Possible Salt Bridges between Transmembrane α-Helices of the Lactose Carrier of *Escherichia coli*

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Although it is energetically extremely unfavorable to have charged amino acid residues of a polypeptide in the hydrophobic environment of the membrane phospholipid bilayer, a few such charged residues are found in membrane-spanning regions of membrane proteins. Ion pairs (salt bridges) would be much more stable in low dielectric media than single ionized residues. This paper provides indirect evidence for a salt bridge between Asp-240 and Lys-319 in the lactose carrier of *Escherichia coli*. When Asp-240 was changed to alanine by site-directed mutagenesis, there was a loss of the ability to accumulate methyl-β-thiogalactopyranoside (TMG), melibiose, or lactose. Fast-growing revertants were isolated on melibiose minimal agar plates. Two second-site revertants were isolated: Asp-240 → Ala plus Gly-268 → Val and Asp-240 → Ala plus Lys-319 → Gln. These revertants showed extremely poor accumulation of TMG, melibiose, and lactose, but showed significant “downhill” lactose entry into β-galactosidase-containing cells with sugar concentrations of 2 and 5 mM. It is concluded that there is some important interaction between Asp-240 and Lys-319, possibly a salt bridge.

Since the amino acid sequences of integral membrane proteins have become available it has been clear that they are extremely hydrophobic. Several different methods have been suggested for evaluating the hydrophobicity (1, 2), and these methods have proved useful in predicting the transmembrane domains in integral membrane proteins. Additional methods of determining structure have been the use of chemical modification reagents, proteolytic digestion, immunological methods, genetic techniques (such as pho A fusions), and various physical methods including NMR and x-ray crystallography (see Refs. 3–5, for reviews). Varying degrees of success have been achieved in determining topology of the membrane proteins whose primary structures are now known.

In spite of the unfavorable energetic factors involved in inserting charged amino acids into the hydrophobic environment of membrane phospholipids, there appear to be such charged residues in membrane-spanning regions in many membrane transport proteins. Honig and Hubbell (6) have pointed out that while 10 kcal/mol is required to insert a charged residue into a region of low dielectric, a salt bridge may require only about 1 kcal/mol, and with a few additional hydrogen bonds the salt bridge may be extremely stable in the hydrophobic environment. There is good evidence for salt bridges in bacteriorhodopsin in which Asp-85 probably salt bridges with Arg-82 and Asp-96 to Arg-227 (7, 8). In addition, it is believed that these 2 aspartic acid residues are directly involved in proton transport and are alternatively protonated and deprotonated. The possibility of a salt bridge within the membrane-spanning region of the anion carrier (band 3) has been suggested by Macara and Cantley (9). They speculate that a negatively charged residue alternately salt bridges with two different positive residues, thus forming two different “gates” which are involved in the transport process for anions.

Salt bridges have also been postulated in the voltage-gated Na⁺ channel (10), the Na⁺/K⁺ -ATPase (11), the Na⁺ channel of toad bladder epithelium (12), and the uncE protein in several microorganisms (13).

Another method of stabilizing a glutamic acid or aspartic acid residue in the membrane-spanning region would be to protonate the carboxyl group. There is evidence that the carboxyl group of Asp-61 of the subunit C of the Fₐ portion of the FoF, ATPase is probably not salt bridged but may be protonated and deprotonated as part of the mechanism of the proton transport process (14).

Although there is too little information available for most membrane proteins (except bacteriorhodopsin) to speculate on the arrangement of charged residues, a few experiments on the lactose carrier of *Escherichia coli* suggest the possibility of salt bridges. The close proximity of His-322 to Ghu-325 in a putative α-helical membrane-spanning region of this protein has suggested the possibility of salt bridging. In a study of site-directed mutants of His-322, Ghu-325 and Arg-302, it was suggested that the 3 residues are important for H⁺-lactose cotransport and may be in close proximity, acting as a charge relay system (15–19).

Evidence for a salt bridge between Asp-237 and Lys-358 of the lactose carrier has recently been presented (20). When a cell containing a plasmid with the mutant Asp-237 → Ala was plated on melibiose minimal agar plates, it failed to grow, and melibiose positive revertants were isolated. These proved to be second-site revertants containing Asp-237 → Ala plus Lys-358 → Gln. Cells with mutant Lys-358 → Thr were similarly found to grow poorly on melibiose, and again, faster growing revertants were isolated. These proved to be Lys-358 → Thr plus Asp-237 substituted by Asn, Gly, or Tyr. Thus, the removal of both a positive and a negative charge resulted in greater transport capacity than the loss of either charge alone. Since this indicated some type of interaction between Asp-237 and Lys-358, it seemed likely that such an interaction was a salt bridge between the two. The present paper presents similar evidence for salt bridging between Asp-240 and Lys-319. A possible role of the salt bridges in cation sugar cotransport is discussed.
Salt Bridges in the Lactose Carrier

TABLE I

<table>
<thead>
<tr>
<th>Genotype of E. coli strains and plasmids</th>
<th>Source</th>
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<tbody>
<tr>
<td>Strains</td>
<td>Wilson and Wilson (21)</td>
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<tr>
<td>DW2</td>
<td>pcn::tn10 from Lopilato (22)</td>
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<tr>
<td>DW2 (pcn)</td>
<td>F' from Hobson et al. (23)</td>
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<td>Plasmids</td>
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<td></td>
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<td>p51R3</td>
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<td>pBR322</td>
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EXPERIMENTAL PROCEDURES

Materials—Melibiose and TMG were purchased from Sigma. [Methyl-14C]TMG was from Du Pont-New England Nuclear. [Glucose-1,14C]lactose was from Amersham Corp. [3H]Melibiose was generously provided by Dr. Gérard Lelblanc (Département de Biologie du CEA, Villefranche sur Mer, France). Radiolabeled sugars were purified by paper chromatography on Whatman No. 3MM chromatography paper using a mixed solvent phase of three parts of 1-propanol to 1 part of water.

Bacterial Strains and Plasmids—All strains are E. coli K12. The genotypes of the strains and plasmids are given in Table I. Cells were grown in LB medium containing ampicillin (0.1 mg/ml) plus tetracycline (0.5 mM) as an inducer of the lactose operon. In one experiment, 0.4% α-methylgalactoside was used as an inducer for the melibiose operon.

Oligonucleotide-directed Mutagenesis—Single-stranded DNA of M13 with a lac Y insert was used as a template for mutagenesis which was carried out by the method of Eckstein et al. (20) as implemented commercially (Amersham Corp.) with exonuclease III. The mutagenic primer was 5'-GCAAACTGGTGGAAAACATCG-3' (mutation in lowercase bold) which resulted in conversion of Asp-240 to Ala. The mutant was rescued from the M13mp18 replicative form by restricting with EcoRI followed by isolation of lac Y and ligation into the dephosphorylated EcoRI site of pBR322. The ligated DNA was initially introduced into TGI, and transformants were selected as clones resistant to ampicillin and tetracycline. Plasmid DNA was isolated from several clones. A clone with the lac Y insert orientated as in the parental Y gene (pTE18) was identified by restriction mapping with AatII. The plasmid DNA was completely sequenced by the method of Sanger et al. (26) as implemented commercially (Amersham Corp.).

Transport Assays—Cells were grown to mid-log phase (three to four doublings), centrifuged, and washed twice with 100 mM potassium phosphate buffer containing (0.1 mg/ml) plus tetracycline (10 μg/ml). In some experiments isopropyl-thiogalactoside (0.5 mM) was used as an inducer of the lac operon. In one experiment, 0.4% α-methylgalactoside was used as an inducer for the melibiose operon.

RESULTS

Asp-240 → Ala Mutant—The lactose carrier mutant Asp-240 → Ala was constructed by oligonucleotide-directed mutagenesis. The plasmid p51 was placed in the lac-deleted strain DW2, and the amount of carrier in the membrane was assayed with a monoclonal antibody against the lactose carrier by the method of Lokema et al. (33). DW2 containing a plasmid with the normal lac Y gene (pTE18) served as a positive control. The quantity of carrier protein in DW2/pTE18 was 110% of that found with DW2/pTE18.

The mutant Asp-240 → Ala shows a very severe defect in the ability to accumulate several different sugars against a concentration gradient. Cells containing a normal lac Y gene (pTE18) accumulated thiomethylgalactoside (TMG) to an internal concentration 30 times higher than the external additions of 5 mM KOH. Sugar-induced proton uptake was initiated by the addition of 25 μl of an anaerobic 1 M solution of sugar (pH 7). The "oxygen pulse" was initiated by the addition of 50 μl of 120 mM KC1, 30 mM KSCN equilibrated with room air. The oxygen dissolved in this sample was sufficient to produce a burst of respiration and proton extrusion from the cell with an acidification of the external medium. When the oxygen was completely exhausted, external protons reentered the cells and the pH of the medium returned to the initial value. Anaerobic 20 mM KOH (20 μl) was added at the end of each experiment to provide calibration.

Measurement of ΔpH—The distribution of radioactive benzoic acid may be used to determine the ΔpH across the cell membrane (30). Because the membrane is more permeable to the protonated species than the ionized form, benzoic acid accumulates on the alkaline side of a biological membrane. Cells were incubated at a density of 0.1 mg of protein/ml in 100 mM potassium phosphate buffer, pH 5.9. This pH was used to create the maximum pH gradient in the normal cell (31). Cells were preincubated with or without sugar (5 mM) for 20 min and then exposed to [14C]benzoic acid (0.1 μCi/ml) and [3H]lactate (0.6 μCi/ml) for 10 min. A sample (1 ml) of this mixture was placed above 0.5 ml of silicone oil in a 1.6-ml microfuge tube and centrifuged for 5 min (32). The cells pass through the silicone carrying a small volume of extracellular fluid which was determined quantitatively from the value for [3H]lactate which is restricted to the extracellular space. After aspirating the aqueous supernatant and most of the silicone oil, the tip of the microfuge (containing the cell pellet) was cut off with a razor blade. This pellet was placed into a counting vial, 4 ml of counting fluid was added, and the vial vortexed vigorously prior to counting.

For the estimation of the cell water space, cells were preincubated with and without sugar (5 mM) for 20 min and then incubated with [3H]H2O (1.4 μCi/ml) and [3H]lactate (0.2 μCi/ml). After 10 min of incubation at room temperature, 1 ml of cells was placed on 0.5 ml of silicone oil and centrifuged. The samples were handled as indicated above and the cell pellet counted. From the taurine counts in the pellet and supernatant fluid, the extracellular water space was calculated. From the radioactive water counts, the total pellet water space was determined. The total pellet water minus extracellular space was taken as the intracellular water space (for general method see Ref. 32).

The abbreviation used is: TMG, methyl-β-D-thiogalactopyranoside.
medium (Fig. 1). On the other hand, cells harboring the plasmid with the Asp-240 \rightarrow \text{Ala} mutation showed no accumulation (the internal concentration was less than that of the external concentration). Similarly, the mutant failed to accumulate melibiose (the parent showed a 31-fold accumulation) (Fig. 2). The rate of uptake of o-nitrophenylgalactoside by the mutant was 2% of that of the parent (data not shown).

Lactose transport by the mutant Asp-240 \rightarrow \text{Ala} showed an interesting diction between the ability to accumulate the disaccharide in a \(\beta\)-galactosidase-negative cell and the ability to enter the cell containing \(\beta\)-galactosidase. The plasmid containing Asp-240 \rightarrow \text{Ala} was placed in a strain with a deletion of the lac Z and lac Y genes (DW2(pcn)). When this cell was exposed to radioactive lactose, it failed to accumulate the sugar to a concentration greater than the external medium. Under the same conditions the cell containing a plasmid with the normal lac Y gene (pTE18) accumulated lactose 26-fold in 2 min (Fig. 3).

In another series of experiments, the mutant plasmid was placed into a cell with a lac Z gene and when the cell was induced, lactose uptake was measured into this cell containing \(\beta\)-galactosidase. Under these conditions, lactose entering the cell would be metabolized. Thus, a positive result in this assay requires the carrier to show sugar recognition and transport but does not require energy-dependent accumulation. Under these conditions, entry of sugar is thermodynamically "downhill." Lactose uptake experiments were carried out at three different sugar concentrations (0.1, 2, and 5 mM), and significant lactose uptake was observed by the Asp-240 \rightarrow \text{Ala} mutant, especially at the higher sugar concentrations (Fig. 4).

An independent experiment confirmed this observation of lactose entry into \(\beta\)-galactosidase-containing cells. DW2/F\(^{10}\)Z\(^{-}\)Y\(-\)/p51 (Asp-240 \rightarrow \text{Ala}) fermented lactose on lactose MacConkey indicator plates (red clones) in a manner similar to cells with a normal lac Y gene. Such plates contain a high concentration (30 mM) of sugar. In addition, the downhill entry of melibiose was positive by this fermentation assay. DW2 (melA\(^\Delta DB\)/p51 gave clones with large red centers on melibiose MacConkey plates.

Since the mutant Asp-240 \rightarrow \text{Ala} failed to accumulate three different sugars but can transport downhill the normal energy coupling to proton entry was defective. It was considered of interest to measure proton uptake induced by melibiose. A continuous pH recording was made of a concentrated suspension of cells of DW2/p51 and DW2/pTE18 which were incubated anaerobically in an unbuffered solution of KCl. Addition of 25 \(\mu\)l of 1 M anaerobic melibiose to the suspension of DW2/pTE18 caused an alkalinization of the medium due to proton uptake by the cell. Cells containing the mutant plasmid (Asp-240 \rightarrow \text{Ala}) showed a greater proton uptake than normal (Fig. 5, panel A). Proton uptake induced by the addition of thelactoside (another substitute for the lactose carrier) was also greater than normal (data not shown). When lactose was added to such cells, the proton uptake was about the same as that found with the normal carrier (data not shown). Proton uptake with melibiose and lactose was an unexpected finding since energy coupling (sugar accumulation) is defective.

Another type of assay for proton entry is the measurement of the pH gradient across the membrane in aerobic cells. A modest uptake of protons with sugar can be compensated for by the proton pumping of the respiratory chain, but a large proton uptake may exceed the rate of proton extrusion and the protonmotive force may be reduced. When aerobic cells are suspended in a buffered medium at pH 5.9, there is
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A) Melibiose-induced H⁺ uptake

B) Oxygen Pulse before and after Melibiose addition

Fig. 4. Downhill lactose transport by lactose carrier mutants. DW2/F'p51R2 containing a plasmid for normal or mutant carrier was grown in LB containing isopropyl-1-thio-β-D-galactopyranoside. Washed cells were incubated in the presence of [¹⁴C] lactose 0.1 mM (panel A), 2 mM (panel B), and 5 mM (panel C).

Fig. 5. Melibiose-induced proton uptake and the effect of melibiose on the shape of the oxygen pulse curve. Panel A, the effect of the addition of 25 µl of 1 M anaerobic melibiose to anaerobic cells. Panel B, the oxygen pulse curve before and after addition of sugar. Fifty µl of KCl (120 mM) containing dissolved oxygen was injected immediately before the rise of each curve. The solid line indicates the pH curve before addition of melibiose and the dashed line, after the addition of melibiose.

Fig. 6. The effect of sugars on the pH gradient across the cell membrane. Cells were incubated in the presence or absence of sugar for 20 min. The pH gradient was estimated with the benzoic acid technique (see “Experimental Procedures”).

A similar observation has been made for the double mutant Ala-177 → Val/Lys-319 → Asn. In this case it was suggested by Brooker (34) that sugar causes proton “leak” because sugar enters the cell with protons but returns on the carrier to the external surface in the absence of protons. This would result in net proton uptake without net sugar uptake. The assay to measure a proton leak was to generate a brief pH gradient across the membrane due to an oxygen pulse and then observe the return of protons across the membrane down their electrochemical gradient. This was accomplished by adding a small amount of oxygen to anaerobic cells (which generate a transient pH gradient) and then observing the rate of reentry of protons into the cell. This reentry of protons is by diffusion and possibly by carrier mediated processes. This leak of protons back into the cell following an oxygen pulse was increased by melibiose in the double mutant studied by

normally a pH gradient of 1.2–1.5 units (inside alkaline). A convenient method for assaying the pH gradient is to measure the accumulation of a weak acid whose protonated form is lipid soluble such as benzoic acid. A 10-fold accumulation of benzoic acid results from an H⁺ gradient of 10-fold (i.e. an internal pH of 1 unit more alkaline than that of the medium). When cells with the normal lac Y gene were incubated in the presence or absence of melibiose or thiogalactoside, no significant difference in benzoic acid accumulation was observed (Fig. 6). However, the addition of either sugar to cells with the Asp-240 → Ala mutation reduced the benzoic acid accumulation to about half of that observed in the absence of sugar (Fig. 6). Thus, we have the unexpected observation that on the one hand, the mutant fails to accumulate and must have a defect in energy coupling, while on the other hand, addition of sugar causes rapid proton entry.
Brooker (34). This assay for sugar-induced proton leak was carried out with cells containing the plasmid with normal or mutant lac Y gene. Fig. 5, panel B, shows that the shape of the oxygen pulse curve in the normal cell was the same before and after addition of melibiose. In the mutant Asp-140 → Ala on the other hand, the proton leak following melibiose addition was more rapid than before sugar addition.

**Melibiose-positive Revertants**—DW2(pcn) containing the mutant plasmid grew much more slowly on melibiose (0.2%) minimal plates than the similar cell containing a plasmid with the normal lac Y gene. When such melibiose minimal plates containing DW2(pcn)/p51 were incubated 3-4 days at 37 °C, a few larger colonies appeared. One clone from each of several plates was restreaked to purify. Plasmid DNA was isolated and used to transform competent cells of DW2(pcn). The transformants grew as large colonies on melibiose minimal plates indicating that the mutation was associated with the plasmid. Plasmid DNA from the transformant was isolated and sequenced. Of the seven mutants that were sequenced, one showed the original Asp-240 and sequenced. All of these revertants retained the Asp-240 + Ala plus a second site mutation Lys-319 → Gln. The downhill melibiose transport by the two revertant cells was greater than that by cells containing p51 although less than the normal (Fig. 7). Lactose accumulation was 5-10% of normal for p51R2 (Asp-240/Val-268) and 5-10% of normal for p51R3 (Asp-240/Gln-319). The downhill melibiose transport by the two revertant cells was greater than that by cells containing p51 although less than the normal (Fig. 7). Lactose accumulation was 5-10% of normal for p51R2 and 10-20% of normal for p51R3 (Fig. 3). On the other hand, the downhill transport of lactose (5 mM) into a β-galactosidase-containing cell was 60% of normal for p51R2 and 100% of normal for p51R3 (Fig. 4). These rates of transport were distinctly higher than those for p51. Thus, the downhill lactose entry is close to normal while the accumulation of three sugars is severely defective.

Melibiose-stimulated proton entry was somewhat faster than normal for both of the revertants (Fig. 5, panel A). The benzoic acid accumulation (pH gradient) was less than normal without added sugar for both revertants. This indicated proton leak through the carrier in the absence of sugar. In the case of p51R2, the addition of melibiose or thiogalactoside further reduced the benzoic acid accumulation. In the case of p51R3, melibiose had little effect and thiogalactoside increased the pH gradient.

The measurement of proton leak following the oxygen pulse was made on the revertants (Fig. 5, panel B). Revertant p51R2 showed a greater H+ leak than the normal in the absence of sugar (consistent with the benzoic acid data). Addition of melibiose increased the proton leak. In the p51R3 revertant addition of melibiose also stimulated proton leak.

**DISCUSSION**

A striking feature of cells containing a plasmid with the mutant Asp-240 → Ala is its complete loss of ability to accumulate substrates of the carrier (lactose, thiomethylgalactoside, and melibiose). On the other hand, sugar recognition and ability to transport lactose downhill into β-galactosidase-containing cells is retained to a significant extent. Lactose fermentation, as indicated by a red clone on lactose MacConkey plates, is exhibited by DW2/pcn/p51. Although lactose entry at a concentration of 30 mM (used in the indicator plates) is sufficiently rapid to produce a red color, at lower lactose concentrations (5, 2, and 0.1 mM) cells show progressively less uptake (Fig. 4). Thus, the affinity of the mutant carrier for lactose is extremely poor. Likewise the downhill transport of melibiose is sufficiently active for DW2/pcn/p51 to show red clones on melibiose MacConkey indicator plates although this mutant fails to accumulate melibiose against a concentration gradient.

An interesting characteristic of Asp-240 → Ala is the abnormally rapid proton entry into anaerobic cells on the addition of 10 mM melibiose. This sugar-induced proton entry was sufficiently rapid to reduce the normal pH gradient across the membrane in aerobic cells (Fig. 6). Furthermore, in the presence of sugar, the carrier permits the "leakage" of protons down their concentration gradient following an oxygen pulse (Fig. 5). Brooker (34) has suggested that under these conditions sugar enters with protons, the protons are discharged on the inside, and the sugar returns on the carrier without protons. Thus, there is a net uptake of protons in the absence of sugar accumulation.

 Attempts were made to isolate revertants that would grow on low concentrations of melibiose on minimal plates. Seven revertants were isolated and the DNA of the lac Y gene sequenced. All of these revertants retained the Asp-240 → Ala mutation. One showed a second site mutation of Gly-268 → Val and six showed a second site mutation of Lys-319 → Gln. Both types of mutants retained the inability to accumulate thiomethylgalactoside against a concentration gradient (Fig. 1). The revertants regained 5-10% of the normal ability to accumulate lactose (Fig. 3) and melibiose (Fig. 2). The downhill lactose entry at 2 and 5 mM concentrations returned toward normal in the revertants (Fig. 4) although the apparent affinity remained impaired. The two revertants showed a proton leak through the carrier in the absence of sugar. Both cells showed an additional melibiose-induced proton leak following the oxygen pulse (Fig. 5, panel B).

Because of the apparent relationship between Asp-240 and Lys-319 it was of interest to know the properties of single mutants with a loss of the positive charge at position 319. Data are available on the physiological properties of single mutants in which Lys-319 was changed to two different neutral amino acids. Kaback (4) has shown that the mutant Lys-319 → Leu has lost the ability for accumulation of TMG and lactose. However, it shows downhill lactose transport and

![Graph](image-url)
FIG. 8. A model of the lactose carrier (20, 35) showing the positions of the mutations.

Brooker\textsuperscript{3} has studied Lys-319 \rightarrow Asn. This cell fails to accumulate TMG and shows only very weak accumulation for lactose and minor downhill transport activity. Thus, the loss of the positive charge at 319 or the negative charge at 240 seriously reduces the ability to accumulate sugar, and the affinity for downhill transport is poor while the simultaneous loss of both positive and negative charges results in significant recovery of downhill transport.

The position of the two second site mutations in relation to Asp-240 is shown in Fig. 8. It should be noted that the 3 residues are located at approximately the same level in three different membrane-spanning segments, thus suggesting a possible interaction between these residues in the three-dimensional structure of the protein. The model shown in the figure is supported by several types of data. Antibodies directed against the COOH-terminal decapetide, a loop 5 decapetide and a loop 7 decapetide, bind to the cytoplasmic surface of the membrane (36-39). Evidence has been presented that the large middle cytoplasmic loop contains a binding site for the regulatory soluble protein \textit{Enz} III (40). And finally, the recent \textit{pho} A fusions of Calamia and Manoil (41) are consistent with this model.

The question of salt bridging within the structure of the lactose carrier was first raised when attention was focused on His-322 and Glu-325 by studies of mutations in these residues (15, 16). The close proximity of these 2 residues in an $\alpha$-helix suggests the possibility of salt bridging. Recent studies (20) have suggested the possibility of a salt bridge between Asp-237 and Lys-358. Asp-237 \rightarrow Asn shows no accumulation of several sugars and low activity toward melibiose. Melibiose-positive revertants showed a second site mutation of Lys-358 changed to Gln. Conversely, the loss of the positive charge at position 358 (Lys-358 \rightarrow Thr) resulted in poor activity and a melibiose-positive revertant showed second site mutations of Asp-237 to neutral amino acids (Asn, Gly, or Tyr). This close functional interaction between Asp-237 and Lys-358 suggests that they are close together in three-dimensional space and reside in membrane-spanning segments and may be ion-paired. The third possible salt bridge is between Asp-240 and Lys-319 as suggested by the data presented in this paper. A possible arrangement of the three salt bridges is given in Fig. 9. Brooker (42) has suggested a rather similar arrangement of $\alpha$-helices based on a consideration of the locations of a variety of sugar recognition mutants.

It is of interest to ask whether one or more of the three salt bridges might have functional as well as structural significance. Since mutations affecting Asp-237, Asp-240, Lys-319, and Lys-358 show marked changes in sugar recognition they are probably a part of a complex sugar recognition site. In addition, since mutations at these four sites all result in loss of normal energy coupling as shown by inability to accumulate sugars it is possible that one or more salt bridges are directly involved in the energy coupling process. One possible role of a salt bridge is given in Fig. 10. In this model the salt bridge is a gate which closes the sugar recognition site near the cytoplasmic surface. Mitchell (43) has proposed what he calls a "proton well" in the proton channel of the $F_oF_1$ ATPase. According to this view the membrane potential (inside negative) draws protons down into the channel resulting in acidification of this microenvironment. The pH gradient across the membrane would give an additional acidification to this region. Thus, if we invoke Mitchell's proton well the pH in the region of the aspartic acid become sufficiently acidic to protonate the carboxyl group. According to this model the gate then opens. In this configuration (the positive charge is no longer salt bridged) the affinity for the sugar falls, and the sugar is deposited on the inside of the cell. The final step in the model is the loss of the proton into the cell.

\textsuperscript{3} R. J. Booker, personal communication.
cytoplasm of the cell and the return of the negative charge, thus closing the gate and completing the cycle.

This very speculative model has the merit that it is consistent with at least some of the data. It is very likely that one or more carboxyl groups are involved in proton translocation as appears to be the case in bacteriorhodopsin and in the F,F, ATPase. The affinity for the sugar is high when the salt bridge is intact and the carrier faces the external medium. The affinity is greatly reduced when one of the two members of the salt bridge has been substituted by a neutral amino acid (this would correspond to the protonated step in the model). In bacteriorhodopsin two salt bridges are believed to be present and the protonation of the aspartic acid portion of the salt bridges is involved in the proton pumping of the carrier (7,8).

It could be argued that when both negative and positive arms of the salt bridge are missing due to their substitution by neutral amino acids, no sugar accumulation could occur according to the model in Fig. 10. The pair Ala-240/Gln-319 fits this prediction rather closely. The two second-site revertants show practically no accumulation although they do permit downhill sugar movement with low affinity for the substrate. With the pair Asp-237/Lys-358, the revertants show moderate to good accumulation for certain concentrations of certain sugars. This suggests the possibility that the Asp-240/Lys-319 salt bridge may be more important in the mechanism of accumulation. In cells lacking a carboxyl group at position 240, the pathway for melibiose-induced proton entry may involve Asp-237 or Gln-269. While we cannot exclude the possibility that the salt bridge Asp-240/Lys-319 stabilizes a sugar and/or proton-binding pocket a more direct role of the salt bridge in proton translocation as indicated in Fig. 13 is an attractive possibility.

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REFERENCES

FIG. 10. Hypothetical mechanism of H+–sugar cotransport. Possible steps in the cotransport mechanism are indicated. In the first step the salt bridge provides the gate that prevents sugar translocation. The protonotive force drives protons into the region of the aspartic acid. Protonation of the carboxyl group releases the constraints of the salt bridge, and the sugar is released to the interior of the cell. The salt bridge is reformed and the cycle is repeated.