Role of Glycine Residues in the Structure and Function of Lactose Permease, an *Escherichia coli* Membrane Transport Protein

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ABSTRACT: By using oligonucleotide-directed, site-specific mutagenesis, the role of 34 Gly residues in the lactose permease of *Escherichia coli* has been studied systematically. Each of 34 out of a total of 36 Gly residues was replaced with Cys in a functional permease mutant devoid of Cys residues (C-less permease), as previous experiments demonstrate that Gly-402 and Gly-404 can be deleted by truncation of the C-terminus with no loss of activity [Roepe, P. D., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86,* 3992; McKenna, E., et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88,* 2969]. Out of the 34 Cys-replacement mutants described, 15 transport lactose with high activity, 16 exhibit decreased but significant ability to catalyze lactose accumulation, and 3 (Gly-64→Cys, Gly-115→Cys and Gly-147→Cys) exhibit no activity whatsoever. The inactive mutants were studied in more detail by replacement of Gly with Ala, Val, or Pro. C-less permease with Gly-115→Ala or Gly-147→Ala transports lactose almost as well as the control, while mutants with Val or Pro in place of Gly have little or no capacity to accumulate the disaccharide. In contrast, mutants with Ala, Val, or Pro in place of Gly-64 are inactive. Strikingly, however, when the mutations are placed in the wild-type background, Gly-64→Ala permease transports lactose, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside, and methyl 1-thio-β,D-galactopyranoside 40−60% as well as wild-type permease, while Gly-64→Val or Gly-64→Pro permease is inactive toward all of these substrates. The results indicate that although none of the Gly residues in lactose permease is mandatory for activity, the bulk of the side chain at positions 64, 115, and 147, rather than conformational flexibility at these positions, is particularly important.

Lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic, plasma membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ [reviewed in Kaback (1983, 1989, 1992)]. The permease is encoded by the *lacY* gene which has been cloned (Teather et al., 1978) and sequenced (Büchel et al., 1980). Moreover, the *lacY* gene product has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (Newman et al., 1981; Viitanen et al., 1986) as a monomer [see Sahin-Tóth et al. (1994a)]. Based on circular dichroism and the hydropathy analysis of the primary amino acid sequence, a secondary-structure model was proposed (Foster et al., 1983) in which the permease is composed of 12 hydrophobic segments in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic domains (loops) with both the N and C termini on the cytoplasmic face (Figure 1). Evidence favoring general features of the model and showing that the C terminus, as well as the second and third cytoplasmic loops, are on the cytoplasmic face of the membrane has been obtained from a variety of experimental approaches [see Kaback (1983, 1989, 1992)]. Moreover, analysis of a large number of lac permease-alkaline phosphatase (*lacY−phoA*) fusions has provided unequivocal support for the topological predictions of the 12-helix model (Calamia & Manoil, 1990). Recently, the combined use of site-directed mutagenesis and site-directed fluorescence labeling has led to a model describing proximity relationships in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

By using site-directed mutagenesis with wild-type permease or Cys-scanning mutagenesis with a functional mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991a), individual amino acid residues in the permease that are essential for activity have been identified [reviewed in Kaback et al. (1994)]. Over 300 of the 417 residues in C-less permease have been mutagenized, mostly by Cys-scanning mutagenesis (Sahin-Tóth & Kaback, 1993; Dunten et al., 1993b; Sahin-Tóth et al., 1994b; Frillingos et al., 1994; Sahin-Tóth et al., 1995; C. Weitzman and H. R. Kaback, manuscript in preparation). Remarkably, less than a half-dozen residues have been identified thus far as being clearly essential for activity, and of the few mutants that do not catalyze active transport, most retain the ability to catalyze partial reactions or bind ligand. More specifically, none of the eight Cys (Trumble et al., 1984; Menick et al., 1985, 1987a; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986; Brooker & Wilson, 1986; van Iwaarden et al. 1991a,b; Jung et al., 1994; Wu & Kaback, 1994), six Trp

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; TMG, methyl 1-thio-β,D-galactopyranoside; IPTG, isopropyl 1-thio-β,D-galactopyranoside; PCR, polymerase chain reaction; KP_i, potassium phosphate.

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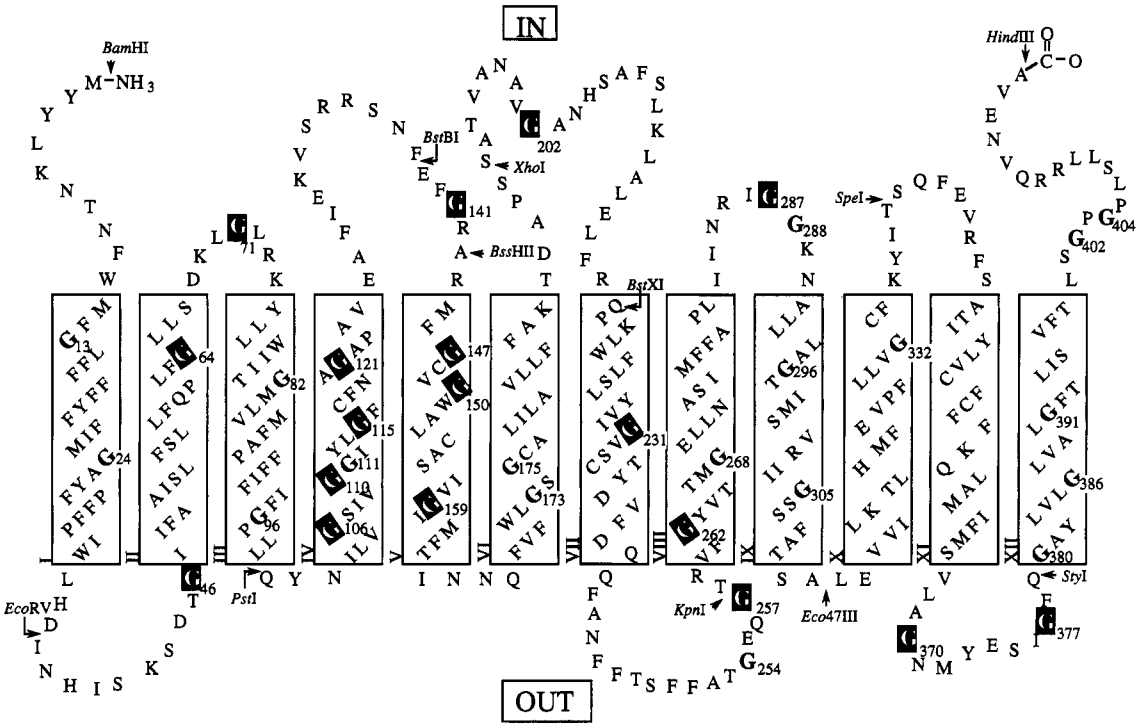

FIGURE 1: Secondary structure model of lac permease. All Gly residues are highlighted and numbered. Identical amino acids according to the alignment of lac, raffinose, and sucrose permeases from *E. coli* and the lac permease from *K. pneumoniae* (Bockmann et al., 1992) are boxed. The location of relevant restriction endonuclease sites in the corresponding DNA sequence are indicated.

(Menezes et al., 1990), or 12 Pro residues (Lolkema et al., 1988; Consler et al., 1991) in the permease is obligatory for active transport. Only one out of four His residues (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988) and possibly one of the 14 Tyr residues (Roepe & Kaback, 1989)[2] are important. On the other hand, Glu-269 (helix VIII) (Ujwal et al., 1994; Franco & Brooker, 1994), Arg-302 (helix IX) (Menick et al., 1987b; Matzke et al., 1992), His-322 (helix X) (Padan et al., 1985; Püttner et al., 1986, 1989; Püttner & Kaback, 1988), and Glu-325 (helix X) (Carrasco et al., 1986, 1989) are essential for active transport and/or binding of substrate.

Gly, with only H for a side chain, is both the smallest residue and the only amino acid with a symmetrical α-carbon. Although Gly is thought to be a strong "helix-breaker" that is found frequently in β-turns (Chou & Fasman, 1974; Merutka et al., 1990; O'Neil & DeGrado, 1990; Luy et al., 1990), it has been suggested that this residue may play a role in conformational flexibility in membrane proteins and that its behavior is environmentally dependent (Li & Deber, 1992a,b). Previous studies (Roepe et al., 1989; McKenna et al., 1991) demonstrate that Gly-402 and Gly-404 can be deleted with the C-terminal tail of the permease without effecting activity. In the experiments described in this paper, the remaining 34 Gly residues in lac permease were studied systematically, initially by Cys-scanning mutagenesis in C-less permease and then by site-directed mutagenesis of wild-type permease. The results demonstrate that none of the Gly residues in lac permease is obligatory for activity but suggest that the protein at positions 64, 115, and 147 is particularly sensitive to the size of the side chain.

[2] Although Tyr→Phe replacements indicate that three Tyr residues are important for activity, Cys-scanning mutagenesis has revealed that Tyr-26 and Tyr-236 can be replaced with Cys with retention of significant activity.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose, [^{35}S] methionine, and [α-^{35}S]-ATP were purchased from Amersham, Arlington Heights, IL. [^{14}C]Methyl 1-thio-β,D-galactopyranoside (TMG) was from Du Pont NEN, Boston, MA. [^{3}H]β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) was synthesized by Yu-Yen Liu under the supervision of Arnold Leibman in the Isotope Synthesis Group of Hoffmann-La Roche, Nutley, NJ. 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., 1984) was prepared by BabCo (Richmond, CA). All restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were from New England Biolabs, Beverly, MA. 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, l$^-$/F$^-$*] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described and for detection of lac permease activity on MacConkey plates 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 of the permease from the *lac* promoter/operator by induction with isopropyl 1-thio-β,D-galactopyranoside (IPTG). A cassette *lacY* gene (EMBL-X56095) containing the *lac* promoter/operator was used for all *lacY* gene manipulations. For overexpression via the T7 promoter (Tabor & Richardson, 1985), *E. coli* T184 were cotransformed with plasmid pGP1-2 encoding T7 RNA polymerase.

Table 1: Mutagenic Oligonucleotides Used To Change Gly into a Cys at Given Positions in the Cassette *lacY* Gene Encoding C-less Permease

mutant		mutagenic oligonucleotide[a]	codon change
G46C	sense	AGTGATACG**TGT**ATTATTTTTGCC	GGT → TGT
G46C	antisense	AAAAATAAT**ACA**CGTATCACTTTTGCT	
G64C	sense	CCGCTGTTTT**GT**CTGCTTTCTGAC	GGT → TGT
G64C	antisense	GTCAGAAAGCAG**ACA**AAACAGCGG	
G71C	sense	GACAAACTC**TGT**CTACGCAAATACCTG	GGT → TGT
G71C	antisense	GTATTTGCGTAG**ACA**GAGTTTGTC	
G82C	sense	ATTATTACC**TGC**ATGTTAGTG	GGC → TGC
G82C	antisense	CACTAACAT**GCA**GGTAATAAT	
G96C	sense	TTTATCTTC**TGC**CCACTGCTGCAG	GGG → TGC
G96C	antisense	CAGCAGTGG**GCA**GAAGATAAAAATAAAGAACGG	
G106C	sense	ATTTTAGTA**TGC**TCGATTGTTGGT	GGA → TGC
G106C	antisense	AACAATCGA**GCA**TACTAAAATGTT	
G110C	sense	TCGATTGTT**TGT**GGTATTTATCTA	GGT → TGT
G110C	antisense	ATAAATACC**ACA**AACAATCGATCC	
G111C	sense	ATTGTTGGT**TGT**ATTTATCTAGGC	GGT → TGT
G111C	antisense	TAGATAAAT**ACA**ACCAACAATCGA	
G115C	sense	ATTTATCTA**TGC**TTTAGTTTTAAC	GGC → TGC
G115C	antisense	AAAACTAAA**GCA**TAGATAAATACC	
G121C	sense	TTTAACGCC**TGT**GCGCCAGCA	GGT → TGT
G121C	antisense	TGCTGGCGC**ACA**GGCGTTAAAAC	
G141A	sense	AGTAATTTCGAATTTT**GT**CGCGCGCGG	GGT → TGT
G147C	sense	TTTGGTCGCGCGCGGATGTTTT**GC**AGTGTTGGCTGGG	GGC → TGC
G202C	sense	AATGCGGTA**TGT**GCCAACCATTCG	GGT → TGT
G202C	antisense	ATGGTTGGC**ACA**TACCGCATTGGC	
G257C	sense	ACCGGTGAACAG**TGT**ACCCGCGTA	GGT → TGT
G257C	antisense	TACGCGGGT**ACA**CTGTTCACCGGT	
G262C	sense	GGTGAACAGGGTACCCGCGTATTT**TGT**TACGTAACG	GGC → TGT
G268C	sense	ACGACAATG**TGC**GAATTACTTAACGCC	GGC → TGC
G268C	antisense	GTTAAGTAATTC**GCA**CATTGTCGT	
G287C	sense	AATCGCATC**TGT**GGGAAGAATGCC	GGT → TGT
G287C	antisense	ATTCTTCCC**ACA**GATGCGATTAATGATCAG	
G288C	sense	AATCGCATCGGT**TGT**AAGAATGCC	GGG → TGT
G288C	antisense	CAGGGCATTCTT**ACA**ACCGATGCG	
G370C	sense	GTACTGGCG**TGC**AATATGTATGAAAGC	GGC → TGC
G370C	antisense	TTCATACATATT**GCA**CGCCAGTAC	
G377C	sense	TATGAAAGCATA**TGT**TTCCAAGGC	GGT → TGT
G377C	antisense	AGCGCCTTGGAAA**CA**TATGCTTTC	
G380C	sense	GGTTTCCAA**TGT**GCTTATCTGGTG	GGC → TGT
G380C	antisense	CACCAGATAAGC**ACA**TTGGAAACC	
G386C	sense	GGTTTCCAAGGCGCTTATCTGGTGCTG**TGT**CTGGTGGCG	GGT → TGT
G391C	sense	GTGGCGCTG**TGT**TTCACCTTAATT	GGC → TGT
G391C	antisense	AATTAAGGTGAAA**CA**CAGCGCCAC	

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

Table 2: Mutagenic Oligonucleotides Used To Replace Gly at Positions 64, 115, and 147 with Ala, Pro, or Val in the Cassette *lacY* Gene Encoding C-less Permease

mutant		mutagenic oligonucleotide[a]	codon change
G64A	sense	CCGCTGTTT**GCT**CTGCTTTCTGAC	GGT → GCT
G64A	antisense	GTCAGAAAGCAG**AGC**AAACAGCGG	
G64P	sense	CCGCTGTTT**CCT**CTGCTTTCTGAC	GGT → CCT
G64P	antisense	GTCAGAAAGCAG**AGG**AAACAGCGG	
G64V	sense	CCGCTGTTT**GTT**CTGCTTTCTGAC	GGT → GTT
G64V	antisense	GTCAGAAAGCAG**AAC**AAACAGCGG	
G115A	sense	ATTTATCTA**GCC**TTTAGTTTTAACGCC	GGC → GCC
G115A	antisense	AAAACTAAA**GGC**TAGATAAATACC	
G115P	sense	ATTTATCTA**CCC**TTTAGTTTTAACGCC	GGC → CCC
G115P	antisense	AAAACTAAA**GGG**TAGATAAATACC	
G115V	sense	ATTTATCTA**GTC**TTTAGTTTTAACGCC	GGC → GTC
G115V	antisense	AAAACTAAA**GAC**TAGATAAATACC	
G147A	sense	TTTGGTCGCGCGCGGATGTTT**GCC**AGTGTTGGC	GGC → GCC
G147P	sense	TTTGGTCGCGCGCGGATGTTT**CCC**AGTGTTGGC	GGC → CCC
G147V	sense	TTTGGTCGCGCGCGGATGTTT**GTC**AGTGTTGGC	GGC → GTC

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

Oligonucleotide-Directed Site-Specific Mutagenesis. The cassette *lacY* gene (EMBL X-56095) encoding C-less permease (van Iwaarden et al., 1991a) was inserted 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 in Tables 1 and 2. The polymerase chain reaction (PCR) overlap extension method (Ho et al., 1989) was employed to create each mutant. PCR products were purified in agarose gels, purified, and digested with appropriate restriction endonucleases (see Figure 1 for restriction sites). The DNA fragments were isolated from low melting point agarose

gels and ligated to similarly treated pT7-5/*lacY* vector.

DNA Sequencing. Mutations were verified by sequencing of the length of the PCR-generated segment through the ligation junctions in double-stranded plasmid DNA 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 lac 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. The cells were grown in Luria broth containing 10 μg/mL streptomycin and 100 μg/mL ampicillin aerobically at 37 °C. Over night precultures were diluted 10-fold and allowed to grow for 2 h before induction with IPTG. After further growth for 2 h, cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i, pH 7.5)/ 10 mM $MgSO_4$, and then adjusted with the same buffer to an OD_{420} of 10 ($d = 1$ cm). Transport of [1-^{14}C]lactose (2.5 Ci/mol; final concentration 0.4 mM), [^{14}C]TMG (2.5 Ci/mol; final concentration 0.4 mM), and [^{3}H]TDG (100Ci/mol; final concentration 0.01 mM) was assayed by rapid filtration as described (Consler et al., 1991).

Immunological Analysis. For Western blot analysis, T184 cells grown as described above were adjusted to an OD_{420} of 100 ($d = 1$ cm; ca. 7 mg of protein/mL). A 50 μL aliquot of the suspension was sonicated. Unlysed cells were removed by low-speed centrifugation, and membranes were collected at 250000g_{max} for 1 h at 4 °C in a Beckman Optima TL ultracentrifuge. Membranes were solubilized in an equal volume of 2-times concentrated protein sample buffer (Laemmli, 1970). Equal amounts of protein were subjected to sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis as described (Newman et al., 1981). Proteins were electroblotted and immunoblots were probed with site-directed polyclonal antibody against the C-terminus of lac permease (Herzlinger et al., 1985).

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

Protein Determinations. Protein was assayed as described (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Construction and Verification of Mutants. Initially, the Gly residues in C-less permease were replaced individually with Cys. The mutants were constructed by using synthetic oligonucleotide primers (Table 1) and PCR overlap extension (Ho et al., 1989). PCR products were cloned into C-less permease using the appropriate restriction endonuclease sites (see Figure 1 for location of sites). Replacement of Gly-64, Gly-115, or Gly-147 with Ala, Val or Pro was carried out in the same manner by using the synthetic oligonucleotide primers given in Table 2. In addition, each of the Gly-64 mutations was cloned into the wild-type *lacY* gene by using the *Bam*HI and *Pst*I restriction endonuclease sites. All mutations were verified by double-stranded DNA sequencing, and, except for the desired base changes summarized in Table 1 and 2, the sequences were identical to those of the cassette *lacY* gene encoding C-less permease (van Iwaarden et al., 1991a) and wild-type *lacY*, respectively. Gly-402 and Gly-404 are not included because it was shown earlier (Roepe et al., 1989; McKenna et al., 1991) that truncation of the C-terminus at position 401 results in little or no alteration in permease activity.

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 sugar 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 mutants G96C,[3] G121C, G141C,[4] G159C,[5] G262C,[6] G268C, G287C, G288C, G296C (Sahin-Tóth & Kaback, 1993), G332C (Sahin-Tóth & Kaback, 1993), G380C, and G386C appear as red colonies with white halo. Cells expressing mutants G64C, G115C, and G147C grow as white colonies. All of the other mutants with Gly in place of Cys [G13C, G24C, G46C, G71C, G82C, G106C, G110C, G111C, G150C,[5] G173C,[7] G175C,[7] G202C, G231C, G254C, G257C (Frillingos et al. 1994), G305C (Sahin-Toth & Kaback 1993), G370C, G377C, and G391C] grow as red colonies indistinguishable from C-less permease.

Lactose Transport. Transport of lactose by cells expressing the mutants was assayed more quantitatively in *E. coli* T184 (Z^-Y^-) which lacks β-galactosidase and cannot metabolize lactose. Lactose transport for each mutant, as well as C-less permease (positive control) and pT7-5 with no *lacY* gene (negative control), was followed for 1 h, and both initial rates (calculated from the initial linear portion of the time course between 0 and 30 s) and steady-state levels of accumulation were determined in at least two independent experiments. The values are presented as a percentage of C-less lac permease in Table 3 (see Figure 2 in addition). Four categories of mutants emerge from the analysis. The first group includes 15 mutants that exhibit both initial rates of lactose transport and steady-state levels of accumulation that are comparable to the C-less control (70−100% of C-less permease). The second group includes three mutants in which the initial rate is compromised significantly, but the steady-state level of accumulation approaches that of C-less permease (Table 3 and Figure 2A,B: G106C, G231C, and G257C). The third group includes 13 mutants in which the initial rate and the steady-state level of accumulation are decreased, but significant ability to accumulate lactose is clearly retained (Table 3 and Figure 2A,B: G96C, G121C, G141C, G150C; G159C, G262C, G268C, G287C, G288C, G296C, G332C, G380C, and G386C). Finally, the fourth

[3] 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. The sequence is followed by a second letter denoting the amino acid replacement at this position.

[4] Mutant G141C was constructed by K. Zen.

[5] Mutants G150C and G159C were constructed and characterized by C. Weitzman (C. Weitzman and H. R. Kaback, manuscript in preparation).

[6] Mutant G262C was constructed by M. L. Ujwal.

[7] Mutants G173C and G175C were constructed by M. Sahin-Tóth.

Table 3: Relative Transport Activity and Expression of Gly Mutants[a]

mutant	initial rate (% C-less)	steady state (% C-less)	expression[g]
G13C[b]	88	129	+++
G24C[b]	112	104	+++
G46C	87	118	+++
G64C	2.5	6.7	+++
G71C	92	97	+++
G82C	112	96	+++
G96C	41	37	+++
G106C	58	87	+++
G110C	117	96	+++
G111C	89	83	+++
G115C	2.5	6.4	+
G121C	17.5	47	+++
G141C	11.7	22	+++
G147C	4.1	6.8	+++
G150C[c]	9.9	41	+++
G159C[c]	62.8	21	+++
G173C	73	135	+++
G175C	108	77	+++
G202C	100	96	+++
G231C	27	106	+++
G254C[d]	79	95	+++
G257C	74	59	+++
G262C	24	18	+++
G268C	15	18	+++
G287C	13	30	+++
G288C	37	43	+++
G296C[e]	5	30	−
G305C[e]	78	120	+++
G332C[e]	5	33	+++
G370C	85	71	+++
G377C	128	144	+++
G380C	6.3	10.8	+
G386C	6.8	57	+
G391C	98	95	+++
G402[f]			
G404[f]			

[a] Data are presented as percentages of the initial rate and steady-state level of lactose accumulation relative to *E. coli* strain T184 pT 7-5/C-less expressing C-less permease. An initial rate of 20 nmol lactose/(mg of protein·min) and a steady-state of 115 nmol of lactose/mg of protein of C-less permease is set as 100%. The initial rate of transport by T184 cells harboring pT7-5 (vector with no *lacY* gene) was 0.5 nmol/(min·mg of protein) (i.e., 2.6% of C-less permease), and the steady-state level was 7.0 nmol/mg of protein (i.e., 6% of C-less permease). Experiments were performed as described under Materials and Methods. [b] Data from Sahin-Tóth et al. (1994). [c] Data from C. Weitzman and H. R. Kaback (manuscript in preparation). [d] Data from Frillingos et al. (1994). [e] Data from Sahin-Tóth and Kaback (1993). [f] No mutants were constructed based on the findings of Roepe et al. (1989) and McKenna et al. (1991) that lac permease truncated at residue 401 shows 70−100% wild-type activity. [g] Expression of the mutants as judged from Western blot analysis: +++, 90−100%; +, 5−10%; −, no expression. Emboldened mutants are Gly residues that are conserved as identical residues in all four members of cluster 5 of the major facilitator superfamily [see Bockman et al. (1992), Figure 1].

group of mutants is completely inactive (Table 3 and Figure 2A: G64C, G115C, and G147C).

Expression of Mutant Permeases. In order to test whether the alterations in activity are due to differences in the amount of permease in the membrane, immunoblots were carried out on membrane preparations from *E. coli* T184 transformed with plasmids encoding each of the mutants. Thirty-one of the mutants are expressed in amounts comparable to C-less permease (Table 3). Expression of G115C is significantly lower than C-less permease, and mutants G296C, G380C, and G386C are hardly detectable. As described previously (Sahin-Tóth & Kaback, 1993), although G296C permease

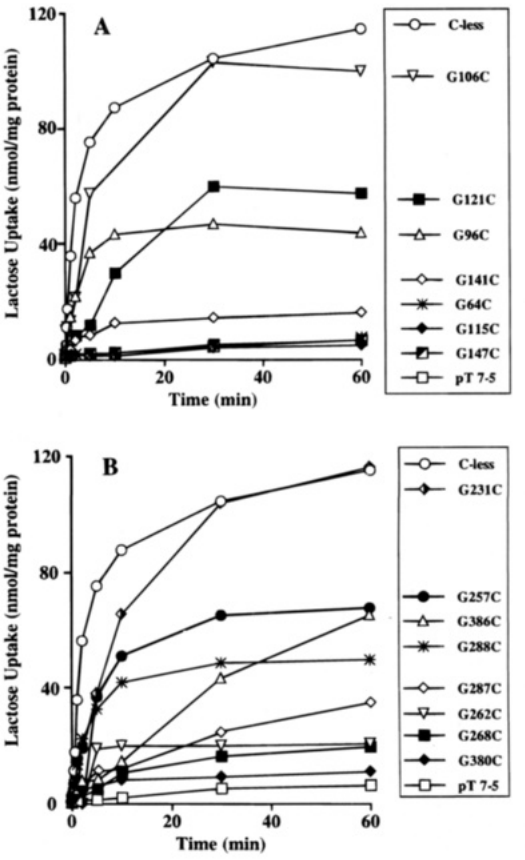

FIGURE 2: Time course of lactose transport by Cys-replacement mutants in Gly residues that exhibit low activity. (A) Mutants in helices I to V. (B) Mutants in helices VI−XII. *E. coli* T184 harboring plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding C-less permease, or pT7-5 encoding mutant permeases with given Gly replacements were grown at 37 °C, and 50 μL aliquots of cell suspension (containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed for lactose uptake at 25 °C as described under Materials and Methods. The final concentration of [^{14}C]lactose (2.5 Ci/mol) was 0.4 mM.

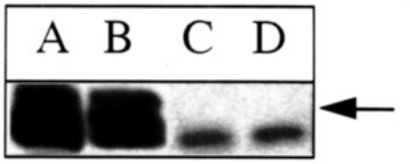

FIGURE 3: Western blot of membranes from mutants G380C and G386C overexpressed from the T_7 promoter. *E. coli* T184 were cotransformed with pGP1-2 encoding T_7 RNA polymerase under the control of a heat-shock promoter and pT7-5 encoding G380C and G386C permeases, respectively. Cells were grown at 30 °C and heat shocked for 20 min at 42 °C. Membranes were prepared from the heat shocked cultures and subjected to electrophoresis and Western blotting as described under Materials and Methods. (Lanes A and B) 100 μg of G380C and 100 μg of G386C membranes, respectively, expressed from the T_7 promoter. (Lanes C and D) 100 μg of G380C and 100 μg of G386C membranes, respectively, expressed from the *lac* promoter/operator by IPTG induction. The arrow indicates the position of the marker protein carbonic anhydrase (32.5 kDa).

is hardly detectable when expressed from the *lac* promoter/operator, the mutant is expressed at a high level in a stable form from the T7 promoter after heat shock. Similarly, when mutants G380C and G386C are overexpressed from the T7 promoter, as opposed to the *lac* promoter/operator, much more permease is observed in Western blots (Figure 3).

One explanation for the behavior of G380C and G386C is that their lifetimes in the membrane are diminished due

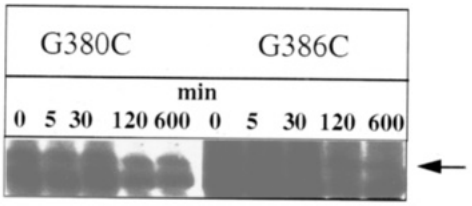

FIGURE 4: [^{35}S]Methionine labeling and pulse−chase with the mutants G380C and G386C expressed from the T7 promoter. Specific labeling of lac permease with [^{35}S]methionine was carried out in the presence of rifampicin (McKenna et al., 1991). Cultivation and heat shock conditions were the same as described in Figure 3. After incubation with [^{35}S]methionine at 30 °C for 10 min, an aliquot was removed as the zero-min time point. A 200-fold excess of cold methionine was then added, and aliquots were removed for the membrane preparation at 5, 30, 120, and 600 min. Membranes (approximately 50 μg of the mutants proteins) were subjected to polyacrylamide gel electrophoresis, and the dried gel was exposed to a phosphoimager screen for 20 h. The arrow indicates the position of the marker protein carbonic anhydrase (32.5 kDa).

to enhanced rates of proteolysis (Roepe et al., 1989; McKenna et al., 1991, 1992). Under these circumstances, little or no permease is observed when synthesis takes place at relatively low rates via the *lac* promoter/operator, but the mutant proteins are present upon synthesis at high rates 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 G380C or G386C permease remains relatively constant for 10 h after addition of unlabeled methionine (Figure 4). Thus, as described for mutations in the interacting pair Asp-237 and Lys-358 (Dunten et al., 1993a), as well as G296C permease (Sahin-Tóth & Kaback, 1993), mutants G380C and G386C appear to be defective in a step between translation and insertion into the membrane.

Properties of Other Amino Acid Replacements for G64, G115, or G147. Gly-64, Gly-115, and Gly-147 appear to be residues that are essential for permease function as judged by Cys-scanning mutagenesis in C-less permease. Therefore these residues were examined further by replacement with Ala, Val, or Pro. C-less permease containing each of these replacements at position 64 is inactive (Figure 5A). Lack of activity is not due to a defect in insertion or stability after insertion, since Western blots reveal no difference in expression between the Gly-64 mutants and C-less permease (Figure 6A). Replacement of Gly-115 with Ala yields permease with activity comparable to C-less permease (90% of the rate and 80% of the steady-state), while G115P permease exhibits significant but low activity (10% of the rate and 41% of the steady-state), and G115V permease has marginally significant activity (5% of the rate and 16% of the steady-state) (Figure 5B). Interestingly, G115C permease is present in the membrane at significantly lower levels than the other replacement mutants (Figure 6B). In a similar fashion, permease with Ala in place of Gly-147 exhibits relatively high activity relative to C-less permease (23% of the rate and 80% of the steady-state), but replacement with Pro or Val yields permease with little or no activity, respectively (Figure 5C), and the mutants are expressed at levels comparable to C-less permease (Figure 6C).

Gly-64 Mutations in the Wild-Type Background. Since the *lacY* gene encoding C-less permease contains eight mutations, it is important to test mutations in the wild-type background before it can be concluded that a particular

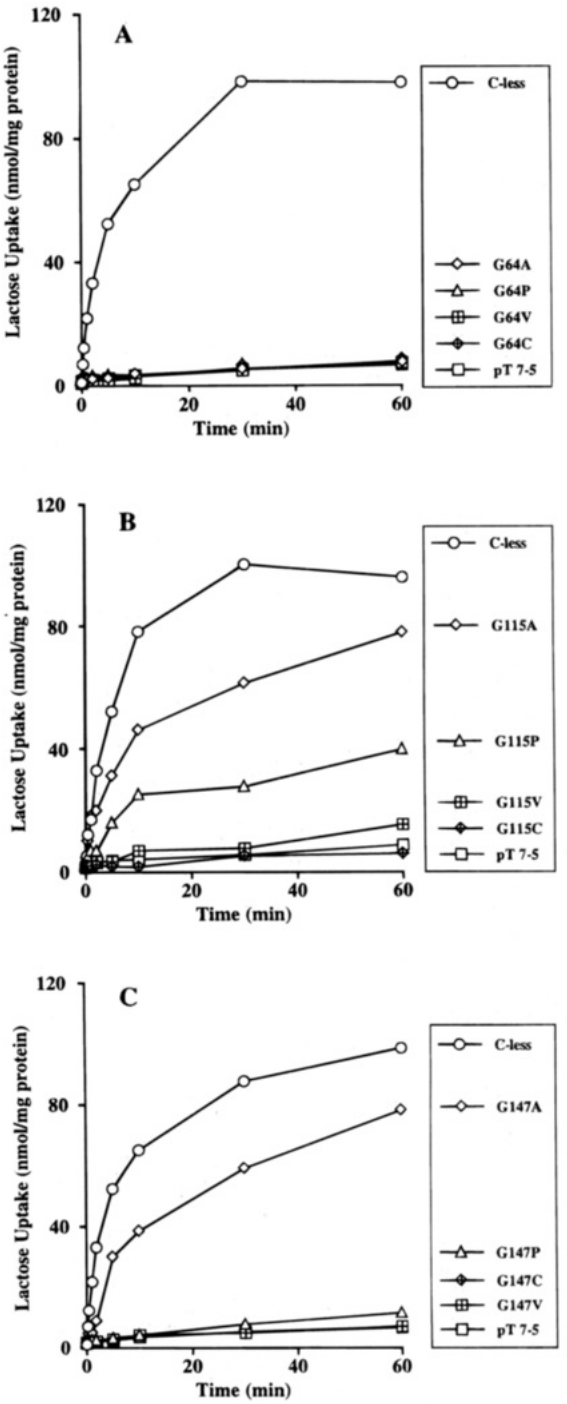

FIGURE 5: Time course of lactose transport by cells expressing Ala, Val, or Pro replacements for Gly-64, Gly-115, or Gly-147. *E. coli* T184 harboring plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding C-less permease, or pT7-5 encoding permeases G64A, G64V, or G64P (A), G115A, G115V, or G115P (B), and G147A, G147V, or G147P (C) were grown and assayed for lactose uptake at 25 °C as described in the legend to Figure 2.

residue is essential for function. Therefore, the mutations in *lacY* encoding C-less G64A, G64V, or G64P permease were cloned into the wild-type *lacY* gene, and each mutant was expressed in *E. coli* T184 and tested for transport of lactose, TDG, or TMG. Remarkably, G64A in the wild-type background catalyzes lactose (Figure 7A), TDG (Figure 7B), and TMG (Figure 7C) transport with 40−60% of the activity of wild-type permease. On the other hand, mutant G64V or G64P does not exhibit significant activity toward any of these sugars.

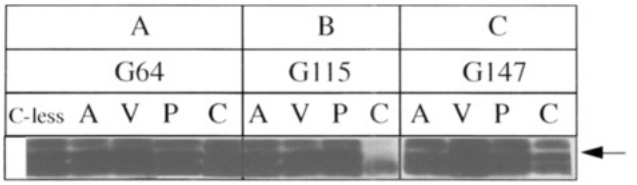

FIGURE 6: Western blot of membranes containing C-less permease and C-less permease with given mutations at Gly-64, Gly-115, and Gly-147. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids and 100 μg of membrane protein was subjected to electrophoresis and Western blotting as described under Materials and Methods. The arrow indicates the position of the marker protein carbonic anhydrase (32.5 kDa).

DISCUSSION

Primary amino acid sequence analysis of cluster 5 of the major facilitator superfamily (Marger & Saier, 1993) which includes the lac, raffinose, and sucrose permeases from *E. coli* and the lac permease from *Klebsiella pneumoniae* reveals that 92 amino acids are identical in the four transporters and that Gly residues, in particular, are highly conserved [see Bockmann et al. (1992)]. Similarly, Gly is a highly conserved residue in the arabinose and xylose permeases from *E. coli* and the glucose facilitator from *Saccharomyces cerevisiae*, human hepatoma cells, human liver cells, and rat adipocytes (Maiden et al., 1987; Henderson 1990). Moreover, conserved sequence motifs, such as the 'R/K-X-G-R-R/K' or the extended 'G-X-X-X-D-R/K-X-G-R-R-/K' motif which contain at least one Gly residue, are found in first and fourth cytoplasmic loops of sugar facilitators from eucaroytes and bacteria (Henderson, 1991; Griffith et al., 1992).

On the basis of its small size and the fact that Gly is the only amino acid which lacks a β-carbon, this residue is thought to play a special role in protein structure. Thus, Gly is frequently found as a cap at the carboxyl termini of α-helices (Richardson & Richardson, 1988b; Aurora et al., 1994). Gly is also found with high frequency in β-turns, particularly in tight β-hairpins (Richardson & Richardson, 1988a), and is thought to be involved in modulating the packing between helices within membranes [e.g., M13 coat protein (Wickner, 1988; Henry & Sykes, 1990) and glycophorin dimers (Lemmon et al., 1992)]. Richardson and Richardson (1988a) have suggested that although Gly is an excellent helix breaker at the beginning or end of a helix, it can be accommodated easily near the middle of a helix. Li and Deber (1992a,b) studied the influence of Gly residues on α-helices in membrane environments by using a series of 20-residue model peptides. Their results are consistent with a role for Gly residues in environment-dependent helix modulation and suggest that Gly may provide the structural basis for conformational transitions within or adjacent to membrane domains such as those accompanying membrane insertion and/or required for transport or signaling functions. In this context, de Cock et al. (1991) have reported that Gly-144 in the outer membrane protein PhoE is required for efficient folding.

With the exception of porins which are β-barrels (Weiss et al., 1991; Cowan et al., 1992), most integral membrane proteins are thought to be largely in α-helical conformation, a notion strongly influenced by the structures of bacteriorhodopsin (Henderson & Unwin, 1975; Henderson et al., 1990) and bacterial photosynthetic reaction centers (Deisenhofer et al., 1985; Komiya et al. 1988). Despite the supposition that Gly is a "helix-breaker" that is found frequently in β-turns, certain transmembrane helices contain many Gly residues [e.g., five Gly residues in helix D of bacteriorhodopsin (Engelman et al., 1980) and four Gly residues in helix D of the L subunit of the photosynthetic

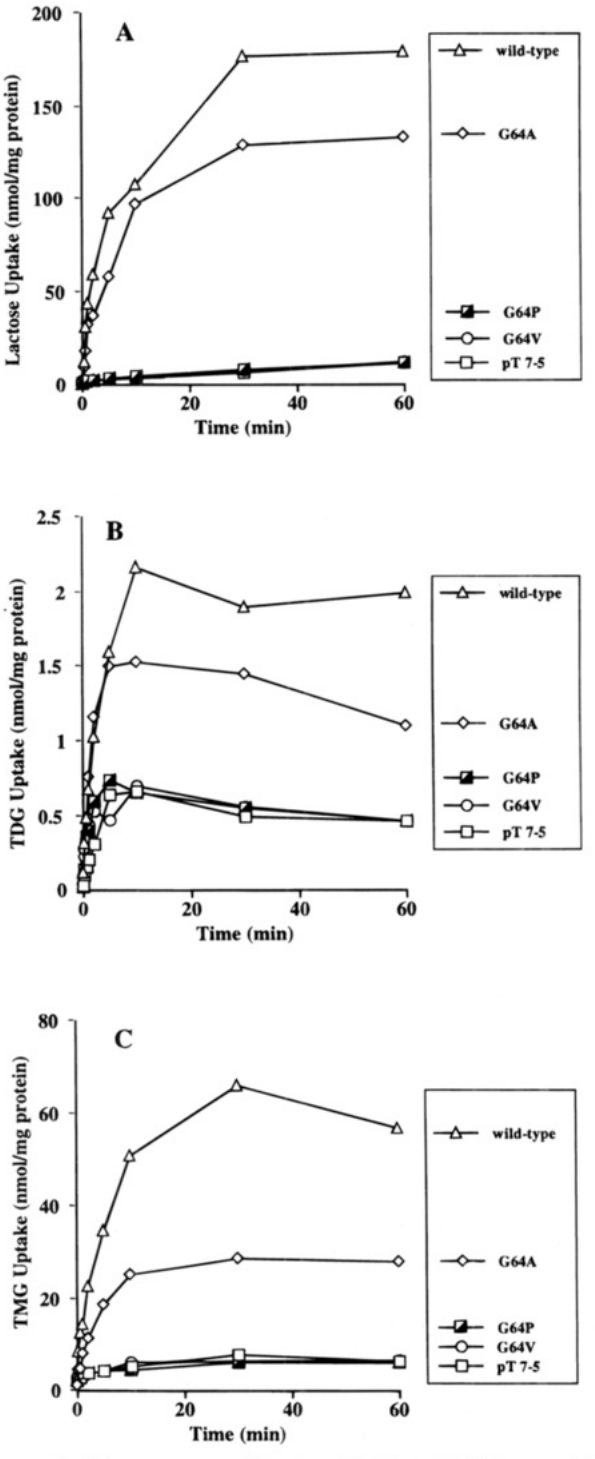

FIGURE 7: Time courses of lactose, TDG or TMG transport by cells expressing Gly-64 mutants in the wild-type background. *E. coli* T184 harboring plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding wild-type permease, or pT7-5 encoding permeases with given amino acid substitutions for Gly-64 were grown and assayed for transport as described in the legend to Figure 2. (A) [^{14}C]Lactose (2.5 Ci/mol) transport was assayed at a final concentration of 0.4 mM; (B) [^{3}H]TDG (100 Ci/mol) transport was assayed at a final concentration of 10 μM; (C) [^{14}C]TMG (2.5 Ci/mol) transport was assayed at a final concentration of 0.4 mM.

reaction center of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985)].

This paper documents a systematic study of Gly residues in lac permease. In an initial round of Cys-scanning mutagenesis on a functional permease mutant devoid of Cys residues, each Gly residue was replaced with Cys, and the expression and activity of the mutants was examined. The great majority of the mutants are expressed normally; only four (G115C, G296C, G380C, G386C) are expressed at significantly low levels as judged by Western blotting. However, Gly-115 mutants with Ala, Val, or Pro at position 115 are expressed normally, and mutants G296C (Sahin-Tóth & Kaback, 1993), G380C, or G386C are inserted into the membrane in a stable form when expressed at a high rate from the T_7 promoter. The results provide a strong indication that conformational flexibility provided by Gly cannot be an important determinant for insertion of the permease into the membrane.

Cys-scanning mutagenesis reveals that out of a total of 36 Gly residues in lac permease, 15 can be replaced with Cys with relatively little or no effect on permease activity, and Gly-402 and Gly-404 can be deleted with the C terminus of the permease with no effect on activity (Roepe et al., 1989; McKenna et al., 1991). Only six residues in this group of mutants are identical residues in cluster 5 of the superfamily [Table 3; see Bockman et al. (1992)], and, with the exception of Gly-110 in putative helix IV, they are located in putative loops (Gly-46, Gly-71, Gly-202, Gly-370, and Gly-377; Figure 1). Cys replacement of nine of the remaining 12 conserved Gly residues (G106C, G121C, G141C, G150C, G159C, G231C, G257C, G262C, and G287C; Figure 1 and Table 3) alters but does not abolish active transport, and mutants G64C, G115C, and G147C are inactive. G262C permease, which exhibits decreased but significant activity, was characterized previously in wild-type permease (Seto-Young et al., 1984; Brooker et al., 1989), and it was postulated that Gly-262 may be close to or part of the sugar recognition domain of the permease. G159C in wild-type permease is a partially uncoupled mutant (Wong et al., 1970; Matos & Wilson, 1994) that exhibits normal exchange and counterflow and may be altered in deprotonation (Herzlinger et al., 1985). In any event, even with mutants G64C, G115C, and G147C which will be discussed, it seems apparent that the alterations in activity observed as a result of Cys or Ala replacement for Gly reflect structural changes that can be tolerated without completely abolishing activity.

Cys replacement for Gly-64, Gly-115, or Gly-147, three conserved residues in cluster 5 (Bockman et al., 1992), completely inactivates C-less permease. However, when Gly-115 or Gly-147 is replaced with Ala (a strong "α-former"), Val (a strong "β-former") or Pro (a strong "α-breaker") (Chou & Fasman, 1974; Chakrabartty et al., 1991; Blaber et al., 1993), G115A or G147A permease exhibits high activity and replacement with Val or Pro yields permease with little or no activity. Gly allows more backbone flexibility than all other residues, and Ala with a methyl group as a side chain does not allow the same degree of flexibility. On the other hand, Ala is small and allows tight packing between helices. It is also relevant that Hecht et al. (1986) demonstrated that replacing Gly with Ala in the lambda repressor increases the stability of the folded protein probably by lowering the entropy of the unfolded state. In brief, therefore, although there is no clear explanation for the observation that Ala is a relatively innocuous replacement for Gly-115 or Gly-147 in lac permease, the most likely requirement at these positions is a small side chain.

Gly-64 is part of a conserved motif (Henderson, 1990; Griffith et al., 1992) that has the following composition in lac permease: G_{64}-L-L-S-D-K-L-G_{71}-R-K. Replacement of Gly-64 in C-less permease with Ala, Val, or Pro inactivates completely. Interestingly, Cys replacement of Gly-71 which is part of the same motif results in permease with wild-type transport activity. Since C-less permease contains eight mutations, each replacement mutant for Gly-64 was cloned into wild-type permease. Clearly, the G64A mutation in the wild-type background exhibits high transport activity with respect to lactose, TDG, and TMG, but no activity is observed with mutant G64V or G64P. Therefore, even Gly-64 does not play an obligatory role in the mechanism of action of the permease, and it appears to be the size of the side chain at this position rather than conformational flexibility that is important for function.

The conserved motif 'GXXXXRXGRR' between hydrophobic domains 2 and 3 has been analyzed in the tetracycline antiporter of *E. coli* (TetA) by site-directed mutagenesis (Yamaguchi et al., 1992) and in lac permease by insertional mutagenesis (McKenna et al., 1992). With the TetA protein, replacement of Gly-62 with Val, Leu, or Asn inactivates completely, and G62A exhibits less than 5% of wild-type activity. On the other hand, replacement of Gly-69 with Ala or Ser yields a TetA protein with up to two-thirds of wild-type activity. With lac permease, insertion of either two or six contiguous His residues into this cytoplasmic loop causes marked inhibition of activity. Although the G64A mutation in wild-type permease does not drastically impair activity, the results presented here for Gly-64 and Gly-71 in lac permease are in general agreement with the previous observations and support the contention that the conserved motif is functionally important. Similarly, the results are consistent with the findings of Loo and Clarke (1994), who demonstrated that Val substitution for the Gly residues in the transmembrane domains of P-glycoprotein inactivates, while Ala replacements yield mutants with significant activity.

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REFERENCES

Aurora, R., Srinivasan, R., & Rose, G. D. (1994) *Science 264*, 1126.

Blaber, M., Zhang, X., & Matthews, B. W. (1993) *Science 260*, 1637.

Bockmann, J., Heuel, H., & Lengler, J. W. (1992) *Mol. Gen. Genet. 235*, 22.

Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459.

Brooker R. J., & Wilson T. H. (1986) *J. Biol. Chem. 261*, 11765.

Brooker, R. J., Myster, S. H., & Wilson, T. H. (1989) *J. Biol. Chem. 264*, 8135.

Büchel, D. E., Groneborn, B., & Müller-Hill, B. (1980) *Nature 283*, 541.

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672.

Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486.

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.

Chakrabartty, A., Schellman, J. A., & Baldwin, R. L. (1991) *Nature 351*, 586.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222.

Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291.

Cowan, S. W., Schirmer, T., Rummer. G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., & Rosenbusch, J. P. (1992) *Nature 358*, 727.

de Cock, H., Quaedvlieg, N., Bosch, D., Scholten, M., & Tommassen, J. (1991) *FEBS Lett. 279*, 285.

Deisenhofer, J., Epp, O., Miki, R., Huber, R., & Michel, H. (1985) *Nature 318*, 618.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993a) *Biochemistry 32*, 3139.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 12644.

Engelman, D. M., Henderson, R., McLachlan, A. D., & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2023.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31.

Fox, C. F., & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 891.

Franco, P. J., & Brooker, R. J. (1994) *J. Biol. Chem. 269*, 7379.

Frillingos, S., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Biochemistry 33*, 8074.

Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A., & Henderson, P. J. (1992) *Curr. Opin. Cell Biol. 4*, 684.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232.

Hecht, M., Sturtevant, J., & Sauer, R. (1986) *Proteins 1*, 43.

Henderson, P. J. F. (1990) *J. Bioenerg. Biomembr. 22*, 525.

Henderson, P. J. F. (1991) *Curr. Opin. Struct. Biol. 1*, 590.

Henderson, P. J. F., & Unwin, P. N. T. (1975) *Nature 257*, 28.

Henry, G. D., & Sykes, B. D. (1990) *Biochem. Cell. Biol. 68*, 318.

Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273.

Jung, H., Jung, K., & Kaback, H. R. (1994) *Biochemistry 33*, 12160.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95.

Kaback, H. R. (1989) *Harvey Lectures 83*, 77.

Kaback, H. R. (1992) *International Review of Cytology 137A* (Jeon, K. W., & Friedlander, M., Eds.) pp 97−125, Academic Press, Inc., New York.

Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G. (1993) *J. Bioenerg. Biomembr. 25*, 627.

Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Zen, K. (1994) *J. Exp. Biol.* (in press).

Komiya, H., Yeates, T. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012.

Laemmli, U. K. (1970) *Nature 227*, 680.

Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B., Dempsey, C. E., & Engelmann, D. M. (1992) *J. Biol. Chem. 267*, 7683.

Li, S.-C., & Deber, C. M. (1992a) *FEBS Lett. 311*, 217.

Li, S.-C., & Deber, C. M. (1992b) *Int. J. Pept. Res. 40*, 243.

Lolkema, J., Püttner, I. B., & Kaback, H. R. (1988) *Biochemistry 27*, 8307.

Loo, T. W., & Clarke, D. M. (1994) *J. Biol. Chem. 269*, 7243.

Luy, P. C., Liff, M. I., Marky, L. A., & Kallenbach, N. R. (1990) *Science 250*, 669.

Marger, M. D., & Saier, M. H. (1993) *Trends Biochem. Sci. 18*, 13

Maiden, M. C. J., Davis, E. O., Baldwin, S. A., Moore, D. C. M., & Henderson, P. J. F. (1987) *Nature 325*, 641.

Matos, M. E., & Wilson, T. H. (1994) *Biochem. Biophys. Res. Commun. 200*, 268.

Matzke, E. A., Stephenson, L. J., & Brooker, R. J. (1992) *J. Biol. Chem. 267*, 19095.

McKenna, E., Hardy, D., Pastore, J. C., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2969.

McKenna, E., Hardy, D., & Kaback, H. R. (1992) *J. Biol. Chem. 267*, 6474.

Menezes, M. E.; Roepe, P. D., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1638.

Menick, D. R., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1985) *Biochem. Biophys. Res. Commun. 132*, 162.

Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H., & Kaback, H. R. (1987a) *Biochemistry 26*, 1132.

Menick, D. R., Carrasco, N., Antes, L. M., Patel, L., & Kaback, H. K. (1987b) *Biochemistry 26*, 6638.

Merutka, G., Lipton, W., Shalongo, W., Park, S.-H., & Stellwagen, E. (1990) *Biochemistry 29*, 7511.

Neuhaus, J. M., Soppa, J., Wright, J. K., Reide, I., Blocker, H., Frank, R., & Overath, P. (1985) *FEBS Lett. 185*, 83

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804.

O'Neil, K. T., & DeGrado, W. F. (1990) *Science 250*, 646.

Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.

Püttner, I. B., & Kaback, H. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1467.

Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483.

Püttner, I. B., Sarkar, H. K., Padan, E., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry 28*, 2525.

Richardson J., & Richardson, D. (1988a) in *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G. D., Ed.) pp 1−98, Plenum Press, New York and London.

Richardson, J., & Richardson, D. (1988b) *Science 240*, 1648.

Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 6127.

Roepe, P. D., Zbar, R. I. S., Sarkar, H. K., & Kaback, H. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3992.

Sahin-Tóth, M., & Kaback, H. R. (1993) *Protein Sci. 2*, 1024.

Sahin-Tóth, M., Lawrence, M. C., & Kaback, H. R. (1994a) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Sahin-Tóth, M., Persson, B., Schwieger, J., Cohan, M., & Kaback, H. R. (1994b) *Protein Sci. 3*, 240.

Sahin-Tóth, M., Frillingos, S., Bibi, E., Gonzalez, A., & Kaback, H. R. (1995) *Protein Sci.* (in press).

Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.

Sarkar, H. K., Menick, D. R., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1986) *J. Biol. Chem. 261*, 8914.

Seto-Young, D.; Bedu, S., & Wilson, T. H. (1984) *J. Membr. Biol. 79*, 185.

Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074.

Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G., & Overath, P. (1978) *Mol. Gen. Genet. 159*, 239.

Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223.

Trumble, W. R., Viitanen, P. V., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1984) *Biochem. Biophys. Res. Commun. 119*, 860.

Ujwal, M. L., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Membr. Mol. Biol. 1*, 9.

van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991a) *Biochemistry 30*, 9595.

van Iwaarden, P. R., Driessen, A. J. M., Menick, D. R., & Kaback, H. R. (1991b) *J. Biol. Chem. 266*, 15688.

Viitanen, P. V., Menick, D. R., Sarkar, H. K., Trumble, W. R., & Kaback, H. R. (1985) *Biochemistry 24*, 7628.

Viitanen, P. V., Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 429.

Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., & Schultz, G. E. (1991) *Science 254*, 1627.

Wickner, W. (1988) *Biochemistry 27*, 1081.

Wong, P. T. S., Kashket, E. R., Wilson, T. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 63.

Wu, J., & Kaback, H. R. (1994) *Biochemistry* (in press).

Yamaguchi, A., Someya, Y., & Sawai, T. (1992) *J. Biol. Chem. 267*, 19155.

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