, whereas the activity of the other Cys mutants is not altered significantly by NEM. Finally, the rate of inactivation of G96C permease is enhanced in the presence of the substrate analogue, β-galactopyranosyl 1-thio-β,D-galactopyranoside.

Results

Construction and verification of mutants

The region between residues Leu 76 and Leu 98 (Fig. 1) was deleted (Δ_{23}) or replaced with 23 Ala (Ala_{23}), Phe (Phe_{23}), or Leu (Leu_{23}) residues using synthetic linkers. Double-stranded linkers with the appropriate sequence (Table 1) were phosphorylated and ligated into the *Acc* I and *Pst* I sites of pT7-5/cassette *lacY* (Fig. 1).

Individual Cys-replacement mutants were constructed in C-less permease from residue Tyr 75 to Leu 99 (Fig. 1) by oligonucleotide-directed, site-specific mutagenesis. Mutations were introduced by PCR using pT7-5/*lacY*/C-less as template as described in the Materials and methods. The PCR fragments were restricted with *Acc* I and *Pst* I (Fig. 1) and ligated into the pT7-5/cassette *lacY* encoding C-less permease. Because mutation of Leu 99 destroys the *Pst* I site, the *Acc* I and *Nae* I sites were used for subcloning this mutant. After propagation in *E. coli* HB101, recombinant plasmid DNA was isolated and mutations were verified by sequencing the *Acc* I–*Pst* I fragments (*Acc* I–*Nae* I fragment for L99C) through the ligation junctions as described in the Materials and methods. Except for the base changes summarized in Table 1, the sequences were identical to that of the C-less cassette *lacY* (EMBO-X56095).

For construction of mutant p(wt)L76C (the L76C mutation in the wild-type background), the *Acc* I–*Pst* I restriction fragment of L76C was isolated and ligated to the similarly treated pT7-5/cassette wild-type *lacY* vector. Sequencing of the subcloned fragment verified the presence of mutation CTG(Leu) → TGT(Cys) at position 76.

Colony morphology

As a preliminary, qualitative assay of transport activity, each mutant was transformed into *E. coli* HB101 and grown on MacConkey indicator plates containing 25 mM lactose. HB101 ($lacZ^+Y^-$) expresses active β-galactosidase but carries a defective *lacY* gene. Cells expressing 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. Cells that are impermeable to lactose grow as white colonies, and permease mutants with low activity grow as red colonies with a white halo.

Mutants Δ_{23}, Ala_{23}, and Phe_{23} grow as white colonies. In marked contrast, cells expressing Leu_{23} permease form significantly smaller, red colonies, indicating that growth of bacteria expressing this mutant is retarded, but some ability to translocate lactose down a concentration gradient is retained. Interestingly, after 30–36 h incubation at 37 °C, fast-growing red revertants appear on the indicator plates containing Leu_{23} permease. DNA-sequence analysis of plasmids isolated from the revertants reveals relatively large deletions in helix III; Leu_{23} was reduced to Leu_{11} or Leu_8.

With the exception of L76C (but not p(wt)L76C), which yields red colonies with a white halo, the Cys-replacement mutants grow as red colonies indistinguishable from cells expressing C-less permease.

Active lactose transport

As a more quantitative measure of function, the ability of each mutant to accumulate lac against a concentration gradient was assayed in *E. coli* T184 (Z^-Y^-). Mutants Δ_{23}, Ala_{23}, Phe_{23}, and Leu_{23} or its fast-growing revertants Leu_8 and Leu_{11} are unable to catalyze active accumulation of lactose above background levels (data not shown). In contrast, the majority of the Cys-replacement mutants transport lactose at high rates (Fig. 2A) to significant steady-state levels of accumulation (Fig. 2B). When rates of lactose transport are measured at 1 min, 18 of 25 mutants exhibit rates of 75–100% or more of C-less, 6 mutants transport at rates that are between 40 and 70% of C-less, and the rate of transport by L76C permease is below 20% of C-less (Fig. 2A). Steady-state levels of lac accumulation by the great majority of the mutants also approximate or exceed that of C-less permease (Fig. 2B). Twenty-one mutants accumulate to levels of 75–100% or more of C-less, and 3 mutants achieve steady states that are between 40 and 60% of C-less. In contrast, mutant L76C accumulates lactose to a level that is only 18% of the C-less.

Time courses of lactose accumulation for the mutants with low or intermediate levels of activity are shown in Figure 3. Mutants L84C and L98C transport the sugar to approximately 40% of the level achieved by C-less permease, and mutant G96C catalyzes close to 60% of C-less accumulation. Clearly, L76C permease also retains some ability to accumulate lactose against a concentration gradient but reaches only about 18% of the C-less steady state. In order to investigate the properties of the L76C mutant further, the mutation was transferred into the wild-type background (Fig. 4). As shown, in the presence of the 8 native Cys residues, the L76C mutant transports lactose at a highly significant rate (55% of wild type) and level of accumulation (65% of wild type). The results demonstrate that Leu 76 is not essential for transport and that the low activity observed for mutant L76C can be attributed to an interaction between this mutation and the 8 other mutations in C-less permease.

Expression of mutants

One possible explanation for the inability of the synthetic helix replacement mutants to accumulate lactose is unstable expression. However, [^{35}S]methionine labeling and pulse-chase experiments demonstrate that Ala_{23}, Phe_{23}, and Leu_{23} are all inserted in the membrane in a stable form (not shown).

In order to test the effect of the Cys replacements on expression, immunoblots were carried out on membrane preparations from *E. coli* T184-harboring plasmids encoding each mutant.

[1] 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 the wild-type lac permease and then a second letter denoting the amino acid replacement at this position.

Table 1. *DNA sequence analysis of Cys-replacement mutants in the C-less cassette lacY gene*

Mutant		Mutagenic oligonucleotide[a]	Codon change
Δ_{23}	sense	CTACGCAAATACCTGCA	
	antisense	GGTATTTGCGT	
Ala$_{23}$	sense	CTACGCAAATAC**GCG(GCC)$_2$GCA(GCG)$_4$GCTGCAGCGGCCGCA(GCG)$_6$GCAGCC(GCG)$_2$**CTGCA	
	antisense	G**(CGC)$_2$GGCTGC(CGC)$_6$TGCGGCCGCTGCAGC(CGC)$_4$TGC(GGC)$_2$CGC**GTATTTGCGT	
Phe$_{23}$	sense	CTACGCAAATAC**(TTC)$_{12}$TTT(TTC)$_{10}$**CTGCA	
	antisense	G**(GAA)$_{10}$AAA(GAA)$_{12}$**GTATTTGCGT	
Leu$_{23}$	sense	CTACGCAAATAC**(CTG)$_5$(CTC)$_2$(CTG)$_6$TTA(CTG)$_9$**CTGCA	
	antisense	G**(CAG)$_9$TAA(CAG)$_6$(GAG)$_2$(CAG)$_5$**GTATTTGCGT	
Y75C	sense	GACAAACTCGGTCTACGCAAA**TGC**CTGTGG	TAC → TGC
L76C	sense	GACAAACTCGGTCTACGCAAATAC**TGT**CTGTGGATTATTACC	CTG → TGT
L77C	sense	CGCAAATACCTG**TGT**TGGATTATTACC	CTG → TGT
	antisense	GGTAATAATCCA**ACA**CAGGTATTTGCG	
W78C	sense	TACCTGCTG**TGT**ATTATTACCGGC	TGG → TGT
	antisense	GGTAATAAT**ACA**CAGCAGGTA	
I79C	sense	CTGCTGTGG**TGT**ATTACCGGC	ATT → TGT
	antisense	GCCGGTAAT**ACA**CCACAGCAG	
I80C	sense	CTGTGGATT**TGT**ACCGGCATG	ATT → TGT
	antisense	CATGCCGGT**ACA**AATCCACAG	
T81C	sense	CTGTGGATTATT**TGC**GGCATGTTAGTG	ACC → TGC
	antisense	CACTAACATGCC**GCA**AATAATCCACAG	
G82C	sense	ATTATTACC**TGC**ATGTTAGTG	GGC → TGC
	antisense	CACTAACAT**GCA**GGTAATAAT	
M83C	sense	ATTACCGGC**TGC**TTAGTGATGTTTGCG	ATG → TGC
	antisense	CATCACTAA**GCA**GCCGGTAATAAT	
L84C	sense	ACCGGCATG**TGT**GTGATGTTTGCG	TTA → TGT
	antisense	AAACATCAC**ACA**CATGCCGGT	
V85C	sense	GGCATGTTA**TGT**ATGTTTGCG	GTG → TGT
	antisense	CGCAAACAT**ACA**TAACATGCC	
M86C	sense	ATGTTAGTG**TGT**TTTGCGCCG	ATG → TGT
	antisense	CGGCGCAAA**ACA**CACTAACATGCC	
F87C	sense	TTAGTGATG**TGT**GCGCCGTTC	TTT → TGT
	antisense	GAACGGCGC**ACA**CATCACTAACAT	
A88C	sense	GTGATGTTT**TGT**CCGTTCTTTATT	GCG → TGT
	antisense	AAAGAACGG**ACA**AAACATCACTAA	
P89C	sense	ATGTTTGCG**TGT**TTCTTTATTTTT	CCG → TGT
	antisense	AATAAAGAA**ACA**CGCAAACATCAC	
F90C	sense	TTTGCGCCG**TGC**TTTATTTTTATC	TTC → TGC
	antisense	AAAAATAAA**GCA**CGGCGCAAACAT	
F91C	sense	GCGCCGTTC**TGT**ATTTTTATCTTC	TTT → TGT
	antisense	GATAAAAAT**ACA**GAACGGCGC	
I92C	sense	CCGTTCTTT**TGT**TTTATCTTCGGG	ATT → TGT
	antisense	GAAGATAAA**ACA**AAAGAACGGCGC	
F93C	sense	TTCTTTATT**TGT**ATCTTCGGG	TTT → TGT
	antisense	CCCGAAGAT**ACA**AATAAAGAA	
I94C	sense	TTTATTTTT**TGC**TTCGGGCCA	ATC → TGC
	antisense	TGGCCCGAA**GCA**AAAAATAAAGAA	
F95C	sense	ATTTTTATC**TGC**GGGCCACTG	TTC → TGC
	antisense	CAGTGGCCC**GCA**GATAAAAATAAA	
G96C	sense	ATTTTTATCTTC**TGT**CCACTGCTGCAG	GGG → TGT
	antisense	CTGCAGCAGTGG**ACA**GAAGATAAAAAT	
P97C	antisense	GTTGTACTGCAGCAG**ACA**CCCGAAGATAAAAAT	CCA → TGT
L98C	antisense	GTTGTACTGCAG**ACA**TGGCCCGAAGAT	CTG → TGT
L99C	sense	GGGCCACTG**TGT**CAGTACAACATT	CTG → TGT
	antisense	GTTGTACTG**ACA**CAGTGGCCC	

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

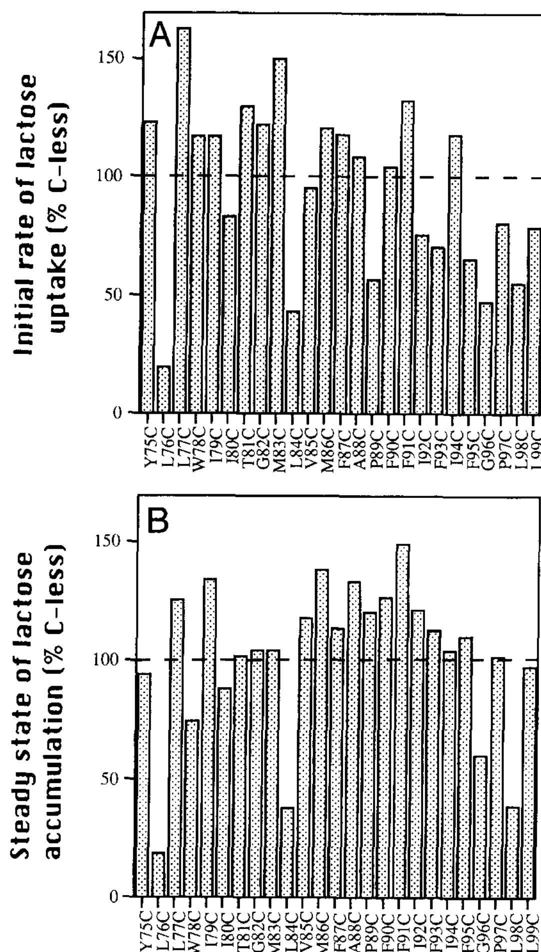

Fig. 2. Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described in the Materials and methods. **A:** Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 35 nmol lactose/min/mg protein. Results are expressed as a percentage of this value. Although not shown (see Fig. 3), T184 cells harboring pT7-5 (vector with no *lacY* gene) transported at a rate of 1.15 nmol lactose/min/mg protein (i.e., 3.3% of C-less). **B:** Steady-state levels of lactose accumulation. Results are expressed as a percentage of the C-less value, which averaged 134 nmol lactose/mg protein. Although not shown (see Fig. 3), T184 cells harboring pT7-5 accumulated 8.5 nmol lactose/mg protein in 1 h (i.e., 6.3% of C-less).

Each mutant is present in the membrane at levels comparable to C-less permease (Fig. 5).

Effect of NEM on transport activity

The effect of NEM, a membrane-permeant sulfhydryl reagent, on the initial rate of lactose transport for each Cys-replacement mutant was tested (Fig. 6). With the exception of G96C, which exhibits approximately 80% inhibition, the activity of the mutants is not affected significantly by the alkylating agent. The time required for 1 mM NEM (final concentration) to inactivate G96C permease by 50% (i.e., $t_{1/2}$) is about 10–15 s (data not shown), a rate significantly faster than that reported for wild-type permease (Viitanen et al., 1985). When cells expressing G96C permease are incubated with NEM under conditions where only partial inactivation occurs (0.1 mM NEM for 10 min; Fig. 7), the substrate analogue TDG enhances the effect of the alkylating agent. Although not shown, in the presence of 10 mM TDG (final concentration), the rate of NEM inactivation increases (i.e., $t_{1/2}$ decreases) about 2-fold.

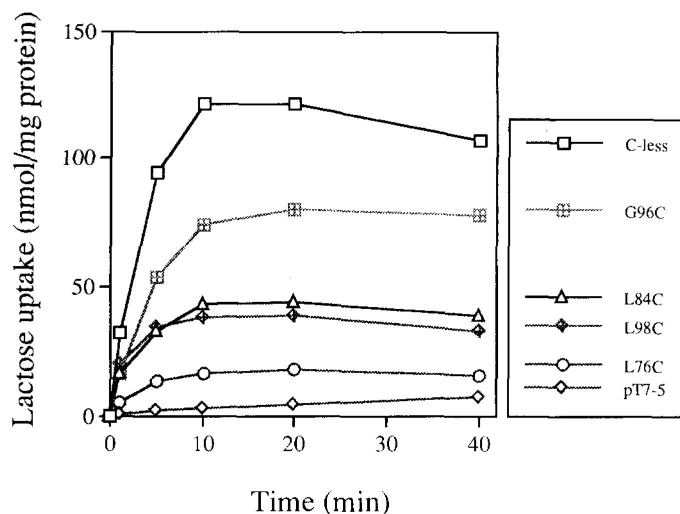

Fig. 3. Time courses of lactose transport by single-Cys mutants with low or intermediate activity. *E. coli* T184 transformed with plasmid pT7-5 (vector with no *lacY* gene), pT7-5-encoding C-less permease, or pT7-5-encoding given Cys-replacement mutants were grown and assayed as described in the Materials and methods.

Discussion

Results from extensive Cys-scanning mutagenesis on lac permease indicate that very few residues are critically involved in the

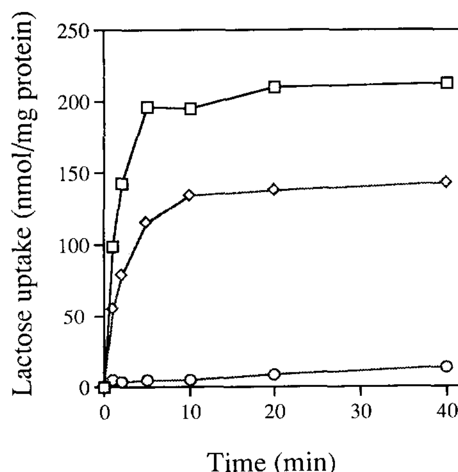

Fig. 4. Time course of lactose transport by mutant p(wt)L76C (i.e., L76C permease in the wild-type background). *E. coli* T184 transformed with plasmid pT7-5 (vector with no *lacY* gene) (○), pT7-5-encoding wild-type permease (□), or pT7-5-encoding mutant p(wt)L76C (◇) were grown and assayed as described in the Materials and methods.

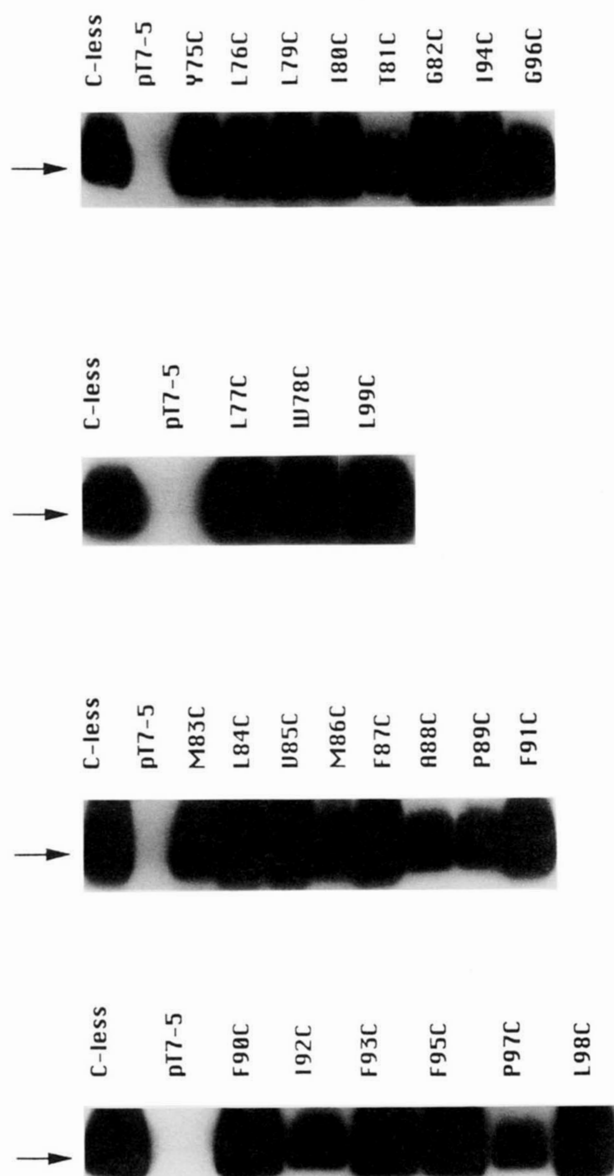

Fig. 5. Western blots of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of T184 harboring given plasmids, as described in the Materials and methods. Samples containing approximately 200 μg of membrane protein were subjected to SDS-PAGE, electroblotted, and the blots were incubated with anti-C-terminal lac permease antibody, followed by incubation with horseradish peroxidase-conjugated protein A (Amersham). The blots were developed with chemiluminescent substrate (Renaissance; New England Nuclear) and exposed to film for 1 min. The arrow on the left indicates the position of the marker protein carbonic anhydrase (32.5 kDa).

mechanism of lac/H^+ symport. Thus, in helices IX and X only Arg 302, His 322, and Glu 325 are essential for activity (Sahin-Tóth & Kaback, 1993), Thr 348 in helix XI is potentially important (Dunten et al., 1993; Frillingos & Kaback, unpubl. obs.) and none of the residues per se in the N-terminus or the first transmembrane domain is required for activity (Sahin-Tóth et al., 1994b). Similar results have been obtained from Cys mutagenesis of helix VII and the flanking cytoplasmic and periplasmic sequences (Frillingos et al., 1994), as well as helix V (C. Weitzman & H.R. Kaback, manuscript in prep.).

In the present study, Cys-scanning mutagenesis was employed to identify functionally important residues in putative helix III. Initially, helix III was deleted or replaced with synthetic helices containing 23 identical "helix-forming" residues (Ala, Phe, or Leu). Both manipulations result in inactive permease, indicating that helix III is important. Therefore, each residue comprising this domain was replaced individually with Cys. Twenty-one of the residues tolerate Cys replacement with relatively little or no effect on transport activity or levels of expression. Three mutants (L84C, G96C, and L98C) exhibit lower, but significant ability to accumulate lactose, and only 1 mutation (L76C) causes more drastic inactivation. However, when transferred into the wild-type background, mutant L76C catalyzes highly significant lactose accumulation.

In an initial screen for accessibility or reactivity of the Cys residues at each position from Tyr 75 to Leu 99, the effect of NEM on lactose transport was tested (Fig. 5). The single-Cys mutants, with exception of G96C, exhibit no significant change in transport activity upon NEM treatment. The simple explanation for the observation is that most of the Cys residues are inaccessible to NEM and do not react. Although this is a possibility, it seems unlikely because NEM is relatively permeant, and a number of Cys mutants in helices VII, X, and XI located in the middle of the membrane or disposed toward the inner surface are readily inhibited (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; Frillingos et al., 1994). Interestingly, G96C is more rapidly inactivated by NEM than wild-type permease (Viitanen et al., 1985), suggesting that this residue is more accessible and/or reactive than Cys 148 (helix V) in wild-type permease. In contrast to wild-type permease (Fox & Kennedy, 1965), however, the substrate analogue TDG does not protect against NEM inactivation but enhances the rate of inactivation.

Permease with a single Cys residue in place of Val 315 (helix X) is inactivated by NEM at least 10 times faster in the presence of TDG or $\Delta\bar{\mu}_{H^+}$ (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a), and it has been suggested that ligand binding or $\Delta\bar{\mu}_{H^+}$ may induce a similar conformational change in the permease. More recently, by using site-directed fluorescence labeling of a number of single-Cys mutants, it has been shown that ligand binding probably induces widespread changes in the tertiary structure without affecting secondary structure (Jung et al., 1994c; Wu & Kaback, 1994). In this context, the finding that NEM inactivation of G96C permease is enhanced by TDG suggests that helix III may also be involved in ligand-induced conformational change.

In conclusion, as judged by Cys-scanning mutagenesis, none of the 25 residues comprising putative helix III is important per se for permease activity. However, Leu_{23}, Ala_{23}, or Phe_{23} permease is unable to catalyze lactose accumulation to any extent whatsoever. The observations taken as a whole indicate that certain physicochemical features (e.g., surface contour, hydrophobicity) rather than individual amino acid side chains are important in this region of the permease. Thus, replacement of a single residue with Cys does not significantly disrupt the functionally competent structure of the protein and is well tolerated, whereas more drastic manipulations (e.g., replacement of the helix) result in impaired function.

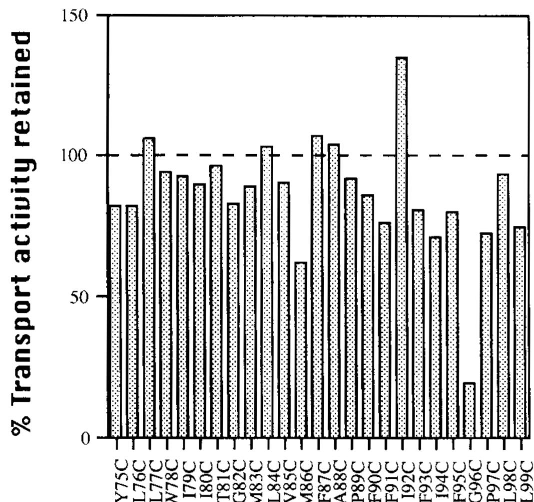

Fig. 6. Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 1 mM NEM (final concentration) at room temperature for 15 min, reactions were quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lactose transport in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971). Rates are presented as a percentage of the rate measured in the absence of NEM.

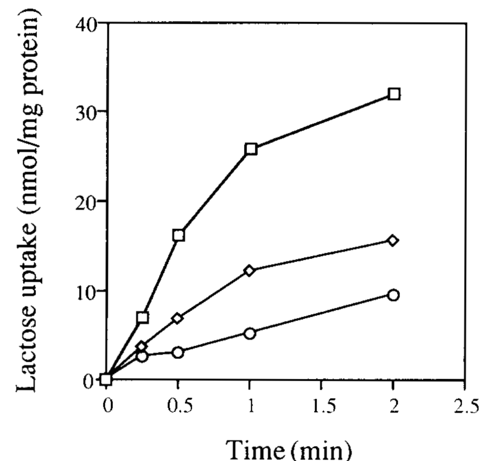

Fig. 7. Effect of TDG on the NEM inactivation of lactose transport by mutant G96C. *E. coli* T184 transformed with pT7-5/*lacY*/C-less encoding mutation G96C was treated with 0.1 mM NEM (final concentration) in the absence or presence of 10 mM TDG (final concentration) for 10 min. The reaction was stopped by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lac uptake in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971). Shown are the time courses of lactose transport obtained for nontreated cells (□), cells treated with NEM only (◇), and cells treated with NEM in the presence of TDG (○).

Materials and methods

Materials

[1-^{14}C]Lactose and [α-^{35}S]dATP were purchased from Amersham (Arlington Heights, Illinois). Deoxyoligonucleotides 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., 1984b) was prepared by BabCo (Richmond, California). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Beverly, Massachusetts). Taq DNA polymerase was from Promega Corporation (Madison, Wisconsin). Sequenase was from United States Biochemical (Cleveland, Ohio). 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

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-agar indicator plates (Difco Laboratories) containing 25 mM lac. *E. coli* T184 ($lacI^+O^+Z^-Y^-(A)$, *rspL*, met^-, thr^-, *recA*, *hsdM*, *hsdR/F'*, $lacI^qO^+Z^{D118}$ (Y^+A^+)) (Teather at al., 1980) harboring plasmid pT7-5/*lacY* with given mutations was used for expression of lac permease from the *lac* promoter. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (van Iwaarden et al., 1991) containing the *lac* promoter/operator was used for site-directed mutagenesis.

Oligonucleotide-directed site-specific mutagenesis

Cys replacement mutants were constructed by a 2-stage PCR method (PCR overlap-extension; Ho et al., 1989) using a pT7-5/*lacY*/cassette encoding C-less permease (van Iwaarden et al., 1991) as template. Sequences of the synthetic mutagenic primers used are given in Table 1. The PCR products were digested with *Acc* I and *Pst* I restriction endonucleases (see Fig. 1 for location of sites) and ligated to a similarly treated pT7-5/*lacY*/C-less vector. Mutants Y75C and L76C, or P97C and L98C were constructed by 1-stage PCR from mutagenic primers that included the sequence of the *Acc* I site or the *Pst* I site, respectively (see Table 1). For construction of mutant L99C, the PCR product was restricted at the *Nae* I site because *Pst* I is destroyed by the mutation (Table 1).

Finally, for construction of mutant p(wt)L76C (the L76C mutation in the wild-type background), the *Acc* I–*Pst* I restriction fragment of pL76C was isolated and ligated to similarly treated pT7-5/cassette wild-type *lacY* vector.

DNA sequencing

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

Growth of bacteria

E. coli HB101 (Z^+Y^-) or T184 (Z^-Y^-) transformed with each of the plasmids described was grown aerobically at 37 °C in Luria-Bertini medium containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). HB101 cell cultures were used for

preparation of plasmid DNA. Dense cultures of T184 cells were diluted 10-fold and allowed to grow for another 2 h, before induction with 1 mM IPTG. After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

Active lactose transport

Cells were washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10 at 420 nm (approximately 0.7 mg protein/mL). Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 mCi = 37 MBq), at a final concentration of 0.4 mM, was assayed by rapid filtration as described by Consler et al. (1991).

Membrane preparation

Crude membrane fractions from T184 cells were prepared essentially as described by Sahin-Tóth and Kaback (1993). Briefly, cells were lysed in ice-cold osmotic shock buffer (25 mM Tris-HCl [pH 8.0]/45% sucrose/1 mM EDTA), pelleted, resuspended in ice-cold water, allowed to stand for 10 min on ice, and then incubated with lysozyme (0.13 mg/mL) for 30 min. Suspensions were sonicated and, after removal of unlysed cells, membranes were harvested by ultracentrifugation.

Immunological analyses

Membrane fractions were subjected to 12% SDS-PAGE, as described (Newman et al., 1981). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-PVDF, Millipore) and probed with a site-directed polyclonal antibody against the C-terminus of lac permease (Carrasco et al., 1984b; Herzlinger et al., 1985).

Protein determinations

Protein was assayed in the presence of SDS by a modified Lowry method (Peterson, 1977), using bovine serum albumin as standard.

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References

Bibi E, Stearns SM, Kaback HR. 1992. The N-terminal 22 amino acid residues in the lactose permease of *Escherichia coli* are not obligatory for membrane insertion or transport activity. *Proc Natl Acad Sci USA 89*:3180–3184.

Boyer HW, Roulland-Dussoix D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol 41*:459–472.

Brooker RJ. 1990. Characterization of the double mutant, Val-177/Asn-322, of the lactose permease. *J Biol Chem 265*:4155–4160.

Brooker RJ. 1991. An analysis of lactose permease "sugar specificity" mutations which also affect the coupling between proton and lactose transport. I. Val^{177} and Val^{177}/Asn^{319} permeases facilitate proton uniport and sugar uniport. *J Biol Chem 266*:4131–4138.

Calamia J, Manoil C. 1990. *Lac* permease of *Escherichia coli*: Topology and sequence elements promoting membrane insertion. *Proc Natl Acad Sci USA 87*:4937–4941.

Carrasco N, Antes LM, Poonian MS, Kaback HR. 1986. *Lac* permease of *Escherichia coli*: His-322 and Glu-325 may be components of a charge-relay system. *Biochemistry 25*:4486–4488.

Carrasco N, Herzlinger D, Mitchell R, DeChiara S, Danho W, Gabriel TF, Kaback HR. 1984a. Intramolecular dislocation of the C-terminus of the *lac* carrier protein in reconstituted proteoliposomes. *Proc Natl Acad Sci USA 81*:4672–4676.

Carrasco N, Püttner IB, Antes LM, Lee JA, Larigan JD, Lolkema JS, Roepe PD, Kaback HR. 1989. Characterization of site-directed mutants in the *lac* permease of *Escherichia coli*. 2. Glutamate-325 replacements. *Biochemistry 28*:2533–2539.

Carrasco N, Tahara SM, Patel L, Goldkorn T, Kaback HR. 1982. Preparation, characterization and properties of monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*. *Proc Natl Acad Sci USA 79*:6894–6898.

Carrasco N, Viitanen P, Herzlinger D, Kaback HR. 1984b. Monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*. I. Functional studies. *Biochemistry 23*:3681–3687.

Consler TG, Tsolas O, Kaback HR. 1991. Role of proline residues in the structure and function of a membrane transport protein. *Biochemistry 30*:1291–1297.

Costello MJ, Escaig J, Matsushita K, Viitanen PV, Menick DR, Kaback HR. 1987. Purified lac permease and cytochrome *o* oxidase are functional as monomers. *J Biol Chem 262*:17072–17082.

Danho W, Makofske R, Humiec F, Gabriel TF, Carrasco N, Kaback HR. 1985. Use of site-directed polyclonal antibodies as immunological probes for the *lac* permease of *Escherichia coli*. In: Deber CM, Hruby VJ, Kopple KD, eds. *Peptides: Structure & function*. Pierce Chemical Co. pp 59–62.

Dornmair K, Corin AS, Wright JK, Jahning F. 1985. The size of the lactose permease derived from rotational diffusion measurements. *EMBO J 4*:3633–3638.

Dunten RL, Sahin-Tóth M, Kaback HR. 1993. Cysteine scanning mutagenesis of putative helix XI in the lactose permease of *Escherichia coli*. *Biochemistry 32*:12644–12650.

Foster DL, Boublik M, Kaback HR. 1983. Structure of the *lac* carrier protein from *Escherichia coli*. *J Biol Chem 258*:31–34.

Fox CF, Kennedy EP. 1965. Specific labeling and partial purification of the M protein, a component of the β-galactoside transport system of *Escherichia coli*. *Proc Natl Acad Sci USA 54*:891–899.

Franco PJ, Brooker RJ. 1994. Functional roles of Glu-269 and Glu-325 within the lactose permease of *Escherichia coli*. *J Biol Chem 269*:7379–7386.

Frillingos S, Sahin-Tóth M, Persson B, Kaback HR. 1994. Cysteine-scanning mutagenesis of putative helix VII in the lactose permease of *Escherichia coli*. *Biochemistry 33*:8074–8081.

Goldkorn T, Rimon G, Kaback HR. 1983. Topology of the *lac* carrier protein in the membrane of *Escherichia coli*. *Proc Natl Acad Sci USA 80*:3322–3326.

Hattori M, Sakaki Y. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal Biochem 152*:232–238.

Herzlinger D, Carrasco N, Kaback HR. 1985. Functional and immunochemical characterization of a mutant of *Escherichia coli* energy uncoupled for lactose transport. *Biochemistry 24*:221–229.

Herzlinger D, Viitanen P, Carrasco N, Kaback HR. 1984. Monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*. II. Binding studies with membrane vesicles and proteoliposomes reconstituted with purified *lac* carrier protein. *Biochemistry 23*:3688–3693.

Hinkle PC, Hinkle PV, Kaback HR. 1990. Information content of amino acid residues in putative helix VIII of lac permease from *Escherichia coli*. *Biochemistry 29*:10989–10994.

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene (Amsterdam) 77*:51–59.

Jung H, Jung K, Kaback HR. 1994a. A conformational change in the lactose permease of *Escherichia coli* is induced by ligand binding or membrane potential. *Protein Sci 3*:1052–1057.

Jung H, Jung K, Kaback HR. 1994b. Cysteine 148 in the lactose permease of *Escherichia coli* is a component of a substrate binding site: I. Site-directed mutagenesis studies. *Biochemistry 33*:12160–12165.

Jung K, Jung H, Kaback HR. 1994c. Dynamics of lactose permease of *Escherichia coli* determined by site-directed fluorescence labeling. *Biochemistry 33*:3980–3985.

Jung K, Jung H, Colacurcio P, Kaback HR. 1994d. *Biochemistry*. Forthcoming.

Jung K, Jung H, Wu J, Privé GG, Kaback HR. 1993. Use of site-directed

fluorescence labeling to study proximity relationships in the lactose permease of *Escherichia coli. Biochemistry 32*:12273–12278.

Kaback HR. 1989. Molecular biology of active transport: From membranes to molecules to mechanism. *Harvey Lect 83*:77–103.

Kaback HR. 1992. In and out and up and down with lac permease. In: Jeon JW, Friedlander M, eds. *International review of cytology vol 137A*. San Diego, California: Academic Press, Inc. pp 97–125.

Kaback HR, Jung K, Jung H, Wu J, Privé GG, Zen K. 1993. What's new with lac permease? *J Bioenerg Biomembr 25*:627–636.

King SC, Hansen CL, Wilson TH. 1991. The interaction between aspartic acid 237 and lysine 358 in the lactose carrier of *Escherichia coli. Biochim Biophys Acta 1062*: 177–186.

King SC, Wilson TH. 1989a. Galactoside-dependent proton transport by mutants of the *Escherichia coli* lactose carrier. Replacement of histidine 322 by tyrosine or phenylalanine. *J Biol Chem 264*:7390–7394.

King SC, Wilson TH. 1989b. Galactoside-dependent proton transport by mutants of the *Escherichia coli* lactose carrier: Substitution of tyrosine for histidine-322 and of leucine for serine-306. *Biochim Biophys Acta 982*: 253–264.

Konings WN, Barnes EM, Kaback HR. 1971. Mechanisms of active transport in isolated vesicles. III. The coupling of reduced phenazine methosulfate to the concentrative uptake of β-galactosides and amino acids. *J Biol Chem 246*:5857–5861.

Matzke EA, Stephenson LJ, Brooker RJ. 1992. Functional role of arginine 302 within the lactose permease of *Escherichia coli. J Biol Chem 267*:19095–19100.

Menezes ME, Roepe PD, Kaback HR. 1990. Design of a membrane transport protein for fluorescence spectroscopy. *Proc Natl Acad Sci USA 87*:1638–1642.

Menick DR, Carrasco N, Antes L, Patel L, Kaback HR. 1987a. *Lac* permease of *Escherichia coli*: Arginine-302 as a component of the postulated proton relay. *Biochemistry 26*:6638–6644.

Menick DR, Lee JA, Brooker RJ, Wilson TH, Kaback HR. 1987b. The role of cysteine residues in the lac permease of *Escherichia coli. Biochemistry 26*:1132–1136.

Newman MJ, Foster DL, Wilson TH, Kaback HR. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli. J Biol Chem 256*:11804–11808.

Padan E, Sarkar HK, Viitanen PV, Poonian MS, Kaback HR. 1985. Site-specific mutagenesis of histidine residues in the *lac* permease of *Escherichia coli. Proc Natl Acad Sci USA 82*:6765–6768.

Page MGP, Rosenbusch JP. 1988. Topography of lactose permease from *Escherichia coli. J Biol Chem 263*:15906–15914.

Peterson GL. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem 83*:346–356.

Püttner IB, Kaback HR. 1988. *Lac* permease of *Escherichia coli* containing a single histidine residue is fully functional. *Proc Natl Acad Sci USA 85*:1467–1471.

Püttner IB, Sarkar HK, Padan E, Lolkema JS, Kaback HR. 1989. Characterization of site-directed mutants in the *lac* permease of *Escherichia coli*. 1. Replacement of histidine residues. *Biochemistry 28*:2525–2533.

Püttner IB, Sarkar HK, Poonian MS, Kaback HR. 1986. *Lac* permease of *Escherichia coli*: Histidine-205 and histidine-322 play different roles in lactose/H^+ symport. *Biochemistry 25*:4483–4485.

Roepe PD, Kaback HR. 1989. Site-directed mutagenesis of tyrosine residues in the lac permease of *Escherichia coli. Biochemistry 28*:6127–6132.

Sahin-Tóth M, Dunten RL, Gonzalez A, Kaback HR. 1992. Functional interactions between putative intramembrane charged residues in the lactose permease of *Escherichia coli. Proc Natl Acad Sci USA 89*:10547–10551.

Sahin-Tóth M, Kaback HR. 1993. Cysteine scanning mutagenesis of putative transmembrane helices IX and X in the lactose permease of *Escherichia coli. Protein Sci 2*:1024–1033.

Sahin-Tóth M, Lawrence MK, Kaback HR. 1994a. Properties of permease dimer, a fusion protein containing two lactose permeases of *Escherichia coli. Proc Natl Acad Sci USA 91*:5421–5425.

Sahin-Tóth M, Persson B, Schwieger J, Cohan P, Kaback HR. 1994b. Cysteine scanning mutagenesis of the N-terminal 32 amino acid residues in the lactose permease of *Escherichia coli. Protein Sci 3*:240–247.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Seckler R, Möröy T, Wright JK, Overath P. 1986. Anti-peptide antibodies and proteases as structural probes for the lactose/H^+ transporter of *Escherichia coli*: A loop around amino acid residue 130 faces the cytoplasmic side of the membrane. *Biochemistry 25*:2403–2409.

Seckler R, Wright JK. 1984. Sidedness of native membrane vesicles of *Escherichia coli* and orientation of the reconstituted lactose:H^+ carrier. *Eur J Biochem 142*:269–279.

Seckler R, Wright JK, Overath P. 1983. Peptide-specific antibody locates the COOH terminus of the lactose carrier of *Escherichia coli* on the cytoplasmic side of the plasma membrane. *J Biol Chem 258*:10817–10820.

Stochaj V, Bieseler B, Ehring R. 1986. Limited proteolysis of the lactose permease from *Escherichia coli. Eur J Biochem 158*:423–428.

Teather RM, Bramhall J, Riede I, Wright JK, Fürst M, Aichele G, Wilhelm V, Overath P. 1980. Lactose carrier protein of *Escherichia coli*. Structure and expression of plasmids carrying the Y gene of the lac operon. *Eur J Biochem 108*:223–231.

Trumble WR, Viitanen PV, Sarkar HK, Poonian MS, Kaback HR. 1984. Site-directed mutagenesis of Cys_{148} in the lac carrier protein of *Escherichia coli. Biochem Biophys Res Commun 119*:860–867.

Ujwal ML, Sahin-Tóth M, Persson B, Kaback HR. 1994. Role of glutamate 269 in the lactose permease of *Escherichia coli. Mol Membr Biol 11*:9–16.

van Iwaarden P, Pastore JC, Konings WN, Kaback HR. 1991. Construction of a functional lactose permease devoid of cysteine residues. *Biochemistry 30*:9595–9600.

Viitanen PV, Menick DR, Sarkar HK, Trumble WR, Kaback HR. 1985. Site-directed mutagenesis of cysteine-148 in the *lac* permease of *Escherichia coli*: Effect on transport, binding, and sulfhydryl inactivation. *Biochemistry 24*:7628–7635.

Vogel H, Wright JK, Jähnig F. 1985. The structure of the lactose permease derived from Raman spectroscopy and prediction methods. *EMBO J 4*:3625–3631.

Wu J, Kaback HR. 1994. Cysteine 148 in the lactose permease of *Escherichia coli* is a component of a substrate binding site: II. Site-directed fluorescence studies. *Biochemistry 33*:12166–12171.

Zen KH, McKenna E, Bibi E, Hardy D, Kaback HR. 1994. Expression of lactose permease in contiguous fragments as a probe for membrane-spanning domains. *Biochemistry 33*:8198–8206.