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Biochemistry

Isolation and nucleotide sequencing of lactose carrier mutants that transport maltose

(lactose permease/membrane protein/substrate specificity mutants)

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ABSTRACT The wild-type lactose carrier of *Escherichia coli* has a poor ability to transport the disaccharide maltose. However, it is possible to select lactose carrier mutants that have an enhanced ability to transport maltose by growing *E. coli* cells on maltose minimal plates in the presence of isopropyl thiogalactoside (an inducer of the *lac* operon). We have utilized this approach to isolate 18 independent lactose permease mutants that transport maltose. The relevant DNA sequences have been determined, and all of the mutations were found to be single base pair changes either at triplet 177 or at triplet 236. The nucleotide changes replace alanine-177 with valine or threonine, or tyrosine-236 with phenylalanine, asparagine, serine, or histidine. Transport experiments indicate that all of the mutants have faster maltose transport compared with the wild-type strain. Position 177 mutants retain the ability to transport galactosides, such as lactose and melibiose, at rates similar to the rate of the wild-type strain. In contrast, the position 236 mutants are markedly defective in the ability to transport galactosides. With regard to secondary structure, alanine-177 and tyrosine-236 are located on adjacent hydrophobic segments of the lactose carrier that are predicted to span the membrane. Thus, the results of this study indicate that the substrate recognition site of the lactose carrier is located within the plane of the lipid bilayer. In addition, a tertiary structure model is proposed that suggests how certain transmembrane segments might be localized relative to one another.

The lactose carrier, located in the *Escherichia coli* cytoplasmic membrane, functions to transport galactosides from the extracellular medium into the cytoplasm (see refs. 1 and 2 for reviews). As proposed by Mitchell (3), galactoside transport via the lactose carrier is coupled to the transport of H^+, thus enabling the cell to utilize the protonmotive force to accumulate galactosides against a concentration gradient. Transport studies with whole cells (4, 5) and membrane vesicles (6–9) strongly support this theory. The lactose carrier has been inserted into proteoliposomes by the octyl glucoside dilution method (10) and the protein has been purified (11). The *lacY* gene, which codes for the lactose carrier, has been cloned on multicopy vectors (12, 13) and the entire gene has been sequenced (14). From the DNA sequence, the lactose carrier is predicted to be composed of 417 amino acid residues and have a molecular weight of 46,504.

For cation–substrate cotransport proteins, little information is available concerning the specific amino acid residues that are involved with substrate recognition and transport. In the case of the lactose carrier, it has long been established that *N*-ethylmaleimide reacts with the protein to inhibit transport activity in a substrate-protectable manner (15). The essential cysteine has been identified by Beyreuther *et al.* (16) to be at position 148. In a more recent study, Trumble *et al.* (17) have utilized the technique of site-specific mutagenesis to change Cys-148 to glycine. This amino acid substitution resulted in a decrease to 1/4th in the initial rate of lactose transport. In addition, Mieschendahl *et al.* (18) screened a collection of *lacY* mutants (19) and found 18 that grew on lactose at 100 mM but not at 5 mM. Seventeen of these "K_m mutants" had mutations that mapped between codons 191 and 360, suggesting that this region of the protein contains the disaccharide recognition site.

A useful approach to investigate the relationship between protein structure and function involves the isolation of strains with mutations that lead to known alterations in substrate specificity. Shuman and Beckwith have described the isolation of lactose carrier mutants that transport the disaccharide maltose (20). In this case, the mutant proteins had acquired the ability to transport a disaccharide that has a poor affinity for the wild-type lactose carrier (21). In the present study, we have utilized this approach to isolate 18 lactose carrier mutants that transport maltose. To identify the nature of the mutants' defects, we have subjected all 18 mutants to DNA sequencing. The results provide important insight as to the location of the substrate recognition site by identifying two amino acid residues that are a part of or very close to that site.

MATERIALS AND METHODS

Reagents. Lactose, melibiose, and maltose were purchased from Sigma. [^{14}C]Lactose and deoxyadenosine 5′-[α-^{32}P]triphosphate were from Amersham. [^{14}C]Maltose and [^{3}H]raffinose were from New England Nuclear. [^{3}H]Melibiose was prepared from [^{3}H]raffinose by the method of Tanaka *et al.* (22). DNA polymerase I (Klenow fragment), isopropyl β-D-thiogalactoside (iPrSGal), and 5-bromo-4-chloro-3-indolyl β-D-galactoside were obtained from Boehringer Mannheim. Restriction enzymes, T4 DNA ligase, phage M13mp8, and other reagents required for DNA sequencing were obtained from New England Biolabs. All other chemicals were analytical grade.

Bacterial Strains and Methods. Bacterial strains and their relevant genotypes are listed in Table 1.

For mating experiments (HS4006 × T184 and HS4006 × MAA23), female cells were grown in YT medium (ref. 23, p. 433) at 37°C with shaking. Male cells were grown under similar conditions without shaking. Equal amounts of male and female cells were mixed and allowed to mate overnight with no shaking. After mating, cells were plated on minimal glucose (1%) plates plus streptomycin (0.1 mg/ml). HS4006 requires proline for growth; T184 requires methionine and threonine for growth; and MAA23 is streptomycin sensitive. Thus, only mated cells (HS4006/F′$I^QZ^-Y^-$ and HS4006/F′$I^QZ^+Y^-$) could grow, since they are streptomycin-resistant (gene on chromosome) and proline independent (proAB$^+$ on F′ factor).

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Abbreviation: iPrSGal, isopropyl β-D-thiogalactoside.

Table 1. Bacterial strains and their relevant genotypes

Strain	Relevant genotype (chromosome/F′/plasmid)	Ref. or source
HS4006	F^- Δ(*lac–pro*)XIII Δ*malB101 rpsL rpoB*/—/—	Shuman and Beckwith (20)
T184	*lacI*$^+$ *lacO*$^+$ *lacZ*$^-$ *lacY*$^-$/*lacI*^{Q} *lacO*$^+$ *lacZ*^{U118} (*lacY*$^+$)/—	Teather *et al.* (13)
HS4006/F′I^{Q}Z$^-$Y$^-$	Δ(*lac–pro*)XIII Δ*malB101 rpsL rpoB*/*lacI*^{Q} *lacO*$^+$ *lacZ*^{U118} (*lacY*$^+$)/—	By mating HS4006 to T184
HS4006/F′I^{Q}Z$^-$Y$^-$/pTE18	Δ(*lac–pro*)XIII Δ*malB101 rpsL rpoB*/*lacI*^{Q} *lacO*$^+$ *lacZ*^{U118} (*lacY*$^+$)/Δ(*lacI*) *lacO*$^+$ Δ(*lacZ*) *lacY*$^+$ Amp^R Tet^R	By transforming HS4006/F′I^{Q}Z$^-$Y$^-$ with the pTE18 plasmid
MAA23	Δ(*lac–pro*)/*lacI*^{Q} *lacO*$^+$ *lacZ*$^+$ *lacY*$^-$/—	Hobson *et al.* (19)
HS4006/F′I^{Q}Z$^+$Y$^-$	Δ(*lac–pro*)XIII Δ*malB101 rpsL rpoB*/*lacI*^{Q} *lacO*$^+$ *lacZ*$^+$ *lacY*$^-$/—	By mating HS4006 to MAA23

Δ before a bracketed symbol indicates a deletion. *lacZ*^{U118} is a polar nonsense mutation that results in a LacZ$^-$ LacY$^-$ phenotype (12). Amp^R, ampicillin-resistant; Tet^R, tetracycline-resistant.

Plasmid DNA, isolated by the NaOH/sodium dodecyl sulfate method (24), was introduced into bacterial strains by the $CaCl_2$ transformation procedure of Mandel and Higa (25).

Growth Media. Plasmid-carrying strains were isolated and identified on rich indicator agar plates (ref. 23, p. 54) containing 1% melibiose (1%), ampicillin (100 μg/ml), and tetracycline (10 μg/ml). For transport assays, cells were grown to midlogarithmic phase at 37°C in YT media containing ampicillin, tetracycline, and 0.5 mM iPrSGal.

For mutant isolation, nonmutagenized individual colonies of HS4006/F′I^{Q}Z$^-$Y$^-$/pTE18 were picked from melibiose indicator agar plates and either restreaked on minimal maltose plates or inoculated into flasks containing 40 ml of liquid minimal maltose medium. To ensure independent mutations, no more than one mutant from each original clone was studied. Minimal maltose plates were composed of minimal medium 63 (ref. 26) with the following additions: agar (1.6 %), maltose (0.4%), 0.1 mM iPrSGal, vitamin B_1 (5 μg/ml), ampicillin (100 μg/ml), and tetracycline (10 μg/ml). Liquid minimal maltose medium was made up in an identical fashion except that no agar was added.

Transport Assays. Intact cells were washed and resuspended in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4. To measure the transport of maltose, lactose, and melibiose, cells were first equilibrated to 25°C in phosphate buffer, pH 7.0. At time zero, the radioactive sugar was added, and at appropriate time intervals, 0.2-ml aliquots containing approximately 10^9 cells were withdrawn and filtered over a membrane filter (pore size = 0.65 μm). The external medium was then washed away with 5–10 ml of phosphate buffer, pH 7.0, by rapid filtration. Radioactivity was determined by liquid scintillation counting using Liquiscint (National Diagnostics, Somerville, NJ). As a control, cells were also incubated with radioactive sugars in the presence of a high-affinity lactose analogue, thiodigalactoside, to block transport via the lactose carrier. The thiodigalactoside control sample was subtracted from the experimental sample. To verify that the observed maltose transport was via the lactose carrier, the parental strain HS4006/F′I^{Q}Z$^-$Y$^-$ lacking a functional *lacY* gene was also assayed for [^{14}C]maltose transport. In this case, the amount of radioactivity incorporated was identical to the amount incorporated in the presence of thiodigalactoside.

Isolation of Segments of the *lacY* Gene. The plasmid pTE18 (13) contains the entire *lacY* gene on a 2300-base-pair (bp) *Eco*RI fragment. To isolate segments of the *lacY* gene in preparation for DNA sequencing, 10- to 15-ml cultures of plasmid-containing cells were grown overnight and plasmid DNA was isolated by the method of Birnboim and Doly (24). Plasmid DNA was subsequently digested with *Eco*RI and subjected to agarose gel electrophoresis (1% agarose, run at 30 V overnight). The DNA bands were stained with ethidium bromide and visualized briefly under long-wavelength UV light. The 2300-bp band containing the *lacY* gene was excised and the DNA was electroeluted by the method of Zassenhaus *et al.* (27). After electroelution, the DNA was precipitated with ethanol, dried, and resuspended in 50–100 μl of TE buffer (10 mM Tris·HCl, pH 7.5/0.1 mM Na_2EDTA). The resuspended 2300-bp fragment was digested with *Sau*3AI or *Hpa* II, and segments of the *lacY* gene were then separated by acrylamide gel electrophoresis (5% acrylamide, run at 40 V overnight). The DNA fragments were visualized with UV light, excised, electroeluted, precipitated with ethanol, and resuspended in 50 μl of TE buffer.

DNA Sequencing. DNA sequencing was performed by the method of Sanger *et al.* (28). Segments of the *lacY* gene were ligated to the *Bam*HI site or *Acc* I site of phage M13mp8 DNA (29). Mutations at triplets 177 and 236 were identified in both strands.

RESULTS

Construction of Bacterial Strains. To isolate, identify, and determine the DNA sequence of lactose carrier mutants that transport maltose, it is necessary to construct a strain of *E. coli* with the following three properties. (*i*) The strain must have undergone a deletion of the *malB* locus so that the uptake of maltose via the normal maltose transport system is blocked (20). (*ii*) The lactose operon must be under the regulatory control of the *lacI* gene (which codes for the *lac* repressor protein) so that lactose permease mutants can be distinguished from other possible mutants (i.e., mutants in other transport systems) by their inducibility in the presence of iPrSGal. (*iii*) The mutants must be obtained on a multicopy plasmid to facilitate DNA sequencing. To satisfy these requirements, an *E. coli* strain, designated HS4006/F′-I^{Q}Z$^-$Y$^-$/pTE18, was constructed. The parental strain, HS4006 (see Table 1), carries a deletion at the *malB* locus that encompasses several of the genes of the maltose transport system. HS4006, however, does possess the enzymes necessary for maltose metabolism. In addition, HS4006 has a deletion of the entire lactose operon. HS4006 was mated to T184 (see Table 1). The resulting strain, HS4006/F′I^{Q}Z$^-$Y$^-$, contains an F′ factor carrying a *lacI* gene (designated *lacI*^{Q}) that leads to overproduction of the *lac* repressor protein (30). Due to a nonsense mutation in the *lacZ* gene, the phenotype is LacZ$^-$ LacY$^-$. Finally, a multicopy plasmid, pTE18 (ref. 13), carrying the wild-type *lacY* gene, was introduced into HS4006/F′I^{Q}Z$^-$Y$^-$ by transformation. This strain, HS4006/F′I^{Q}Z$^-$Y$^-$/pTE18, contains its only functional *lacY* gene on a multicopy plasmid that is under the regulatory control of the *lacI* gene located on the F′ factor.

Isolation of Lactose Carrier Mutants That Transport Maltose. As mentioned previously, the disaccharide maltose is not recognized by the wild-type lactose carrier (21). However, it is possible to isolate lactose carrier mutants that transport maltose by growing cells on minimal maltose under conditions in which the lactose operon is expressed (20). In the present study, two similar mutant isolation procedures were employed. In the first case, individual colonies of

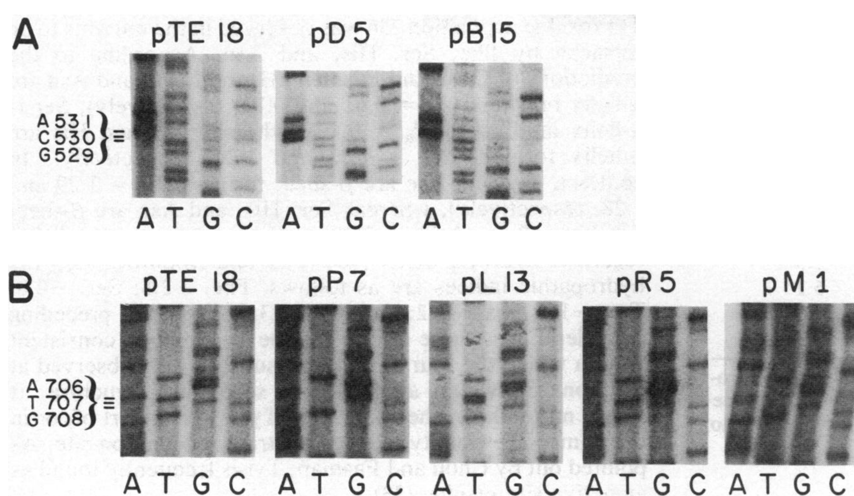

FIG. 1. Autoradiographs of sequencing gels according to Sanger *et al.* (28). (*A*) Antisense strand nucleotides 515–549. (*B*) Sense strand nucleotides 688–723. The wild-type sequence (pTE18) is compared with the sequences from several mutant plasmids: pD5, pB15, pP7, pL13, pR5, and pM1.

HS4006/F′I^{Q}Z$^-$Y$^-$/pTE18 were picked from rich plates and restreaked onto minimal (0.4%) maltose plates containing 0.1 mM iPrSGal, an inducer of the lactose operon. While all cells grew at an extremely slow but detectable rate, much larger "fast-growing" colonies appeared in 5–7 days at 37°C. In general, approximately 1–5 *lacY* mutants were found on each plate. Since 10^7–10^8 bacteria were applied per plate, this suggests a mutation frequency between 10^{-7} and 10^{-8}. However, it must be kept in mind that the wild-type strain is growing at a very slow rate and that mutants continue to appear on maltose plates after several days. Therefore, the preceding frequency is likely to be an underestimate of the true mutation rate. While this is difficult to quantitate accurately, it seems most likely that the mutation frequency is between 10^{-8} and 10^{-9}. In a second method, colonies were picked from rich plates and inoculated into liquid medium containing 0.4% maltose plus 0.1 mM iPrSGal. Growth in liquid medium was allowed to proceed to stationary phase (2–3 days at 37°C). After growth in liquid culture, 0.02–0.2 ml of cells was plated on minimal maltose plates plus iPrSGal. Fast-growers appeared in 2–3 days.

After the isolation of fast-growing mutant colonies, it was necessary to distinguish *lacY* mutants from other possible types of mutants. To accomplish this, mutant colonies were picked and restreaked on minimal maltose plates with and without iPrSGal. *lacY* mutants were identified as those that grew on maltose in the presence of iPrSGal but failed to grow in its absence. In all, 18 independent *lacY* mutants were isolated and saved for further study. It should be mentioned that with the first mutant isolation procedure described above, only a small proportion (1–5%) of the fast-growers required iPrSGal for growth on maltose. In this case, it appeared that the majority of mutants did not have their mutations within the *lacY* gene. In contrast, with the second method, most of the mutant colonies required iPrSGal for growth on maltose minimal plates.

DNA Sequencing on *lacY* Mutants. The main goal of this investigation was to identify amino acids within the protein that are involved with substrate recognition and transport. It seemed reasonable that mutations that alter substrate specificity might do so through amino acid substitutions that are at, or very close to, the substrate recognition site. Therefore, to test this hypothesis, all 18 *lacY* mutants were subjected to DNA sequencing. Quite surprisingly, single base pair substitutions were observed at only two sites, either at triplet 177 or at triplet 236. DNAs from all 18 mutants were sequenced between nucleotides 318 and 844 from the start of the *lacY* gene. Several mutants (pD5, pF1, pM1, and pP7) were sequenced from nucleotide 318 to nucleotide 1314 (this encompasses amino acids 106–417). In addition, the DNAs of two mutants (pD5 and pP7) were sequenced over the entire *lacY* gene in both strands. In all cases, only one base pair change (at triplet 177 or 236) was observed. When restreaked on maltose minimal plates containing 0.1 mM iPrSGal, all mutants (including pD5 and pP7) showed a homogenous population of colonies with no evidence that a second mutation might confer the ability to grow even faster on maltose. Taken together, these results indicate that the ability to transport maltose is due to a single mutation within the *lacY* gene. This conclusion is consistent with the estimated mutation frequency, which was between 10^{-8} and 10^{-9} (also see ref. 20).

The autoradiograph of Fig. 1 shows a short segment of a DNA sequencing run. Fig. 1*A* shows the antisense strand from nucleotide 515 to nucleotide 549, and Fig. 1*B* shows the sense strand from nucleotide 688 to nucleotide 723. The following nucleotide changes can be seen: pD5, G-529 to A; pB15, C-530 to T; pP7, T-707 to G; pL13, A-706 to T; pR5, A-706 to G; and pM1, T707 to A. These substitutions change codon 177 (GCA) to ACA and GUA, and codon 236 (UAC) to UCC, AAC, CAC, and UUC, respectively.

Table 2 summarizes the sequencing data on all 18 mutants. Changing codon 177 (GCA) to GUA and ACA replaces alanine-177 with valine and threonine. The four different nucleotide changes seen at codon 236 replace tyrosine with phenylalanine, serine, histidine, and asparagine. Overall, the sequencing of 18 independent mutations revealed only six different types of amino acid replacements, two at Ala-177 and four at Tyr-236. As can be seen in Table 2, many of the independently isolated mutants were identical. For example, three mutants changed Tyr-236 to Phe and nine mutants changed Ala-177 to Val. Taken together, the results indicate that only a very limited number of amino acid substitutions can confer the ability of the lactose carrier to transport

Table 2. Base changes and deduced amino acid substitutions of the maltose-transport mutants

Plasmids	Total	Nucleotide change	Amino acid substitution
pB15, pE15, pG12, pH2, pI1, pCC2, pDD1, pEE2, pII4	9	G-530 to A	Ala-177 to Val
pD5	1	C-529 to T	Ala-177 to Thr
pE4, pF1, pM1	3	T-707 to A	Tyr-236 to Phe
pL13	1	A-706 to T	Tyr-236 to Asn
pP7	1	T-707 to G	Tyr-236 to Ser
pB14, pR5, pFF1	3	A-706 to G	Tyr-236 to His

Nucleotide changes are in the sense strand.

Table 3. Transport properties of hosts bearing wild-type and mutant plasmids

Plasmid	Transport velocity, nmol/min per mg of protein		
	Maltose	Lactose	Melibiose
pTE18	0.078	8.4	28.0
pB15	0.39	6.1	23.8
pD5	0.37	8.3	22.5
pF1	0.29	<0.5	1.3
pL13	0.092	3.9	5.1
pP7	0.10	0.81	<1.0
pR5	0.20	<0.5	<1.0

For [^{14}C]maltose transport, the designated plasmids were introduced into HS4006/F′I^{Q}Z$^+$Y$^-$ by transformation. For [^{14}C]lactose and [^{3}H]melibiose transport, the plasmids were introduced into MAA23. The substrate concentration was 0.1 mM.

maltose when the mutants are isolated by the methods described.

Transport of Maltose, Lactose, and Melibiose by Wild Type and Mutants. After isolation and DNA sequencing, it was of interest to investigate the transport properties of these mutants. In the experiment of Table 3, whole-cell transport measurements were conducted with three different disaccharides: maltose, lactose, and melibiose. Maltose is an α-glucoside (4-*O*-α-D-glucopyranosyl-D-glucose), lactose is a β-galactoside (4-*O*-β-D-galactopyranosyl-D-glucose), and melibiose is an α-galactoside (6-*O*-α-D-galactopyranosyl-D-glucose). Lactose and melibiose are transported by the wild-type lactose carrier with similar affinities [K_m = 0.1–1.0 mM (31)]. As expected, Table 3 shows that all six of the mutants strains transport maltose at a faster rate than the wild-type strain. The position 177 mutants (pB15 and pD5) are also able to transport galactosides (lactose and melibiose) at a rate similar to that of the wild type. In contrast, however, all four of the position 236 mutants are markedly defective in the ability to transport galactosides.

DISCUSSION

The results presented in this paper demonstrate that it is possible to isolate, identify, and determine the DNA sequence of a relatively large number of lactose carrier mutants that transport maltose. DNA sequencing has revealed that alterations in substrate specificity are the result of single amino acid substitutions within the protein. Most importantly, only two sites (at positions 177 and 236) are seen to be affected, indicating that the maltose-transport phenotype can be brought about by substitutions at only a few discrete locations. It is possible, however, that mutant isolation procedures carried out at higher maltose concentrations might reveal other *lacY* mutants that transport maltose at a reduced rate compared to the ones observed in this study. Transport experiments indicate that the mutant proteins have an enhanced ability to recognize α-glucosides. The position 177 mutants retain the ability to recognize and transport galactosides, whereas the position 236 mutants are defective in the transport of galatosides.

It seems likely that mutations which alter substrate specificity would be localized at, or very close to, the substrate recognition site. Indeed, examples are known for other proteins in which altered substrate specificity appears to be the result of amino acid substitutions at the substrate recognition site (32). Alternatively, however, amino acid replacements that dramatically alter secondary structure (i.e., substituting a proline residue in the center of an α-helix) might be able to change the configuration at the substrate recognition site while being relatively far away. In the present study, the Tyr residue at position 236 was observed in the mutants to be replaced by Phe, Ser, His, and Asn. According to the predictions of Chou and Fasman (33), both Tyr and Asn are α-helix breakers (P_α = 0.61 and 0.73, respectively), Ser is α-helix indifferent (P_α = 0.79), whereas Phe and His are α-helix formers (P_α = 1.12 and 1.24, respectively). In contrast, Tyr and Phe are β-sheet formers (P_β = 1.29 and 1.28, respectively), whereas Ser, His, and Asn are β-sheet breakers (P_β = 0.72, 0.71, and 0.65, respectively). With regard to hydrophobicity [see Kyte and Doolittle (34)], the hydropathic indices are as follows: Phe, +2.8; Ser, −0.8; Tyr, −1.3; His, −3.2; and Asn, −3.5. From the preceding considerations, there appears to be no obvious consistent way in which the four amino acid substitutions observed at position 236 would alter protein secondary structure. It seems more likely, therefore, that Tyr-236 is a part of, or in the immediate vicinity of, the substrate recognition site. As pointed out by Chou and Fasman, Tyr is frequently found as an active site residue (33).

A similar argument can be made about the substitutions observed at position 177. Both Ala and Val are α-helix formers (P_α = 1.45 and 1.14, respectively), whereas Thr is α-helix indifferent (P_α = 0.82). Val and Thr are β-sheet formers (P_β = 1.65 and 1.20, respectively), whereas Ala is β-sheet indifferent (P_β = 0.97). The hydropathic indices are Val, +4.2; Ala, +1.8; and Thr, −0.7. Again, it appears unlikely that the substitutions of Val and Thr would result in a dramatic and consistent change in protein secondary structure. As with Tyr-236, it seems probable that Ala-177 is also a part of, or close to, the substrate recognition site. It should be pointed out that both Val and Thr have side chains that are similar with respect to size [side-chain volume found in known protein conformations = 141.7 and 122.1 Å^3, respectively (35)] and both are significantly larger than Ala (91.5 Å^3). In addition, both Thr and Val have methyl groups attached to the β-carbon atom, whereas Ala has a methyl group attached to the α-carbon atom. While single base substitutions at triplet 177 could have changed Ala-177 to Gly (66.4 Å^3), Ser (99.1 Å^3), Pro (129.3 Å^3), and Glu (155.1 Å^3), the sequencing of 18 mutants did not reveal any of these substitutions. Therefore, the size of the side chain at position 177 as well as the requirement for a methyl group attached to the β-carbon atom may be important in conferring the maltose-transport phenotype. The technique of site-specific mutagenesis should prove useful in resolving these questions.

Several models for the secondary structure of the lactose carrier have been proposed (ref. 36 and K. Beyreuther, quoted in ref. 37). On the basis of α-helical content and the hydropathic profile of the protein, Foster *et al.* (36) have postulated that the lactose carrier consists of at least 12 hydrophobic segments in an α-helical conformation, which traverse the membrane. In their model, the segments of residues 146–163, 169–188, and 222–237 are predicted to be imbedded within the lipid bilayer. While the lengths and exact positions of transmembrane segments should be considered to be tentative, this model predicts that Ala-177 and Tyr-236 are located on hydrophobic transmembrane segments. Therefore, the results of this study provide compelling evidence that the substrate recognition site of the lactose carrier is located within the plane of the lipid bilayer. In addition, the model indicates that Cys-148 (the substrate-protectable *N*-ethylmaleimide-sensitive site) is located on a transmembrane segment as well. Thus, all three residues that have been implicated to be at, or close to, the substrate recognition site are predicted to be on hydrophobic transmembrane segments of the lactose carrier.

As schematically shown in Fig. 2, it can easily be postulated how Cys-148, Ala-177, and Tyr-236 could come to lie close to each other in the tertiary structure of the protein. In this model, lactose carrier is depicted as a protein with 12

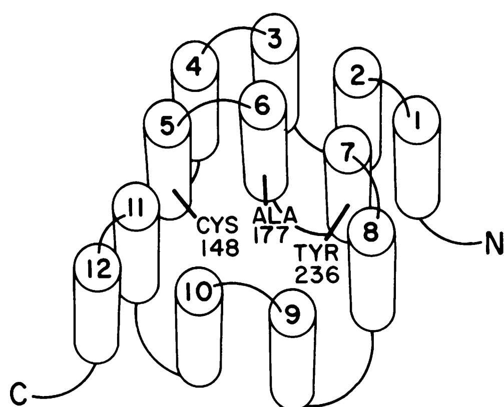

FIG. 2. Hypothetical tertiary structure of the lactose carrier. The cylinders labeled 1 through 12 represent hydrophobic transmembrane segments. Cys-148, Ala-177, and Tyr-236 are located on segments, 5, 6, and 7, respectively.

hydrophobic segments, which traverse the lipid bilayer. Cys-148, Ala-177, and Tyr-236 are located on transmembrane segments labeled 5, 6, and 7, respectively. Assuming that the hydrophilic regions provide the appropriate turns, Fig. 2 depicts how these three transmembrane segments could be oriented so that Cys-148, Ala-177, and Tyr-236 are in the immediate vicinity of one another. In addition, one could imagine how these parallel transmembrane segments might provide a passageway through which H^+ and galactosides could be transported.

While the tertiary model proposed in Fig. 2 is clearly in need of refinement, it provides a reasonable framework on which to design future experiments. Certainly it is possible, if not probable, that other transmembrane segments (besides 5, 6, and 7) may contain amino acid residues that are directly involved with substrate recognition and transport. Since the tertiary model of Fig. 2 postulates that the transmembrane segments labeled 5, 6, and 7 lie close to each other, it would seem that the configuration of the flanking hydrophilic segments would be important in providing the proper orientation of these transmembrane segments. If this is the case, then replacing certain residues within the flanking hydrophilic segments, which are likely to be found in regions of protein coiling (i.e., Pro, Gly, and Asn), might disrupt the proper orientation of hydrophobic segments 5, 6, and 7 and thereby affect the ability to recognize and/or transport galactosides. Particularly interesting residues to replace would include Asn-137, Asn-165, Asn-166, Pro-192, Asn-199, Gly-202, Asn-204, Pro-220, and Asn-245. In this way, the technique of site-specific mutagenesis may help to elucidate these structural aspects of the lactose carrier.

Note. From a collection of mutants isolated by Hobson *et al.* (19) one mutant was identified as being able to grow on 5 mM maltose in the presence of iPrSGal (18). The DNA of this mutant has been sequenced by Markgraf *et al.* (38). Thr-266 was replaced by Ile. With regard to the tertiary structure model proposed in Fig. 2, Thr-266 is located in transmembrane segment 8.

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