# Cysteine-Scanning Mutagenesis of Putative Helix VII 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 helix VII and the flanking cytoplasmic and periplasmic regions (from Leu212 to Glu255) was replaced individually with Cys. Of the 44 single-Cys mutants, 40 exhibit high transport activity, accumulating lactose to >50% of the steady-state observed with C-less permease. In contrast, permease with Cys in place of Ala213 or Tyr236 exhibits low but significant activity, and Cys substitution for Asp237 or Asp240 yields permease molecules with little or no activity due to disruption of charge-neutralizing interactions between Asp237 and Lys358 or Asp240 and Lys319, respectively. Immunological analysis reveals that membrane levels of the mutant proteins are comparable to that of C-less permease with the exception of Tyr228→Cys, which exhibits reduced but significant levels of permease. Finally, the effect of *N*-ethylmaleimide (NEM) was tested on each mutant, and the results indicate that the transport activity of the great majority of the mutants is not affected by the alkylating agent. Remarkably, the six positions where Cys replacements render the permease highly sensitive to inactivation by NEM are confined to the C-terminal half of helix VII, a region that is strongly conserved among transport proteins homologous to lactose permease. The results demonstrate that although no residue *per se* in the region scanned is essential, structural features of the C terminus of helix VII may be important for transport activity.

The lactose (lac)[1] 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 $H^+$ (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 [see Kaback (1989, 1992) and Kaback et al. (1993) for reviews]. Furthermore, evidence has been presented (Dornmair et al., 1985; Costello et al., 1987; Sahin-Tóth et al., 1994b) indicating that the permease is functional as a monomer. On the basis of circular dichroic 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 (Figure 1). Evidence supporting the general features of the model has been obtained from laser Raman spectroscopy (Vogel et al., 1985), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and chemical labeling (Page & Rosenbusch, 1988). Moreover, immunological studies demonstrate that the C terminus (hydrophilic domain 13), as well as hydrophilic domains 5 and 7, is on the cytoplasmic surface of the membrane (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1986; Seckler & Wright, 1984; Herzlinger et al., 1984, 1985; Danho et al., 1985). 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 indicates that very few residues in lac permease are essential for activity (Sahin-Tóth & Kaback, 1993a; Dunten et al., 1993b; Sahin-Tóth et al., 1994a);[2] however, certain functionally important residues have been identified [see Kaback (1989, 1992) and Kaback et al. (1993)]. Site-directed replacement of Arg302 (Menick et al., 1987; Matzke et al., 1992) in putative helix IX or of His322 (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a,b, 1990a,b; Brooker, 1990, 1991) or Glu325 (Carrasco et al., 1986, 1989; Sahin-Tóth & Kaback, 1993a) in helix X indicates that these residues play a particularly important role in lactose/$H^+$ symport and/or substrate recognition. Moreover, Arg302, His322, and Glu325 are the only residues in transmembrane domains IX and X that are important for transport (Sahin-Tóth & Kaback, 1993a). In addition, Glu269 in helix VIII (Figure 1) has been shown to be a functionally important residue (Hinkle et al., 1990; Ujwal et al., 1993).

Recently (Jung et al., 1993), site-directed fluorescence labeling of combinations of paired Cys replacements in a functional permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991) was used to study

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; IPTG, isopropyl 1-thio-β-D-galactopyranoside; $KP_i$, potassium phosphate; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate; TDG, β-D-galactopyransoyl 1-thio-β-D-galactopyranoside; $\Delta\bar{\mu}_{H^+}$, $H^+$ electrochemical gradient across the membrane.

[2] In addition to helices I, VII, IX, X, and XI, Cys-scanning mutagenesis has been carried out with helices III (M. Sahin-Tóth, S. Frillingos, A. Gonzalez, and H. R. Kaback, manuscript in preparation), V (C. Weitzman, M. Sahin-Tóth, and H. R. Kaback, manuscript in preparation), and VI (S. Frillingos, M. Sahin-Tóth, A. Gonzalez, and H. R. Kaback, manuscript in preparation).

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proximity relationships in the lac permease by pyrene excimer fluorescence. The results demonstrate that His322 and Glu325 (putative helix X) are located in a portion of the permease that is in an $\alpha$-helical conformation and that helices VIII (Glu269) and IX (Arg302) are in close proximity to helix X (His322 and Glu325, respectively). These and other findings showing that helix VII (Asp237 and Asp240) is close to helices X (Lys319) and XI (Lys358) (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b; Kaback et al., 1993) 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). Site-directed fluorescence labeling is also being used to study conformational dynamics of the permease, and the results obtained thus far (Jung et al., 1994a; Sahin-Tóth & Kaback, 1993a)[3] are consistent with the interpretation that ligand binding or the $H^+$ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) may induce widespread changes in tertiary structure with little or no alteration in secondary structure.

The model (Jung et al., 1993; Kaback et al., 1993) indicates that helix VII is in close proximity to helices VIII, X, and XI, thereby suggesting that helix VII may contain residues which play an important role in the transport mechanism. Furthermore, recent experiments (Zen et al., 1994) in which lac permease was expressed in contiguous, nonoverlapping peptide fragments suggest that Phe247 is at or near the periplasmic border of helix VII as postulated by King et al. (1991). Therefore, Cys-scanning mutagenesis has been employed to investigate the functional importance of residues in transmembrane helix VII and the flanking cytoplasmic and periplasmic regions (from Leu212 to Glu255). The results demonstrate that all of the singly-Cys mutants, with the exceptions of D237C[4] or D240C (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b), exhibit significant ability to catalyze lactose accumulation. Furthermore, the Cys-replacement mutants that are most sensitive to NEM cluster in a highly conserved region between Tyr236 and Phe247, which is consistent with the idea that the C-terminal half of helix VII may be important structurally.

## MATERIALS AND METHODS

*Materials.* [1-$^{14}$C]Lactose and [$\alpha$-$^{35}$S]dATP were purchased from Amersham, Arlington Heights, IL. 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., 1984a) were prepared by BabCo, Richmond, CA. Restriction endonucleases and T4 DNA ligase were from New England Biolabs, Beverly, MA. Taq DNA polymerase was from Promega Corp., Madison, WI. Sequenase was from United States Biochemical, Cleveland, OH. 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*, $\lambda^-/F^-$] (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 lactose. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*), *rspL*, *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 of lac permease from the *lac* promoter/operator. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (C-less permease; van Iwaarden et al., 1991) containing the *lac* promoter/operator was used for all *lacY* gene manipulations.

*Oligonucleotide-Directed Site-Specific Mutagenesis.* The cassette *lacY* gene encoding C-less permease was inserted into the replicative form of phage M13mp19 or into plasmid pT7-5 and used as a template for mutagenesis. The sequences of all synthetic mutagenic primers used are given in Table 1. Cys replacements for positions 212–215, 217–225, 227–229, and 231–233 were created in M13 phage (Kunkel, 1985) as described by Consler et al. (1991). All remaining Cys replacement mutants were constructed by two-stage polymerase chain reaction (overlap-extension; Ho et al., 1989).

*DNA Sequencing.* Double-stranded plasmid DNA prepared by Magic Minipreps (Promega) was sequenced using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulsen, 1978) 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 $\mu$g/mL) and ampicillin (100 $\mu$g/mL). HB101 cultures were used for preparation of plasmid DNA. Dense cultures of T184 were diluted 10-fold and allowed to grow for another 2 h before induction with 1 mM isopropyl 1-thio-$\beta$-D-galactopyranoside (IPTG). After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

*Transport Assays.* Cells were washed with 100 mM potassium phosphate ($KP_i$, pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10 at 420 nm (approximately 0.7 mg of 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 as described (Consler et al., 1991).

*Membrane Preparation.* Crude membrane fractions from T184 cells were prepared essentially as described (Sahin-Tóth & Kaback, 1993a). Briefly, cells were suspended in ice-cold osmotic shock buffer [25 mM Tris-HCl (pH 8.0)/45% sucrose/1 mM ethylenediaminetetraacetate], 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 sonified, and after removal of unlysed cells, membranes were harvested by ultracentrifugation.

*Immunological Analyses.* Membrane fractions were subjected to 12% sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis, as described (Newman et al., 1981). Proteins were electroblotted to poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984a; Herzlinger et al., 1985).

*Protein Determinations.* Protein was assayed in the presence of $NaDodSO_4$ by a modified Lowry method (Peterson et al., 1977) with bovine serum albumin as standard.

[3] Purified, reconstituted permease with a single Cys in place of Val315 (helix X) exhibits marked increases in reactivity either in the presence of ligand or when $\Delta\bar{\mu}_{H^+}$ is imposed across the membrane of the proteoliposomes (Jung et al., 1994b).

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

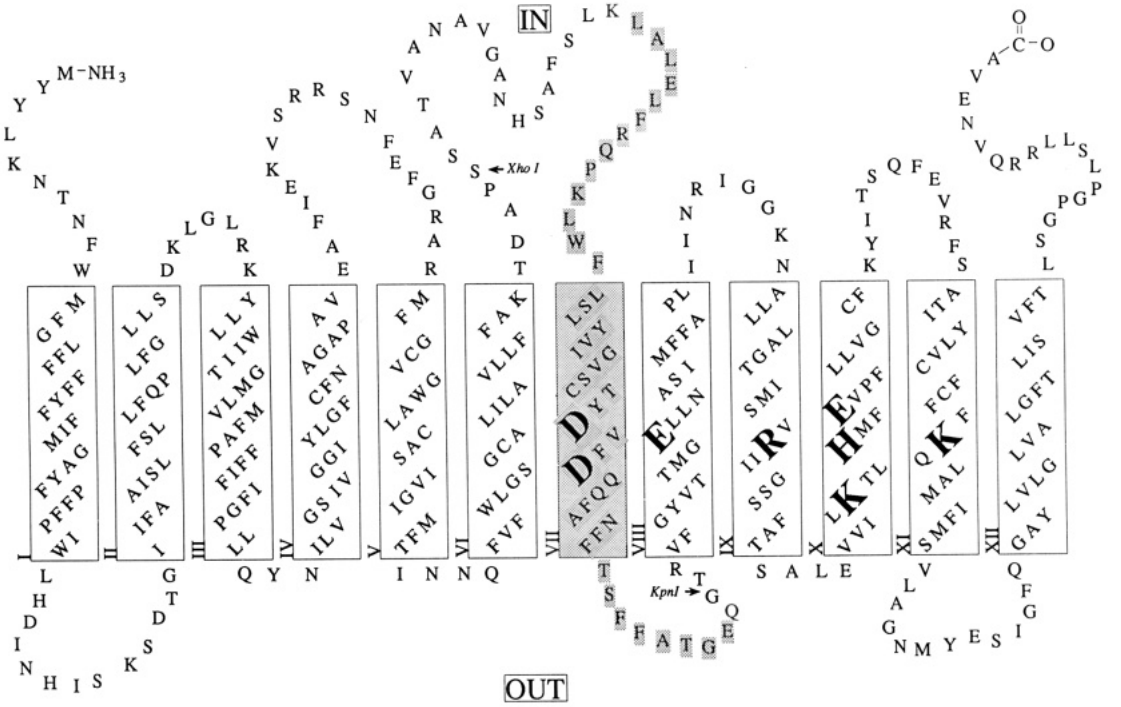


FIGURE 1: Secondary structure model of *E. coli* lac permease, based on hydropathy analysis (Foster et al., 1983). The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. The topology of helix VII is modified according to King et al. (1991), and the results are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

## RESULTS

*Construction and Verification of Mutants.* Each amino acid residue from Leu212 to Glu255, a region containing putative transmembrane helix VII and part of the flanking cytoplasmic and periplasmic loops (Figure 1), was replaced with Cys in C-less permease. The PCR products or the replicative forms of verified M13 clones were restricted with *Xho*I and *Kpn*I and the DNA 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 mutations were verified by sequencing the length of subcloned double-stranded DNA through the ligation junctions (*Xho*I–*Kpn*I). Except for the base changes introduced (Table 1), the sequences are identical to that of C-less cassette *lacY*. Mutants D237C and D240C were constructed and verified previously (Sahin-Tóth et al., 1992).

*Colony Morphology.* As a preliminary, qualitative assay of transport activity, each mutant was transformed into *E. coli* HB101, and colonies were 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 the external lactose to cytosolic β-galactosidase, and subsequent metabolism of the sugar leads to acidification and the appearance of red colonies. Cells expressing inactive mutants form white colonies, while mutants with low activity grow as red colonies with a white halo. It is important that indicator plates report "downhill" translocation of lactose and give no indication as to whether or not the cells catalyze lactose accumulation. With the exception of D240C which yields red colonies with a white halo, the other 43 Cys-replacement mutants described grow as red colonies indistinguishable from cells expressing C-less permease. Therefore, judging from indicator plates, all of the mutants appear to retain at least some ability to translocate lactose downhill.

*Active Lactose Transport. E. coli* T184 ($lacZ^-Y^-$) was used to test the ability of the mutants to catalyze active lactose transport. The majority of the 44 mutants transport lactose at very significant rates (Figure 2A). Thirty mutants exhibit rates that are between 65% and 100% or more of C-less permease, while 11 mutants transport at lower but significant rates (>30% of C-less). Only Y228C, D237C, and D240C exhibit markedly diminished rates of accumulation (<20% of C-less). Steady-state levels of lactose accumulation for the great majority of mutants also approximate those of C-less permease (Figure 2B); steady states of 50–100% or more of C-less are achieved by 40 mutants. A213C, Y236C, and D237C accumulate lactose to levels of about 25%, 30%, and 15%, respectively, of C-less, while accumulation by D240C is negligible.

Time courses of lactose transport by mutants with low rates are presented in Figure 3. Despite a low initial rate, mutant Y228C approaches the same steady-state as C-less permease by 1 h. Accumulation by mutants A213C or Y236C, on the other hand, reaches a lower but significant level (25–30% of C-less) which represents about an 8–10-fold concentration gradient. Therefore, as shown for Tyr26 (Sahin-Tóth et al., 1994a), although replacement of Tyr236 with Phe abolishes active transport (Roepe & Kaback, 1989), replacement with Cys leaves significant activity. Finally, low activity observed for D237C and lack of activity with D240C confirm previous findings (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b).

*Expression of Permease Mutants.* Western blot analysis of membrane fractions prepared from *E. coli* T184 expressing individual Cys-replacement mutants demonstrates that all of the mutants are present in the membrane at levels comparable to C-less permease with the exception of Y228C (Figure 4). Y228C is expressed at a significantly reduced level, which is consistent with the finding that this mutant exhibits a low rate of transport, but essentially a normal steady-state level

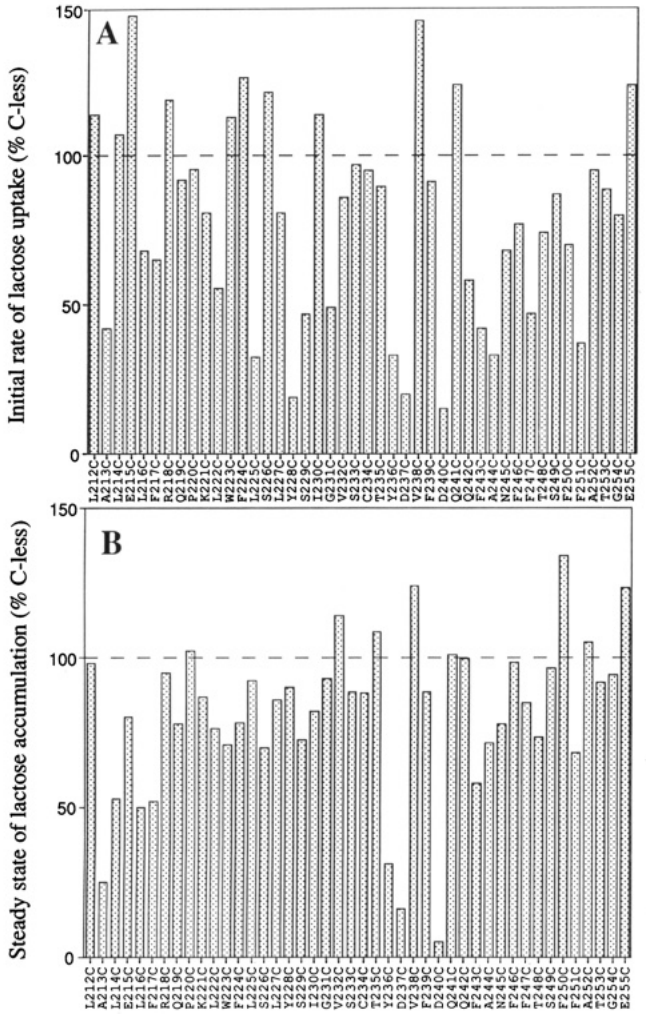


FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants or C-less permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM $KP_i$ (pH 7.5)/10 mM $MgSO_4$ were assayed as described under Materials and Methods. (A) Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 50 nmol $min^{-1}$ (mg of protein)$^{-1}$. Results are expressed as a percentage of this value. Although not shown (see Figure 3), T184 cells harboring pT7-5 (vector with no *lacY* gene) transported at a rate of 2 nmol $min^{-1}$ (mg of protein)$^{-1}$ (i.e., 4% of C-less). (B) Steady-state levels of lactose accumulation. Results are expressed as a percentage of the C-less value, which was 170 nmol of lactose/mg of protein, on average. Although not shown (see Figure 3), T184 cells harboring pT7-5 accumulated 10 nmol of lactose/mg of protein in 1 h (i.e., 5.9% of C-less).

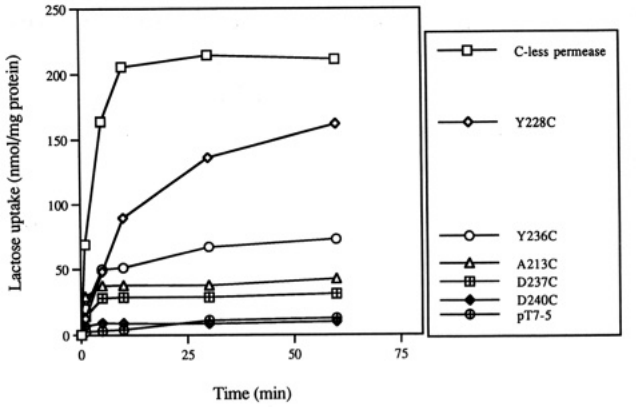


FIGURE 3: Time courses of lactose transport by single-Cys mutants with low activity. *E. coli* T184 transformed with plasmid pT7-5 (vector with no *lacY* gene), PT7-5 encoding C-less permease, or pT7-5 encoding given Cys-replacement mutants were grown and assayed as described under Materials and Methods.

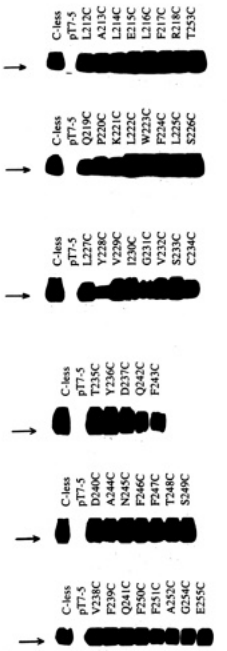

FIGURE 4: Western blots of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of T184 harboring given plasmids, as described under Materials and Methods. Samples containing approximately 200 μg of membrane protein were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and 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. Membranes prepared from cells harboring pT7-5 with no *lacY* gene exhibited no immunoreactive material. The arrow on the left indicates the migration position of the marker protein carbonic anhydrase (32.5 kDa).

of accumulation (Figures 2 and 3).

*Effect of NEM on Lactose Transport Activity*. The effect of NEM, a membrane-permeable thiol reagent, on the initial rate of lactose transport for each mutant is shown in Figure 5. The activity of most of the Cys-replacement mutants is not altered significantly by treatment with the alkylating agent. The six Cys-replacement mutants that are inhibited by 80% or more by NEM lie between Tyr236 and Phe247. When viewed on a helical wheel plot (Figure 6), four of the sensitive positions (Y236C, F243C, A244C, and F247C) cluster on the face of helix VII that is thought to lie in close proximity to helices VIII, X, and XI (Jung et al., 1993; Kaback et al., 1993).

## DISCUSSION

The results presented here extend a series of ongoing observations that have led to the conclusion that remarkably few residues in lac permease are directly involved in the mechanism of lactose/$H^+$ symport. Thus, Cys-scanning mutagenesis of helices IX and X has revealed that Arg302, His322, and Glu325 are important for activity (Sahin-Tóth & Kaback, 1993a), Thr348 in helix XI is potentially important (it has been replaced with Cys only; Dunten et al., 1993b), and none of the residues in the N terminus or the first transmembrane domain (Sahin-Tóth et al., 1994a) is essential for activity. Moreover, none of the residues in helices III, V, or VI is critical as judged by Cys-scanning mutagenesis.[2]

In the present study, Cys-scanning mutagenesis has been employed to identify important residues in putative trans-

Table 1: DNA Sequence Analysis of Cys-Replacement Mutants in the C-less Cassette *lacY* Gene

| mutant | | mutagenic oligonucleotide[a] | codon change |
|---|---|---|---|
| L212C | antisense | TTCCAGTGCGCACTTAAGGC | CTG→TGC |
| A213C | antisense | CAGTTCCAGGCACAGCTTAAG | GCA→TGC |
| L214C | antisense | GAACAGTTCGCATGCCAGCTTA | CTG→TGC |
| E215C | antisense | TCTGAACAGGCACAGTGCCAG | GAA→TGC |
| L216C | sense | GCACTGGAATTCTTCAGACAGCCA | CTG→TGC |
| | antisense | CTGTCTGAAGCATTCCAGTGC | |
| F217C | antisense | TGGCTGTCTGCACAGTTCCAG | TTC→TGC |
| R218C | antisense | TTTTGGCTGGCAGAACAGTTC | AGA→TGC |
| Q219C | antisense | CAGTTTTGGGCATCTGAACAG | CAG→TGC |
| P220C | antisense | CCACAGTTTGCACTGTCTGAA | CCA→TGC |
| K221C | antisense | AAACCACAGGCATGGCTGTCT | AAA→TGC |
| L222C | antisense | CAAAAACCAGCATTTTGGCTG | CTG→TGC |
| W223C | antisense | TGACAAAAAGCACAGTTTTGG | TGG→TGC |
| F224C | antisense | CAGTGACAAACACCACAGTTT | TTT→TGT |
| L225C | antisense | ATACAGTGAGCAAAACCACAG | TTG→TGC |
| S226C | sense | TGGTTTTTGTGCCTGTATGTTATTGGC | TCA→TGC |
| | antisense | AACATACAGGCACAAAAACCA | |
| L227C | antisense | AATAACATAGCATGACAAAAA | CTG→TGC |
| Y228C | antisense | GCCAATAACACACAGTGACAA | TAT→TGT |
| V229C | antisense | AACGCCAATACAATACAGTGA | GTT→TGT |
| I230C | sense | CTGTATGTTTGTGGCGTTTCC | ATT→TGT |
| | antisense | GGAAACGCCACAAACATACAG | |
| G231C | antisense | GGAGGAAACGCAAATAACATA | GGC→TGC |
| V232C | antisense | GGTGGAGGAACAGCCAATAAC | GTT→TGT |
| S233C | antisense | GTAGGTGGAGCAAACGCCAAT | TCC→TGC |
| C234C[b] | sense | GGCGTTTCCTGCACCTACGAT | TCC→TGC |
| | antisense | ATCGTAGGTGCAGGAAACGCC | |
| T235C | sense | GTTTCCTCCTGCTACGATGTT | ACC→TGC |
| | antisense | AACATCGTAGCAGGAGGAAAC | |
| Y236C | sense | TCCTCCACCTGCGATGTTTTT | TAC→TGC |
| | antisense | AAAAACATCGCAGGTGGAGGA | |
| D237C | sense | TCCACCTACTGTGTTTTTGACCAA | GAT→TGT |
| | antisense | GTCAAAAACACAGTAGGTGGA | |
| V238C | sense | CCACCTACGATTGTTTTGACCAAC | GTT→TGT |
| | antisense | TTGGTCAAAACAATCGTAAGGT | |
| F239C | sense | TACGATGTTTGTGACCAACAG | TTT→TGT |
| | antisense | CTGTTGGTCACAAACATCGTA | |
| D240C | sense | GATGTTTTTTGCCAACAGTTTGC | GAC→TGC |
| | antisense | AAACTGTTGGCAAAAAACATC | |
| Q241C | sense | CGATGTTTTTGACTGCCAGTTTGCT | CAA→TGC |
| | antisense | AGCAAACTGGCAGTCAAAAACATCG | |
| Q242C | sense | GTTTTTGACCAATGCTTTGCTAATTTC | CAG→TGC |
| | antisense | GAAATTAGCAAAGCATTGGTCAAAAAC | |
| F243C | sense | TTTGACCAACAGTGTGCTAATTTC | TTT→TGT |
| | antisense | GAAATTAGCACACTGTTGGTCAAA | |
| A244C | sense | GACCAACAGTTTTGTAATTTCTTT | GCT→TGT |
| | antisense | AAAGAAATTACAAAACTGTTGGTC | |
| N245C | sense | CAGTTTGCTTGTTTCTTTACTTCG | AAT→TGT |
| | antisense | CGAAGTAAAGAAACAAGCAAACTG | |
| F246C | sense | CAGTTTCGTAATTGTTTTACTTCG | TTC→TGT |
| | antisense | CGAAGTAAAACAATTAGCAAACTG | |
| F247C | sense | GCTAATTTCTGTACTTCGTTCTTT | TTT→TGT |
| | antisense | AAAGAACGAAGTACAGAAATTAGC | |
| T248C | sense | GCTAATTTCTTTTGTTCGTTCTTT | ACT→TGT |
| | antisense | AAAGAACGAACAAAAGAAATTAGC | |
| S249C | sense | TTCTTTACTTGTTTCTTTGCTACC | TCG→TGT |
| | antisense | GGTAGCAAAGAAACAAGTAAAGAA | |
| F250C | sense | TTCTTTACTTCGTGTTTTGCTACC | TTC→TGT |
| | antisense | GGTAGCAAACACGAAGTAAAGAA | |
| F251C | sense | ACTTCGTTCTGTGCTACCGGTGAA | TTT→TGT |
| | antisense | TTCACCGGTAGCACAGAACGAAGT | |
| A252C | sense | TCGTTCTTTTGTACCGGTGAACAG | GCT→TGT |
| | antisense | CTGTTCACCGGTACAAAAGAACGAAG | |
| T253C | sense | TCGTTCTTTGCTTGTGGTGAACAG | ACC→TGT |
| | antisense | CTGTTCACCACAAGCAAAGAACGA | |
| G254C | sense | TTCTTTGCTACCTGTGAACAGGGT | GGT→TGT |
| | antisense | ACCCTGTTCACAGGTAGCAAAGAA | |
| E255C | sense | GCTACCGGTTGTCAGGGTACCCGC | GAA→TGT |
| | antisense | CGCCCTACCCTGACAACCGGTAGC | |

[a] Sequences of mutagenic primers are presented in 5′→3′ order with altered codons in boldface type. [b] The C-less cassette *lacY* gene codes for Ser at position 234.

membrane helix VII and the flanking cytoplasmic and periplasmic regions. Thirty-nine of the residues between Leu212 and Glu255 tolerate replacement with Cys with relatively little or no significant effect on transport activity or levels of expression. Although Y228C permease exhibits a somewhat reduced level of expression in the membrane and

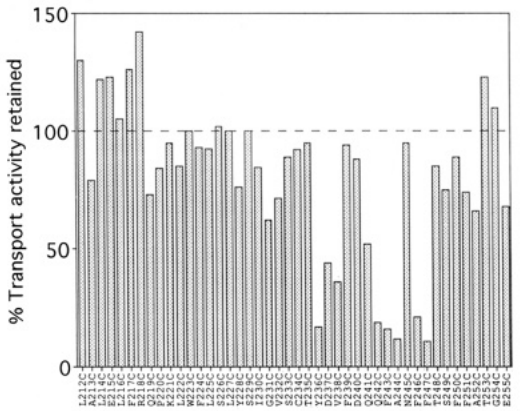


FIGURE 5: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 2 mM NEM (final concentration) at room temperature for 10 min; the reaction was quenched by addition of 20 mM dithiothreitol (final concentration) and assayed for initial rates of lactose uptake 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. The value for mutant D240C was determined using the active mutant D240C/K319A (Sahin-Tóth et al., 1992).

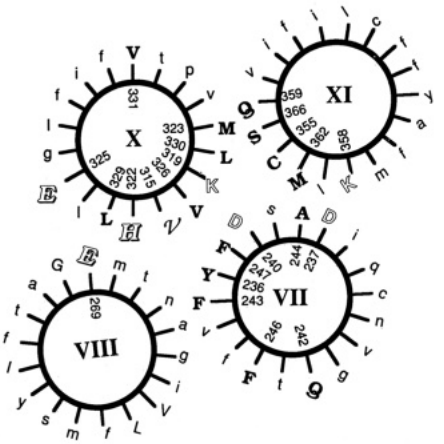


FIGURE 6: Relationship between helices VII, VIII, X, and XI in lac permease. Eighteen residues of each putative transmembrane helix are shown on helical wheels viewed from the periplasmic surface of the membrane. Helix VII includes residues from Ile230 to Phe247 (King et al., 1991; Zen et al., 1994). Residues shown as filled capital letters in helices X (Sahin-Tóth & Kaback, 1993a), XI (Dunten et al., 1993b), and VII represent positions where a Cys-replacement mutant is significantly inactivated by treatment with NEM (>60% inhibition of initial rate; see Figure 5). Also shown (open capital letters) are the interactions between Asp237 and Lys358 and between Asp240 and Lys319 (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b; Kaback et al., 1993). Positions 322 (His) and 269 (Glu), as well as 325 (Glu) and 322 (His) (shadowed open capitals), have been shown to be in close proximity by site-directed excimer fluorescence (Jung et al., 1993; Kaback, 1993), and permease with Cys in place of Val315 ($\nu$) exhibits markedly enhanced rates of NEM inactivation in the presence of TDG or $\Delta\bar{\mu}_{H^+}$ (Sahin-Tóth & Kaback, 1993a).[3]

a low initial rate of transport, lactose accumulation eventually reaches significant levels that are comparable to C-less permease. The finding is consistent with a previous observation (Roepe & Kaback, 1989) demonstrating that Phe replacement for Tyr228 results in wild-type expression and activity. Interestingly, like Y228C, Cys replacement for Tyr350 (helix XI) also results in a small decrease in expression (Dunten et al., 1993b).

A213C and Y236C permeases exhibit low but significantly ability to catalyze lactose accumulation. With regard to Tyr236, previous studies demonstrate that replacement with Phe abolishes transport (Roepe & Kaback, 1989); therefore, the hydroxyl group at position 236 may be required for H-bonding. In agreement with this notion, Cys replacement for Tyr236 results in significant transport activity. More dramatically, Phe replacement for Tyr26 (helix I) completely inactivates the permease, while Y26C permease is fully active (Sahin-Tóth et al., 1994a). Thus, in both instances, the thiol group of Cys appears to be able to mimic the hydroxyl group of Tyr.

On the basis of the observations that Phe, His, Asn, or Ser replacements alter the sugar-specificity of the permease, Tyr236 has been suggested to play a direct role in substrate recognition (Brooker & Wilson, 1985; Olsen et al., 1993). However, Tyr236 in *E. coli* lac permease is conserved in the lac permease from *Klebsiella pneumoniae* (McMorrow et al., 1988), as well as the raffinose permease from *E. coli* (Aslandis et al., 1989) and an *E. coli* sucrose permease (Bockmann et al., 1992; Lengeler et al., 1992). Therefore, it seems unlikely that Tyr236 plays a direct role in substrate-specificity. Rather, it is more probable that the residue is involved in a common step in the translocation mechanism. Consistently, preliminary studies (not shown) indicate that TDG does not protect Y236C permease against inactivation of NEM. In any case, Tyr236 is not essential, but appears to be required for full activity.

The inactivating effect of Cys replacements for Asp237 or Asp240 has been described previously (Sahin-Tóth et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b) and is due to the disruption of interactions between Asp237 and Lys358 or Asp240 and Lys319, respectively [see King et al. (1991) and Lee et al. (1992) in addition]. Neutralization of either member of the pairs leads to the presence of an unpaired charge which inactivates the permease, while simultaneous neutralization of both members restores activity. Moreover, Asp237 and Lys358 can be reversed without loss of activity, while interchanging Asp240 and Lys319 inactivates the permease. Therefore, none of the four residues nor the interactions *per se* is essential for activity, but the correct polarity between Asp240 and Lys319 is essential.

In an initial screen for accessibility or reactivity of the Cys residues at each position from Leu212 to Glu255, the effect of NEM on lactose transport in each mutant was tested (Figure 5). Most of the mutants are insensitive, but those that are sensitive to the alkylating agent cluster between Tyr236 and Phe247. The simple explanation for the observations 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) single-Cys replacement mutants with Cys at position 237 (Dunten et al., 1993a) or 240 (Sahin-Tóth et al., 1992), which are probably located near the middle of helix VII (King et al., 1991; Zen et al., 1994), react with NEM and/or more polar thiol reagents. Interestingly, the segment containing the NEM-sensitive positions comprises the C-terminal half of helix VII according to the modified secondary structure model of the permease (King et al., 1991). As shown in Figure 7, this region is strongly conserved in 2 other $H^+$-coupled sugar transporters homologous to lac permease, the raffinose permease and the sucrose permease of *E. coli*, and notably, the 12 residues are identical in the lac permeases from *E. coli* and *K. pneumoniae*. Therefore, the data taken as a whole indicate that although none of the residues in helix VII and the flanking cytoplasmic or periplasmic loops is essential in and of itself, the C-terminal half of this helix may play an important structural role. This suggestion is also consistent with observations (Zen et al., 1994) showing that expression of contiguous, nonoverlapping permease fragments with a discontinuity between Gly254 and

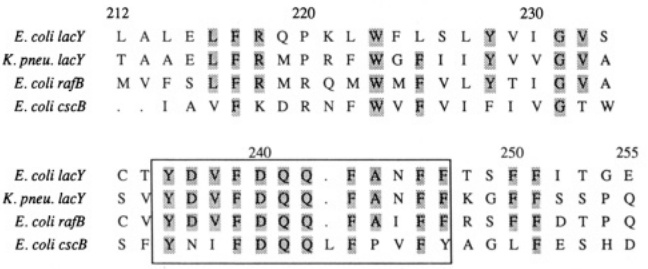


FIGURE 7: Comparison of the sequence Leu212–Glu225 from *E. coli* lac permease (*E. coli lacY*) with the corresponding regions of the lac permease from *Klebsiella pneumoniae* (*K. pneu. lacY*), the raffinose permease (*E. coli rafB*), and the sucrose permease of *E. coli* (*E. coli cscB*). Alignment of the sequences is according to Bockmann et al. (1992), and numbering (upper line) is according to *E. coli lacY*. Identical residues in three or more molecules are shaded. The region containing all of the NEM-inhibitable positions in *E. coli* lactose permease (236–247; see Figure 5) is shown in a box.

Glu255 results in functional complementation, while expression of fragments with a discontinuity between Phe247 and Thr248 does not. In addition, mutants containing insertions of two or six His residues after Ala244 [see Figure 5 in Kaback (1992)] or an in-frame repeat of residues 245–247 (S. Frillingos and H. R. Kaback, unpublished information) are inactive.

As suggested in Figure 6, four of the six NEM-sensitive positions in helix VII (Tyr236, Phe243, Ala244, and Phe247) lie on the same relatively hydrophilic face that contains Asp237 and Asp240 which interact with Lys358 (helix XI) and Lys319 (helix X), respectively (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993a; Sahin-Tóth & Kaback, 1993b, Kaback et al., 1993). Addition of a bulky maleimide might hinder conformational flexibility in this region of the protein, or, alternatively, these residues might provide part of an interface between helices in the C-terminal half of the permease that comprises the pathway for substrate and $H^+$.

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## REFERENCES

Aslanidis, C., Schmid, K., & Schmitt, R. (1989) *J. Bacteriol. 171*, 6753–6763.
Bockmann, J., Heuel, H., & Lengeler, J. W. (1992) *Mol. Gen. Genet. 235*, 22–32.
Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459–472.
Brooker, R. J. (1990) *J. Biol. Chem. 265*, 4155–4160.
Brooker, R. J. (1991) *J. Biol. Chem. 266*, 4131–4138.
Brooker, R. J., & Wilson, T. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3959–3963.
Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937–4941.
Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894–6898.
Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Dahno, W., Gabriel, T. F., & Kaback, H. R. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672–4676.
Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984b) *Biochemistry 23*, 3681–3687.
Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486–4488.
Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 2533–2539.
Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291–1298.
Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R., & Kaback, H. R. (1987) *J. Biol. Chem. 262*, 17072–17082.
Danho, W., Makofske, R., Humiec, F., Gabriel, T. F., Carrasco, N., & Kaback, H. R. (1985) in *Peptides: Structure & Function* (Deber, C. M., Hruby, V. J., & Kropple, K. D., Eds.) pp 59–62, Pierce Chemical Co., Rockford, IL.
Dornmair, K., Corin, A. S., Wright, J. K., & Jähning, F. (1985) *EMBO J. 4*, 3633–3638.
Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993a) *Biochemistry 32*, 3139–3145.
Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 12644–12650.
Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31–34.
Goldkorn, T., Rimon, G., & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3322–3326.
Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232–238.
Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688–3693.
Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221–229.
Hinkle, P. C., Hinkle, P. V., & Kaback, H. R. (1990) *Biochemistry 29*, 10989–10994.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene (Amsterdam) 77*, 51–59.
Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273–12277.
Jung, K., Jung, H., & Kaback, H. R. (1994a) *Biochemistry 33*, 3980–3985.
Jung, H., Jung, K., & Kaback, H. R. (1994b) *Protein Sci.* (in press).
Kaback, H. R. (1989) *Harvey Lect. 83*, 77–105.
Kaback, H. R. (1992) *Int. Rev. Cytol. 137A*, 97–125.
Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. (1993) *J. Bioenerg. Biomembr. 25*, 627–636.
King, S. C., & Wilson, T. H. (1989a) *J. Biol. Chem. 264*, 7390–7394.
King, S. C., & Wilson, T. H. (1989b) *Biochim. Biophys. Acta 982*, 253–264.
King, S. C., & Wilson, T. H. (1990a) *J. Biol. Chem. 265*, 3153–3160.
King, S. C., & Wilson, T. H. (1990b) *Biochim. Biophys. Acta 1022*, 373–380.
King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177–186.
Konings, W. N., Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5857–5861.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Lee, J.-I., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758–20764.
Lengeler, J. W., Bockmann, J., Heuel, H., & Titgemeyer, F. (1992) in *Molecular Mechanisms of Transport* (Quagliariello, E., & Palmieri, Y. F., Eds.) pp 77–85, Elsevier, New York.
Matzke, E. A., Stephenson, L. J., & Brooker, R. J. (1992) *J. Biol. Chem. 267*, 19095–19100.
McMorrow, I., Chin, D. T., Fiebig, K., Pierce, J. L., Wilson, D. M., Reeve, E. C. R., & Wilson, T. H. (1988) *Biochim. Biophys. Acta 945*, 315–323.
Menick, D. R., Carrasco, N., Antes, L., Patel, L., & Kaback, H. R. (1987) *Biochemistry 26*, 6638–6644.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804–11808.
Olsen, S. G., Greene, K. M., & Brooker, R. J. (1993) *J. Bacteriol. 175*, 6269–6275.
Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765–6768.
Page, M. G. P., & Rosenbusch, J. P. (1988) *J. Biol. Chem. 263*, 15906–15914.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483–4485.
Püttner, I. B., Sarkar, H. K., Padan E., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry 28*, 2525–2533.
Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 6127–6132.
Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547–10551.
Sahin-Tóth, M., & Kaback, H. R. (1993a) *Protein Sci. 2*, 1024–1033.
Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 10027–10035.
Sahin-Tóth, M., Persson, B., Schwiger, J., Cohan, M., & Kaback, H. R. (1994a) *Protein Sci. 3*, 240–247.
Sahin-Tóth, M., Lawrence, M. C., & Kaback, H. R. (1994b) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107–110.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5468.
Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 268–279.
Seckler, R., & Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817–10820.
Seckler, R., Möröy, T., & Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403–2409.
Stochaij, V., Bieseler, B., & Ehring, R. (1986) *Eur. J. Biochem. 158*, 423–428.
Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223–231.
Ujwal, M. L., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Membr. Mol. Biol. 1*, 9–16.
van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595–9600.
Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625–3631.
Zen, K., McKenna, E., Bibi, E., Hardy, D., & Kaback, H. R. (1994) *Biochemistry* (in press).