JOURNAL OF BACTERIOLOGY, Aug. 1992, p. 5436–5441
0021-9193/92/165436-06$02.00/0

Vol. 174, No. 16

Amino Acid Substitution in the Lactose Carrier Protein with the Use of Amber Suppressors

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Received 9 March 1992/Accepted 12 June 1992

Five *lacY* mutants with amber stop codons at known positions were each placed into 12 different suppressor strains. The 60 amino acid substitutions obtained in this manner were tested for growth on lactose-minimal medium plates and for transport of lactose, melibiose, and thiomethylgalactoside. Most of the amino acid substitutions in the regions of the putative loops (between transmembrane α helices) resulted in a reasonable growth rate on lactose with moderate-to-good transport activity. In one strain (glycine substituted for Trp-10), abnormal sugar recognition was found. The substitution of proline for Trp-33 (in the region of the first α helix) showed no activity, while four additional substitutions (lysine, leucine, cysteine, and glutamic acid) showed low activity. Altered sugar specificity was observed when Trp-33 was replaced by serine, glutamine, tyrosine, alanine, histidine, or phenylalanine. It is concluded that Trp-33 may be involved directly or indirectly in sugar recognition.

In recent years, it has been possible to study the correlation between structure at the amino acid level and the function of membrane transport proteins. At present, there is a great deal of data available on specific mutants of the lactose carrier of *Escherichia coli*. More than 150 mutants have been isolated and studied for various aspects of transport. Two general techniques have been used to generate these mutants. The first of these has been the isolation of spontaneous mutants by using a variety of special selection techniques (1–3, 16). The second method is that of site-directed mutagenesis (11, 17, 22) or cassette mutagenesis (9). Mutants derived from these procedures have been extremely useful in determining amino acid residues which are essential for normal transport function and may be involved in either sugar recognition or coupling of cation movement to sugar transport (for reviews, see references 1 and 22).

A useful variation on the point mutagenesis theme involves the use of natural as well as synthetic amber suppressor strains (13, 18–20) to extend the phenotypic range that can be made available for study as a consequence of a single mutation (amber) occurring at a known locus. It is now possible to insert 12 or more different amino acids into the position of a single amber stop codon. Here we describe the results of a study in which five *lacY* amber mutants from the collection of Hobson et al. (10) were cloned, sequenced, and functionally expressed in 12 different suppressor strains. Relatively normal physiological properties were observed for many of the 60 phenotypic variants, but a few, especially those involving substitutions of Trp-33, led to abnormalities with respect to sugar recognition.

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MATERIALS AND METHODS

Bacterial strains. The five *lacY*(Am) mutations (Table 1) are from the collection of Hobson et al. (10). The *lac* genes are on an F factor [F′ *lacI*^{q}*Z*$^+$*Y*(Am)]. Each F′ was transferred by conjugation to the suppressor strains, which are Δ(*lac-proAB*) *metB rif*. The mated mixture was spread on glucose-minimal medium plates with methionine, rifampin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The donor was selected by the antibiotic and the recipient-required proline. Blue clones were picked, purified by restreaking, and then tested for growth on lactose (0.2%)-minimal medium plates with methionine and the antibiotic.

Five different natural suppressors were used in this study (Table 2). Su5 contains the *uar* mutation described by Ryden and Isaksson (23). Seven synthetic suppressors were made available from the laboratory of Jeffrey Miller. The plasmid (pGF1B1) containing the suppressor gene was placed into XAC [Δ(*lac-pro*) *metB argE*(Am) *rif*]. The normal *lacY* gene on an F factor was placed into each of the suppressor strains, and the transport activity of the mutants was expressed as a percentage of the normal activity in the same suppressor strain.

Cloning the *lacY* gene. The polymerase chain reaction technique was utilized to obtain the *lacY* gene from the F factor for cloning. One primer (AAGTCATCTGAATTC CATTACCAGTTGGTCTGGTGTC) was complementary to the antisense strand in the latter portion of the *lacZ* gene and also contained an *Eco*RI site. The second primer (AAGT CATCTAAGCTTAAGCGACTTCATTCACCTGAC) was complementary to the sense strand at the C-terminal end of the *lacY* gene and also contained a *Hin*dIII restriction site. F factor DNA was isolated by the method of Wilson (24). This DNA was incubated with the two primers by the polymerase chain reaction technique. Each cycle consisted of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Thirty cycles were used. The product was treated in the Qiagen (Studio City, Calif.) column to remove nucleotides and primers, etc. The DNA was then cut with *Eco*RI and *Hin*dIII. Following treatment with phenol and chloroform, the *lacY* DNA was

TABLE 1. *lacY*(Am) mutants

Strain	Mutation	Relevant genotype
DP90Y$^+$	None	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*$^+$ *proB*$^+$
AA26	Trp-10→Amb	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*(Am) *pro*B$^+$
NE4	Trp-33→Amb	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*(Am) *pro*B$^+$
AG43	Gln-219→Amb	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*(Am) *pro*B$^+$
AA24	Gln-256→Amb	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*(Am) *pro*B$^+$
MAB19	Gln-379→Amb	Δ(*lac-pro*) F′ *lacI*^{q}*Z*$^+$*Y*(Am) *pro*B$^+$

ligated into the plasmid vector pKK223-3 (Pharmacia), which had been previously treated with *Eco*RI and *Hin*dIII. TG1, lacking its F [Δ(*lac-pro*) *supE*], was transformed with the ligated mixture and plated on melibiose-MacConkey agar plates with 10 mM Li^+ (to inactivate the chromosomal melibiose carrier) and ampicillin. The *lacY*(Am) of the plasmid was suppressed by Su2 of TG1, and the lactose carrier accumulated melibiose, which induced the α-galactosidase, so that the cells fermented melibiose and the clones were red on melibiose-MacConkey agar plates.

Double-stranded plasmid DNA was extracted from cells and purified by the Qiagen method according to the directions of the manufacturer. The entire *lacY* gene was sequenced by using T7 polymerase (Sequenase) from the United States Biochemical Corporation. Appropriately spaced oligonucleotides complementary to the *lacY* coding strand were used to synthesize the second strand, which was labeled with α-^{35}S-dATP (>600 Ci/mmol). In each mutant DNA, an amber mutation was found as the sole alteration (Table 1).

Sugar transport assay. Cells were grown overnight in LB medium containing the appropriate antibiotic. The next morning, the cells were diluted 50-fold in the same medium plus 0.5 mM isopropyl-β-D-thiogalactopyranside (IPTG) and grown at 37°C for three or four doublings. Cells were harvested by centrifugation and washed twice with medium 63 (5). The cells were resuspended in the same buffer and used at a final concentration of 2×10^9 cells per ml. Transport assays were carried out at 25°C in medium 63 (pH 7.0). Washed cells were incubated in buffer with [^{14}C]lactose (0.1 mM, 0.1 μCi/ml), [^{14}C]thiomethylgalactoside ([^{14}C]TMG; 0.1 mM, 0.1 μCi/ml), or [^{3}H]melibiose (0.1 mM, 0.2 μCi/ml). Samples were periodically removed and vacuum filtered through 0.65-μm-pore-size filters (Sartorius). After being washed with 5 ml of buffer, the filters were dissolved in 4 ml of Liquiscint (National Diagnostics), and the radioactivity was counted. The initial rates of transport were obtained from the 30-s points, and these data were expressed as a percentage of the normal activity (the normal *lacY* gene transport activity in the corresponding suppressor strain).

Immunochemical assay. The transport protein was assayed immunologically with the use of monoclonal antibody 4A10R (4). This antibody reacts with the carboxyl terminus of the lactose carrier molecule plus one or more additional cytoplasmically disposed epitopes (8). The membrane protein was assayed by the immunoblot method of Lolkema et al. (15).

TABLE 2. Amber suppressor strains

Suppressor	Amino acid inserted	Efficiency[a] (%)
Natural[b]		
Sul (*supD*)	Serine	6–54
Su2 (*supE*)	Glutamine	41–61
Su3 (*supF*)	Tyrosine	11–100
Su5 *uar* (*supG*)	Lysine	6–30
Su6 (*supP*)	Leucine	30–100
Synthetic[c]		
pGFIB:Ala	Alanine	8–83
pGFIB:Cys	Cysteine	17–51
pGFIB:Glu	Glutamic acid (85%), glutamine (15%)	8–100
pGFIB:Gly	Glycine	39–67
pGFIB:His	Histidine	16–100
pGFIB:Phe	Phenylalanine	48–100
pGFIB:Pro	Proline	9–60

[a] Data for percent efficiency was taken from the work of Kleina and Miller (14).
[b] The natural suppressor strains are derived from XAC [Δ(*lac-proB*) *metB rif*].
[c] The synthetic suppressor genes are on the pGFIB1 plasmid (Amp^r) and were placed in strain XAC.

RESULTS

The F factor containing the *lacY*(Am) mutation was placed into each of 12 suppressor strains by conjugation. After isolation of the conjugant on glucose-minimal medium plates (plus rifampin and X-Gal), the cells were streaked onto lactose (0.2%)-minimal medium plates (plus rifampin). The size of each clone obtained on the lactose plates is given in Table 3. Of the 60 amino acid substitutions studied, 33 of the cells grew to 50% or more of the size of the cell containing the normal Y^+ gene and 50 grew to at least 25% of normal. The growth of 10 cells was less than 25% of normal. Three cells (Lys or Pro substitution for Trp-33 and Ser substitution for Gln-379) failed to grow. In the case of Pro substituted for Trp-33, transport of each of the three sugars was zero (see Fig. 2). In the case of Lys substituted for Trp-33, the transport of radioactive lactose was 2% of normal. In these two strains, the possibility that the low carrier activity was due to low efficiency of suppression or to the failure of the carrier to be inserted into the membrane could not be excluded. Ser substituted for Gln-379 will be discussed in a later section.

The efficiency of suppression (e.g., the percentage of protein molecules containing the amino acid substitution) varies with the different suppressors and with various amber mutations (Table 2). In general, Su1 (Ser), Su2 (Gln), and Su6 (Leu) give high efficiency, while that of Su5 (Lys) is considerably lower. The synthetic suppressors give variable efficiency. It has been found that when the amber codon (TAG) is followed by A or G, efficiency is greater than when the codon is followed by T or C (14). Two of the amber mutations studied in this work (NE4 and AG43) are followed by C and thus are expected to show somewhat lower expression than the other mutations (AA26, AA24, and MAB19) whose amber codons are followed by A and G.

An attempt was made to assay the membrane transport protein immunologically in some of the strains and correlate this result with transport activity. The antibody used for this purpose was a monoclonal antibody that recognizes several different epitopes (8). Table 4 shows that in several cases the protein assayed immunologically gave values with the same order of magnitude as the values for transport activity. In several other cases (such as the Glu substitution for Trp-10), the activity of immunoreactive material was far less than the transport activity. Apparently in these latter cases, the amino acid substitution altered the conformation of the protein such that the antibody binding was reduced without

TABLE 3. Growth of mutants on lactose-minimal plates (for 48 h)

Amino acid substitution	Suppressor	Colony diam (mm)					
		Y^+	Trp-10 → Amb	Trp-33 → Amb	Gln-219 → Amb	Gln-256 → Amb	Gln-379 → Amb
Ser	Su1	2.0	1.5	0.2	0.3	1.5	0
Gln	Su2	2.0	1.3	0.2	0.2	1.5	1.5
Tyr	Su3	2.0	2.0	1.8	1.5	2.0	1.5
Lys	Su5RF	1.8	0.2	0	0.5	0.5	0.8
Leu	Su6	1.5	2.0	0.1	0.8	1.0	1.2
Ala	pGFIB:Ala	2.0	1.0	1.2	1.5	0.8	0.3
Cys	pGFIB:Cys	2.5	1.0	0.5	1.0	0.8	0.3
Glu	pGFIB:Glu	2.5	1.5	1.5	1.5	2.0	1.0
Gly	pGFIB:Gly	2.0	0.6	0.5	1.2	2.0	1.0
His	pGFIB:His	2.0	0.9	0.8	1.2	1.5	0.8
Phe	pGFIB:Phe	2.0	0.9	1.0	1.2	1.5	0.8
Pro	pGFIB:Pro	2.0	1.2	0	0.8	1.5	0.5

affecting membrane transport. Thus, the low levels of immunologically reactive material cannot be taken as an accurate measure of carrier protein in the membrane.

In the cases of moderate-to-good growth on lactose plates and good transport activity, it is concluded that suppression efficiency is adequate, the protein is inserted into the membrane, and the carrier is active. This was the case for the majority of the cases studied. On the other hand, when growth and transport are poor (frequently associated with low levels of immunoreactive protein), it is not clear whether suppression efficiency is low, insertion into the membrane is poor, activity of the carrier is reduced, or a combination of all three factors is present. It was therefore decided to study sugar specificity since this aspect of the carrier is not affected by these factors.

TABLE 4. Lactose carrier (assayed with antibody) compared with lactose transport

Position of mutation	Amino acid substitution	Lactose transport (%)	Protein in the membrane by antibody assay (%)
Trp-10 → Amb	Ala	61	18
	Gln	58	21
	Glu	34	0
	Gly	130	128
	Lys	5	1
	Phe	30	25
	Tyr	46	60
Trp-33 → Amb	Ala	26	3
	Gln	15	2
	Glu	6	10
	Gly	37	7
	Lys	2	4
	Phe	18	11
	Ser	34	21
	Tyr	69	14
Gln-379 → Amb	Ala	40	9
	Gly	56	45
	Phe	18	11
Gln-219 → Amb	Ala	2	0
	Gln	14	14
	Gly	60	19
	Phe	15	14
Gln-256 → Amb	Ala	22	6
	Gly	50	79

Experiments were therefore carried out to investigate the possibility of altered sugar specificity due to amino acid substitutions in the transport protein. For each of the 60 substitutions, the transport was tested for three different sugars: lactose, TMG, and melibiose. TMG and melibiose were nonmetabolizable by these cells so that accumulation of sugar was observed when the normal carrier was present. Since these cells contained β-galactosidase, lactose taken up was metabolized and the radioactive products were measured. If the relative transport rates (percentage of normal rates) for the three sugars were the same, a normal sugar recognition was assumed, but if the relative transport rate of one sugar was very different from that of the other two sugars, it was inferred that there was an abnormal recognition site for sugar or a defect in accumulation. Figures 1 to 5 show the transport rates of three sugars for the 60 mutants. The data are expressed as the percentage of the transport rate of the cell with normal carrier in that particular suppressor strain.

The data show that significant transport of the three sugars was observed in most cases. In 36 of 60 substitutions, the lactose transport was greater than 20% of the normal value. In many cases, the relative transport rates for the three sugars were similar. There were, however, cases in which altered sugar recognition was evident. In several of the

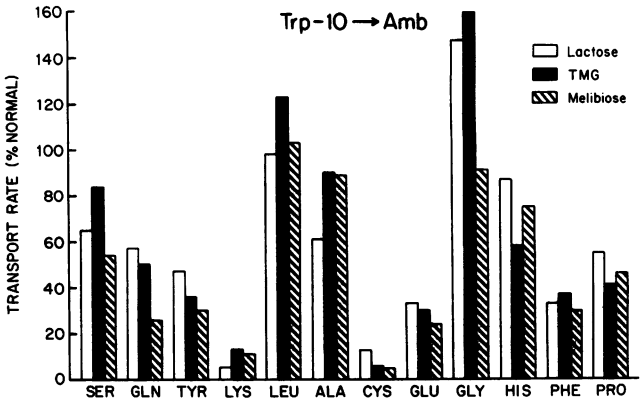

FIG. 1. Transport of lactose, TMG, and melibiose by suppressor strains containing F' Trp-10 → Amb.

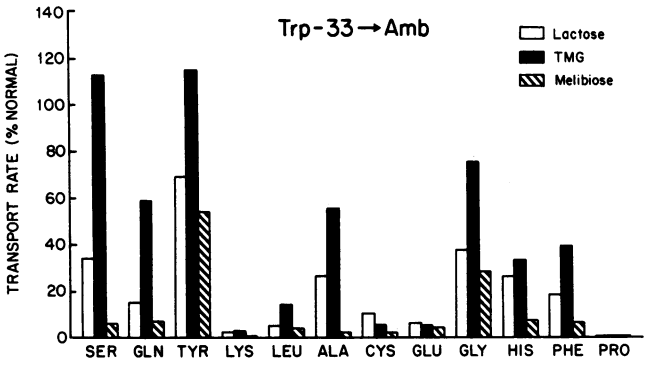

FIG. 2. Transport of lactose, TMG, and melibiose by suppressor strains containing F' Trp-33 → Amb.

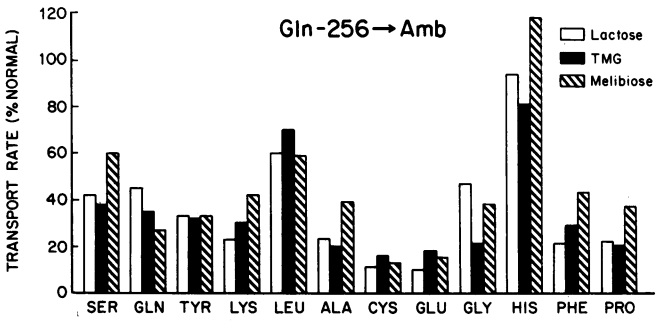

FIG. 4. Transport of lactose, TMG, and melibiose by suppressor strains containing F' Gln-256 → Amb.

substitutions in NE4 (Trp-33 → Amb), the TMG transport was relatively greater than the transport of the other two sugars (Fig. 2). In several cases (Ser, Gln, Ala, His, and Phe substitutions for Trp-33), the melibiose transport was particularly low (Fig. 2).

This question of sugar recognition was studied in more detail with four strains in which the transport of six different sugars was compared. Two of these sugars (lactose and ONPG [*o*-nitrophenyl-β-D-galactopyranoside]) are split by β-galactosidase and are not accumulated. The remaining four sugars are not metabolized and accumulate within the cell. Table 5 shows that in the case of Gln-219 replaced by Ser, the relative rates of transport of the six sugars were approximately equal. On the other hand, the other three substitutions showed a very different pattern. In one case (Ser substituted for Trp-33), the TMG transport rate was 113% of normal, while melibiose uptake was 6% of normal. Trp-33 replaced by Gly showed a TMG transport rate of 75% of normal and an α-PNPG (*p*-nitrophenyl-α-D-galactopyranoside) uptake of 14% of normal. In the case of the Gly substitution for Trp-10, α-PNPG and TMG transport rates were each 160% of normal, while that of melibiose was 91% of normal. These three mutants clearly show altered sugar recognition.

Gln-379 replaced by Ser showed several unusual properties. Although this cell failed to grow on lactose-minimal medium plates (Table 3), washed cells showed [^{14}C]lactose uptake which was 50% of that of the normal cell. The suppressor strain harboring the mutant plasmid (Su1/pMAB19) accumulated lactose 22-fold compared with the norm of 40-fold. In addition, Ser substituted for Gln-379 grew slowly on lactose-MacConkey agar plates, a unique property among all of the mutants. Further evidence for some type of lactose toxicity is the growth inhibition of Ser substituted for Gln-379 by lactose when cells are growing in rich media. In one experiment in which cells grew in LB plus lactose, β-galactosidase was found in the external medium, suggesting lysis of some cells. On the other hand, cells grew well in the presence of melibiose and [^{3}H]melibiose transport was about 50% of normal. The possibility was considered that lactose stimulated a proton "leak" in the manner described by Brooker (2) for the double mutant Val 177/Asn 319. Unlike the Brooker mutant, Su1/pMAB19 failed to show a greater sugar-induced reduction in proton motive force than the parent and failed to show sugar-induced proton leak (proton entry into anaerobic cells following an oxygen pulse).

DISCUSSION

In four of five amber mutations studied, the location of the altered amino acid was presumed to be in one of the loops between transmembrane segments (Fig. 6). For NE4 (Trp-33 → Amb), the tryptophan is believed to be located within the first membrane-spanning α helix. One might predict that the function of the carrier would be less sensitive to amino acid substitutions in the loops than similar changes in membrane-spanning segments. In general, this prediction was supported by the data presented. Amino acid substitutions in Gln-256, located in a periplasmic loop, resulted in the least change in lactose growth function and showed relatively little change in transport of the three sugars. Substitutions at positions Trp-10, Gln-219, and Gln-379 generally resulted in modest-to-good transport activity.

There were two exceptions to the generalization that mutations in the loops gave relatively little change in activ-

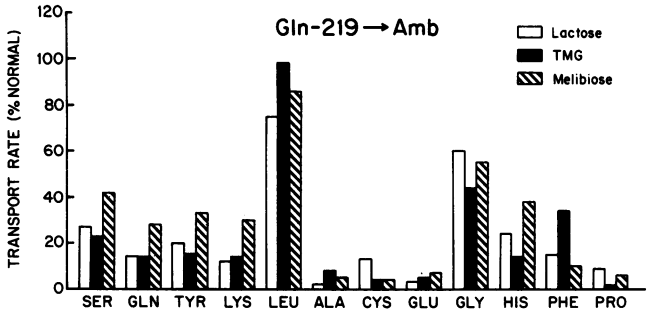

FIG. 3. Transport of lactose, TMG, and melibiose by suppressor strains containing F' Gln-219 → Amb.

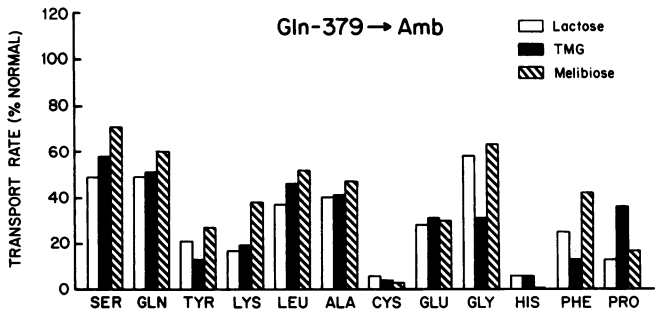

FIG. 5. Transport of lactose, TMG, and melibiose by suppressor strains containing F' Gln-379 → Amb.

TABLE 5. Sugar specificity in four mutants[a]

Sugar	% of normal transport activity			
	Ser/Gln-219 → Amb	Ser/Trp-33 → Amb	Gly/Trp-33 → Amb	Gly/Trp-10 → Amb
Lactose	27	34	37	148
TMG	28	113	75	160
ONPG	35	32	39	109
IPTG	23	48	28	126
Melibiose	41	6	28	91
α-PNPG	35	32	14	160

[a] The F factors [*lacI*^{q}*Z*$^+$*Y*(Am) and *lacI*^{q}*Z*$^+$*Y*$^+$] were placed into each suppressor strain. The transport data are expressed as the percentage of normal transport activity in the same suppressor strain.

ity. Gly substituted for Trp-10 showed two abnormalities. It was the only case in which there was an elevated level of protein as assayed with the antibody (120% of normal). In addition, there was altered sugar specificity. The transport of both TMG and α-PNPG was 160% of normal, while melibiose transport was 91% of normal. It had previously been found by Menezes et al. (17) that site-directed mutation of Trp-10 to phenylalanine resulted in lactose transport that was 75% of normal. In this study, Trp-10 → Amb in the phenylalanine suppressor gave 35% of normal lactose transport. This observation is consistent with data on the site-directed mutant if we assume 48% efficiency of suppression.

A second strain with an alteration in an amino acid located in one of the loops that resulted in unusual properties was Ser substituted for Gln-379. This cell failed to grow on lactose-minimal medium plates in spite of the fact that [^{14}C]lactose uptake in washed cells was about 50% of normal. Other substitutions with this level of lactose transport showed moderate-to-good growth on lactose plates. In addition, Ser substituted for Gln-379 grew poorly on lactose-MacConkey agar plates, a growth medium containing amino acids and thus not requiring lactose metabolism for growth. An abnormally high level of lactose accumulation was excluded as the cause of the lactose toxicity. Cells harboring the mutant plasmid (Sul/pMAB19) accumulated lactose 22-fold compared with the norm of 40-fold. The possibility was considered that lactose stimulated a proton leak in the manner described by Brooker (2) for the double mutant Val-177/Asn-319. No evidence could be obtained for a sugar-induced proton leak. The explanation for the inhibition of growth by lactose is not, at present, understood.

Several mutations of Trp-33 showed abnormal sugar recognition. Unlike other strains in this study, substitution of Trp-33 by Ser, Gln, Tyr, Ala, Gly, and Phe showed proportionally higher transport rates for TMG than for the other two sugars. For Trp-33 replaced by Ser, Gln, Ala, His, and Phe, the melibiose transport was especially low. In two of these mutants, six different sugars were tested. Table 5 shows that Ser substituted for Trp-33 transported TMG at a rate of 113% of normal, melibiose 6% of normal, and the other sugars at an intermediate rate. Trp-33 replaced by Gly transported TMG at a rate of 75% of normal and α-PNPG at 14% of normal. In all of these substitutions of Trp-33, a distinct change in sugar recognition was observed. Menezes et al. (17) found that changing Trp-33 to Phe had little effect on lactose transport. The 20% lactose transport observed in this study for the phenylalanine substitution is probably due to a low level of suppression. The altered relative rates of transport by several Trp-33 mutants suggest that this amino acid may be involved directly or indirectly in sugar recognition. Mutations of Gly-24, Tyr-26, and Pro-28 give greatly reduced (6, 16, 21) transport rates, suggesting that several residues in α-helix 1 of the carrier protein are important for function.

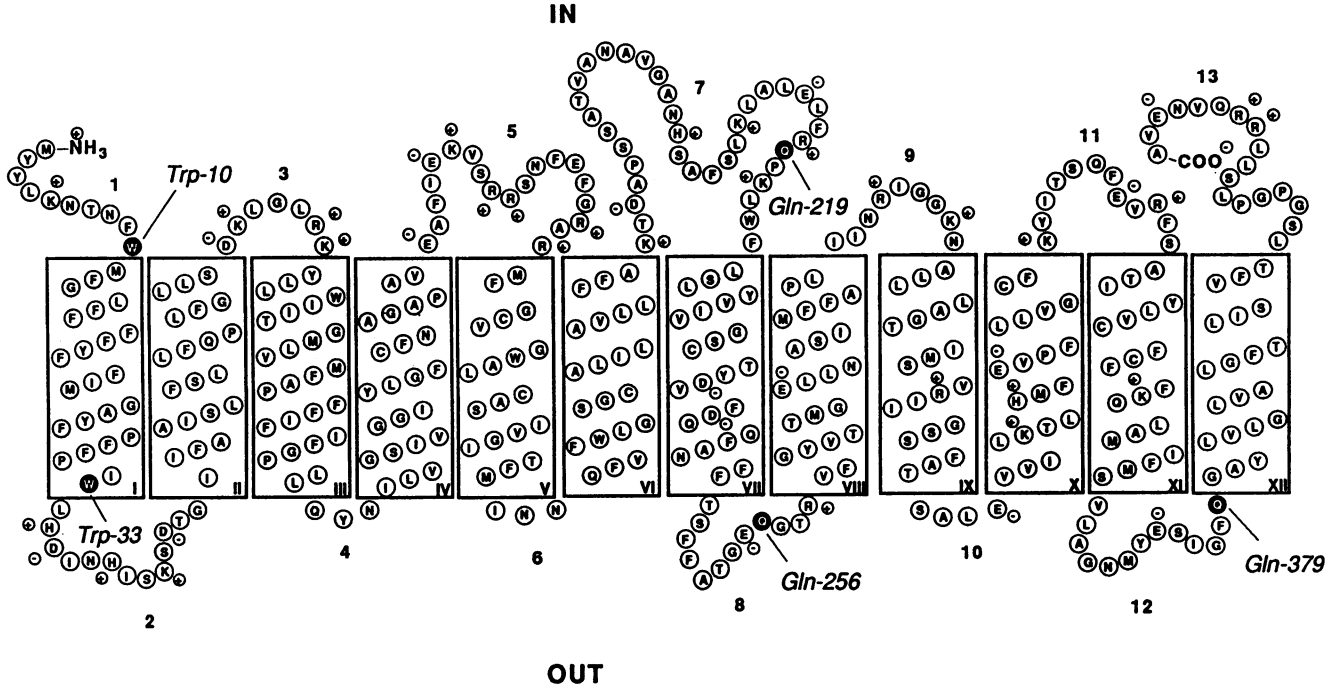

FIG. 6. Positions of amber mutations in a model of the lactose carrier. White letters indicate the position of the amber mutation. The model is that of Foster et al. (7) as modified by King et al. (12).

ACKNOWLEDGMENTS

We thank Beno Müller-Hill for the five amber strains, Jeffrey Miller for the synthetic amber suppressor strains, Ronald Kaback for the monoclonal antibody, and Gerard Leblanc for the [^{3}H]melibiose.

This work was supported by U.S. Public Health Service grant DK 05736 and by National Science Foundation grant DCB 9017255.

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