Exchange, Efflux, and Substrate Binding by Cysteine Mutants of the Lactose Permease of Escherichia coli

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ABSTRACT: In this study, wild-type lac permease and lac permease mutated at each of the eight cysteiny1 residues in the molecule were solubilized from the membrane, purified, and reconstituted into proteoliposomes. Lactose equilibrium exchange and efflux activities of mutants with Ser in place of Cys117, Cys176, Cys234, Cys333, Cys353, or Cys355 are essentially the same as wild-type permease. In contrast, mutants in Cys148 and Cys154 exhibit diminished exchange and efflux activities. These mutants in Cys148 and Cys154, except for the C148S mutant, have previously been shown to show down active transport as well [Van Iwaarden, P. R., Driessen, A. J. M., Menick, D. R., Kaback, H. R., & Konings, W. N. (1991) J. Biol. Chem. 266, 15688-15692]. C148S permease shows monophasic kinetics with a high apparent $K_M$ with respect to external lactose in the exchange reaction under nonequilibrium conditions, whereas wild-type permease exhibits biphasic kinetics with both a high and low $K_M$ component. Moreover, the absence of the low $K_M$ pathway in the C148S permease is correlated with the absence of a high-affinity binding site for p-nitrophenyl $\alpha$-D-galactopyranoside (NPG). Interestingly, the affinity of the permease for NPG appears to increase with the hydrophobicity of the side chain at position 154 (Ser < Cys < Gly < Val). Finally, the presence of a high-affinity binding site for NPG in C154V is consistent with the biphasic exchange kinetics exhibited by this mutant. The results are discussed in the context of a model in which lac permease has two substrate binding sites, a catalytic site and a regulatory site.

The lactose (lac) permease of Escherichia coli is a hydrophobic polytopic cytoplasmic membrane protein that catalyzes concomitant translocation of $\beta$-galactosides and $H^+$ with a stoichiometry of 1:1 (i.e., lactose/H+ cotransport or symport) (for reviews, see Kaback (1990) and Wright et al. (1986)]. This prototypic membrane protein, which is encoded by the lacY gene, has been solubilized from the membrane, purified to homogeneity, and reconstituted into proteoliposomes. It has been demonstrated to be solely responsible for $\beta$-galactoside transport as a monomer. On the basis of circular dichroism with purified permease and hydrophathy analysis of the primary amino acid sequence, a secondary structure has been proposed in which the protein consists of 12 hydrophobic domains in a-helical conformation that traverse the membrane in zigzag fashion, connected by hydrophilic segments with the N- and C-termini on the cytoplasmic surface (cf. Figure 1). The general features of the model are consistent with other spectroscopic measurements, chemical modification, limited proteolysis and immunological studies. Furthermore, more recent studies on an extensive series of lac permease–alkaline phosphatase (lacY-phoA) fusion proteins provide strong and exclusive support for the topological predictions of the 12-helix model (Calamia & Manoil, 1990).

The effects of site-directed mutagenesis on the functional properties of the permease suggest that Arg302 in putative helix IX and His322 and Glu325 in helix X (Menick et al., 1987b; Pütter et al., 1986; Carrasco et al., 1986) play an important role in lactose/H+ symport, possibly as components in a type of charge relay. Recent studies also indicate that the sugar recognition properties of the lac permease are altered by substitutions nearby [Ser306, Lys319 (Collins & Brooker, 1989), and Pro327 (Lolkema and Kaback, unpublished results)] or within the putative charge relay [His-322 (Franco et al., 1989)].

A number of mutants have been constructed in which each cysteine residue was replaced (Trumble et al., 1984; Menick et al., 1985, 1987a; Neuhaus et al., 1985; Viitanen et al., 1985; Brooker & Wilson, 1986; Sarkar et al., 1986; Kaback, 1987; van Iwaarden et al., 1991). These studies revealed that only Cys154 is important for active lactose transport and that disulfide–sulphydryl interconversion is probably not involved in regulation of permease activity (Konings & Robillard, 1982). However, the exchange and efflux reactions catalyzed by the mutants were not analyzed. We now compare these reactions between the different Cys mutants and the wild-type permease. Except for the C148S permease, exchange correlates well with $\Delta$-driven transport.

In addition, new insights into the kinetic mechanism of lactose transport have been obtained recently which suggest that lac permease contains two distinct binding sites for substrate. The binding of $p$-nitrophenyl $\alpha$-D-galactopyranoside (NPG) to purified lac permease reconstituted in proteoliposomes indicates two binding sites per lac permease molecule (Lolkema & Walz, 1990). Furthermore, analysis of lactose out/lactose in exchange under nonequilibrium conditions suggests that one of the two sites might be a regulatory site and the other the catalytic site (Lolkema et al., 1991). In order to gain further evidence for the two substrate binding sites on

1 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. This sequence is followed by a second letter denoting the amino acid replacement at this position (e.g., C148S designates that Cys148 is replaced with Ser).

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lac permease, we studied exchange catalyzed by the C148S' and C154V permeases in more detail, as well as NPG binding. The results suggest that C148S permease is defective in a high-affinity regulatory binding site. In contrast, C154V, like wild-type permease, exhibits biphasic exchange kinetics and high NPG binding. The results provide additional evidence for two binding sites in lac permease.

MATERIALS AND METHODS

Materials. [1-14C]Lactose (57 mCi/mmol) and [14C]urea (1.2 mCi/mL) were obtained from Amersham (Buckinghamshire, U.K.). p-Nitro[6-3H]phenyl α-D-galactopyranoside (4 Ci/mmol) was synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group, Hoffman-La Roche Inc.). All other reagents were of reagent grade and were obtained from commercial sources.

Bacterial Strains, Plasmids, and Growth of Cells. E. coli T206, T184, and plasmid pGM21 encoding wild-type lacY (Teather et al., 1980) or lacY with site-directed mutations in single Cys codons have been described (Trumble et al., 1984; Menick et al., 1985, 1987a; Neuhaus et al., 1985; Viitanen et al., 1985; Brooker & Wilson, 1986; Sarkar et al., 1986). E. coli T184 transformed with the appropriate plasmid was grown and induced with isopropyl 1-thio-β-D-galactopyranoside (IPTG) as described by Teather et al. (1980) with the exception that methionine and threonine in the growth medium were replaced by 0.2–0.5% casamino acids (Page & Rosenbusch, 1988).

Purification and Reconstitution of Lac Permease. Wild-type and mutant lac permeases were solubilized, purified, and reconstituted into proteoliposomes as described (Viitanen et al., 1986). After freeze-thawing, the proteoliposomes were not sonified but extruded through polycarbonate filters of 100-nm pore size using an extrusion device (Lipex Biomembranes, Vancouver, B.C.). This procedure yields proteoliposomes of a relatively uniform size distribution (Hope et al., 1985). The final preparations contained proteoliposomes at 37.5 mg of phospholipid/mL and 50–90 μg of protein/mL in 50 mM potassium phosphate (pH 7.5) and 1 mM DTT. The protein concentration of the proteoliposomes was determined by the method of Schaffner and Weismann (1975).

Preparation of Membrane Vesicles. Right-side-out (RSO) membrane vesicles were prepared by osmotic lysis of lysozyme/ethylenediaminetetraacetic acid-induced spheroplasts as described (Kaback, 1971). For quantitative estimation of NPG binding in RSO membrane vesicles, the membrane vesicles were purified by centrifugation through 60% sucrose as described (Lolkema & Walz, 1990). Protein in RSO membrane vesicles was measured with the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.

Assays. Efflux and equilibrium exchange were performed as described (Viitanen et al., 1986). The $t_{1/2}$ of the efflux or exchange process is defined as the time point where half of the labeled substrate has left the proteoliposomes or vesicles. Measurement of the kinetics of exchange under nonequilibrium conditions was carried out as described previously (Lolkema et al., 1991). Binding of NPG to RSO vesicles was measured by flow dialysis as described (Ramos & Kaback, 1977) or after purification on a 60% sucrose gradient by the centrifugation method described by Lolkema and Walz (1990). [14C]Urea was used as a volume marker with the latter method.

Immunological Analysis. Immunoblots were carried out with monoclonal antibody (Mab) 4A10R as described.
Exchange, Efflux, and Substrate Binding of Lactose Permease

RESULTS

Efflux and Equilibrium Exchange. When proteoliposomes reconstituted with the purified wild-type permease were loaded with 10 mM \([1-\text{\textsuperscript{14}C}]\) lactose in the presence of valinomycin and subsequently diluted into medium containing 10 mM unlabeled lactose, equilibrium exchange occurred at about the same rate as reported previously (Figure 2A, \(\Phi, \Delta\)) or C148S mutant permease (\(\Phi, \Delta\)) were equilibrated for 1 h at room temperature with \([1-\text{\textsuperscript{14}C}]\) lactose (10 mM/mmol; 10 mM final concentration) in 50 mM potassium phosphate, pH 5.5, 1 mM DTT, and 20 mM valinomycin. Aliquots of 1 ¼L were rapidly diluted into 200 ¼L of 50 mM potassium phosphate, pH 5.5 (efflux, triangles), or in the same solution containing 10 mM unlabeled lactose (exchange, circles). (B) Lactose efflux at pH 7.5. Proteoliposomes reconstituted with wild-type permease (\(\Phi\)) or C148S permease (\(\Phi\)) were equilibrated as above, but at pH 7.5, and diluted into potassium phosphate, pH 7.5. In a control experiment, proteoliposomes reconstituted with wild-type permease were diluted after preincubation with 2.5 mM \(p\)-(chloromercuri)benzenesulfonate (\(\Delta\)). (Carrasco et al., 1982) using an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) to detect immune complexes.

Efflux and equilibrium exchange were measured as described in the legend to Figure 2.

FIGURE 2: Lactose equilibrium exchange and efflux. (A) Lactose equilibrium exchange and efflux at pH 5.5. Proteoliposomes reconstituted with purified wild-type permease (\(\Phi\), \(\Delta\)) or C148S mutant permease (\(\Phi\), \(\Delta\)) were equilibrated for 1 h at room temperature with \([1-\text{\textsuperscript{14}C}]\) lactose (10 mM/mmol; 10 mM final concentration) in 50 mM potassium phosphate, pH 5.5, 1 mM DTT, and 20 mM valinomycin. Aliquots of 1 ¼L were rapidly diluted into 200 ¼L of 50 mM potassium phosphate, pH 5.5 (efflux, triangles), or in the same solution containing 10 mM unlabeled lactose (exchange, circles). (B) Lactose efflux at pH 7.5. Proteoliposomes reconstituted with wild-type permease (\(\Phi\)) or C148S permease (\(\Phi\)) were equilibrated as above, but at pH 7.5, and diluted into potassium phosphate, pH 7.5. In a control experiment, proteoliposomes reconstituted with wild-type permease were diluted after preincubation with 2.5 mM \(p\)-(chloromercuri)benzenesulfonate (\(\Delta\)). (Carrasco et al., 1982) using an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) to detect immune complexes.

Table I summarizes the results of the exchange and efflux experiments at pH 7.5 with the other Cys mutants of lac permease. Proteoliposomes reconstituted with purified C117S, C176S, C234S, C333S, and C353S/C355S permease catalyze equilibrium exchange and efflux with rates comparable to those of proteoliposomes reconstituted with wild-type permease, consistent with previous observations demonstrating that these mutants catalyze active transport normally (Menick et al., 1985; Brooker & Wilson, 1986; van Iwaarden et al., 1991). In addition to C148S permease, proteoliposomes reconstituted with C148G, C154G, C154S, or C154V permease also show reduced exchange and efflux activity. However, these mutants,

Table I: Equilibrium Exchange and Efflux at pH 7.5 of the Isolated Cys Mutants and Wild-Type Permease Reconstituted into Proteoliposomes

<table>
<thead>
<tr>
<th>permease</th>
<th>exchange (t_{1/2}) (s)</th>
<th>efflux (t_{1/2}) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>C117S</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>C148S</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>C148G</td>
<td>&gt;60</td>
<td>&gt;5</td>
</tr>
<tr>
<td>C154S</td>
<td>&gt;60</td>
<td>&gt;5</td>
</tr>
<tr>
<td>C154G</td>
<td>&gt;60</td>
<td>&gt;5</td>
</tr>
<tr>
<td>C154V</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>C176S</td>
<td>&lt;5</td>
<td>1</td>
</tr>
<tr>
<td>C234S</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>C333S</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>C353S/C355S</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

\(\Phi\) Exchange and efflux were measured as described in the legend to Figure 2.

FIGURE 3: Kinetics of the lactose\(_{in}\)/lactose\(_{out}\) exchange reaction. Proteoliposomes containing wild-type (\(\Phi\), 85 \(\mu\)g/mL), C154V (\(\Delta\), 65 \(\mu\)g/mL), or C148S (\(\Theta\), 80 \(\mu\)g/mL) permease were loaded with 3 mM (wild-type permease) or 10 mM (C154V and C148S permeases) \([1-\text{\textsuperscript{14}C}]\) lactose at pH 5.5 and subsequently diluted 2000-fold into 50 mM KPi, pH 5.5, containing lactose concentrations ranging from 0.1 to 25 mM. Initial rates of exchange are plotted according to Eadie-Hofstee.

in contrast to C148S, also catalyze \(\Delta\eta\)-driven transport at a reduced rate (Trumble et al., 1984; Menick et al., 1985; Viitanen et al., 1985; Kaback, 1987; van Iwaarden et al., 1991).

Kinetic Analysis of the Exchange Reaction. The above results show that only mutations of Cys148 and Cys154 significantly affect the kinetics of lac permease. The C148S mutation slows down exchange but not active transport; the C154V mutation slows down both exchange and active transport. To gain further insight into the different behavior of these two mutants, we examined the kinetics of the lactose\(_{in}\)/lactose\(_{out}\) exchange reaction in detail. As demonstrated previously (Lolkema et al., 1991), the exchange reaction catalyzed by wild-type permease reconstituted into proteoliposomes is characterized by two kinetic regimes with respect to the external lactose concentration. Thus, when proteoliposomes containing wild-type lac permease are loaded with 3 mM \([1-\text{\textsuperscript{14}C}]\) lactose and the initial rate of exchange is measured at external lactose concentrations ranging from 0.1 to 25 mM lactose, Eadie-Hofstee plots of initial rate data exhibit biphasic exchange kinetics (Figure 3, \(\Phi\)). The kinetic parameters for the two phases resemble those reported (Lolkema et al., 1991, and Table II). C154V permease, which catalyzes equilibrium exchange at a slower rate than wild-type permease, shows essentially the same two kinetic states

Table I: Equilibrium Exchange and Efflux at pH 7.5 of the Isolated Cys Mutants and Wild-Type Permease Reconstituted into Proteoliposomes
with apparent affinity constants, $K_p$, of 0.6 mM and 2.6 mM (Figure 3, A; Table II) at an internal lactose concentration of 10 mM [1-14C]lactose. Over the whole range of external lactose concentrations, the exchange rate catalyzed by the C154V mutant is lower than that observed with the wild-type enzyme.

In this respect, it should be noted that the maximal rates in Table II for wild-type permease should be multiplied by a factor of about 1.5 to correct for the lower internal lactose concentration [the affinity constant for internal lactose in the exchange reaction is 3 mM (Lolkema et al., 1991)]. In contrast to wild-type and C154V permeases, analysis of exchange over the same external concentration range with C148S permease shows only a single phase (Figure 3, O). Both the affinity and the maximal rates resemble those of the high-affinity phase observed for wild-type and C154V permease (Table II). The low-affinity kinetic phase appears to be completely absent.

NPG Binding. Flow dialysis is routinely used to measure binding of NPG to lac permease [see Ramos and Kaback, (1977), Rudnick et al. (1976), and Herzlinger et al. (1985) for examples]. Addition of a large excess of unlabeled substrate to RSO membrane vesicles containing wild-type enzyme equilibrated with [3H]NPG results in an increase in the dialyzable concentration of the ligand. The increase reflects the amount of [3H]NPG displaced from the binding site(s) of the permease by the unlabeled substrate. The same experiment with RSO membrane vesicles containing C148S permease shows no increase in radioactivity in the dialysate upon addition of excess unlabeled NPG, suggesting that the C148S mutant does not bind NPG. Importantly, immunoblot analyses carried out on the RSO membrane vesicles used to assay binding demonstrate that comparable amounts of permease are present (data not shown).

We also analyzed the binding properties of the Cys148 and Cys154 mutants with the more sensitive centrifugation method that was used to demonstrate two NPG binding sites on lac permease (Lolkema & Walz, 1990). The method allows reliable measurement of a few micromolar bound NPG at total NPG concentrations up to 500 μM. As shown previously (Lolkema & Walz, 1990) and in Table III, wild-type lac permease binds NPG with a dissociation constant for the high-affinity binding site of 16 μM in purified RSO membrane vesicles. In spite of the higher sensitivity of the method, we could not detect NPG binding to the RSO membrane vesicles containing the C148S mutant up to concentrations of 500 μM NPG. Similar lack of NPG binding was observed with RSO vesicles containing C154S permease. Thus, replacements of either Cys148 or Cys154 with Ser appears to disrupt the high-affinity binding site for NPG. Very remarkably, replacement of Cys154 for Gly or Val seemed to increase the affinity of the permease for NPG drastically ($K_p = 5 \mu M$ and 0.8 μM, respectively). Binding of NPG to C154G was reported before by Menick et al. (1985).

**DISCUSSION**

*Lac* permease of *E. coli* contains a total of eight cysteine residues (Figure 1). Previous observations [e.g., van Iwaarden et al. (1991)] and those presented here demonstrate that replacement of Cys117, Cys176, Cys234, Cys333 or Cys353, and Cys355 with Ser has no significant effect on active transport, equilibrium exchange, or downhill efflux. Therefore, it seems reasonable to conclude that these Cys residues do not play any role in the catalytic mechanism of the permease.

On the other hand, it is clear that mutations in Cys148 or Cys154 have interesting effects on certain aspects of permease activity.

For a long time, it was believed that a cysteine residue located near or at the substrate binding site played an essential role in permease activity because of the classical experiments of Fox and Kennedy (1965), showing substrate protection against N-ethylmaleimide inactivation of the permease. Later, Cys148 was shown to be the residue that is alkylated by N-ethylmaleimide (Beyreuther et al., 1981). However, replacing Cys148 with Ser does not significantly affect active transport (Neuhaus et al., 1985; Sarkar et al., 1986), and it was shown subsequently that C154 is important for pmf-driven lactose transport. Although replacement of Cys154 with Gly abolishes lactose transport, C154S and C154V exhibit about 10% and 30%, respectively, of wild-type activity (Menick et al., 1985; van Iwaarden et al., 1991). The presence of a Cys residue at neither position 148 nor position 154 is essential for catalytic activity of the carrier. Nevertheless, analysis of certain functional changes caused by mutating Cys148 and Cys154 may provide insight into the kinetic mechanism of the permease.

The binding affinity of lac permease for its natural substrate lactose has been inferred from exchange measurements (Lolkema et al., 1991). The affinity constants are in the millimolar range, which makes it difficult to measure binding stoichiometrically. Instead, the substrate analogue NPG for which the permease has an unusually high affinity (micromolar range), is used for binding studies. The effect of mutations of Cys148 and Cys154 on the affinity for NPG depends strongly on the replacement (Table III). In both cases, replacement of Cys with Ser results in loss of binding to undetectable levels. In marked contrast, replacement of Cys154 with Gly or Val results in increased affinity for NPG. Since the hydrophobicity of the side chains increases in the order Ser < Cys < Gly < Val, the data suggest that the hydrophobic nitrophenyl group of NPG might determine to a large extent the affinity of the permease for NPG. This would imply that both Cys154 and Cys148 are components of the NPG binding site and would provide an explanation for the much higher affinity of the permease for NPG relative to lactose. On the other hand, these observations raise the question of whether or not a change in affinity for NPG necessarily reflects a change in affinity for lactose. The present results suggest that this is not the case. On one hand, loss of

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**Table II: Affinities, $K_p$, and Maximal Rates, $V_{max}$, of the Exchange Reactions Catalyzed by Wild-Type, C148S, or C154V Lac Permease Reconstituted into Proteoliposomes**

<table>
<thead>
<tr>
<th>permease</th>
<th>$K_p$ (μM)</th>
<th>$V_{max}$ (μM/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>C148S</td>
<td>0.6</td>
<td>2.6</td>
</tr>
<tr>
<td>C154V</td>
<td>0.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

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**Table III: NPG Binding and Active Transport of Cys148 and Cys154 Mutants of Lac Permease**

<table>
<thead>
<tr>
<th>permease</th>
<th>$K_p$ (μM)</th>
<th>active transport (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>C148S</td>
<td>b</td>
<td>100</td>
</tr>
<tr>
<td>C154S</td>
<td>b</td>
<td>10</td>
</tr>
<tr>
<td>C154G</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>C154V</td>
<td>0.8</td>
<td>30</td>
</tr>
</tbody>
</table>

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* The experiments are described in the legend to Figure 3. a Internal lactose concentration was 3 mM. b Internal lactose concentration was 10 mM.
affinity for NPG in C148S (Table III) is correlated with the loss of one kinetic phase in the lactose\textsubscript{in}/lactose\textsubscript{out} exchange kinetics (Figure 3), suggesting loss of a lactose binding site. On the other hand, the 20-fold increase in the affinity of the C154V mutant for NPG (Table III) is not paralleled by a significant change in the affinity for lactose in the exchange reaction (Table II).

Binding studies and nonequilibrium exchange measurements have revealed two distinct binding sites per Lac permease molecule (monomer) for NPG and lactose (Lolkema & Walz, 1990; Lolkema et al., 1991). The findings that two kinetic phases are observed with respect to external lactose during exchange but only one phase with respect to internal lactose suggest that one site is located on the periplasmic aspect of the permease. The other site would be the transport site that oscillates between the two sides of the membrane. It was proposed (Lolkema & Walz, 1990) that the fixed site might have a regulatory function. Binding of lactose to the external site would speed up the translocation of the catalytic site. The two kinetic pathways observed in the exchange experiments would reflect exchange through the catalytic site with the regulatory site free or occupied. The regulatory site would exhibit low affinity for lactose but high affinity for NPG. Furthermore, it was postulated (Lolkema & Walz, 1990) that the regulatory site might also provide the explanation for the biphasic nature observed for the kinetics of lactose uptake at intermediate values of the proton motive force (Robertson et al., 1980). Both binding of lactose to the regulatory site and the proton motive force would have the same effect on the kinetics of the permease: increasing the rate of the catalytic cycle. The two effects would be complementary, since the overall maximal rate was shown to be independent of the contributions of the high- and low-affinity pathway (Robertson et al., 1980). The present results support the above model in a number of respects that will be discussed below.

Table III lists the dissociation constants of the high-affinity NPG binding site next to the relative activities for active transport of the wild-type permease and a number of Cys148 and Cys154 replacements. There is no correlation between the effects of the mutations on these two parameters. Although it is possible that the lack of correlation can be explained by a complex relationship between the binding affinity of the transport cycle and turnover through this cycle, it is easily explained by the model described above. The changes in the NPG affinity constants would reflect changes in the regulatory site. Since the regulatory site would be the low-affinity lactose site, it would not be occupied under the assay conditions of active transport. Moreover, the effects of binding to the regulatory site would be silent in any case, since active transport is measured under conditions of high proton motive force.

The strongest support for the model described above comes from the analysis of C148S. C148S catalyzes active transport at normal rates but exhibits decreased equilibrium exchange activity. Furthermore, it does not bind NPG with high affinity, which suggests that the regulatory site is defective. The loss of a regulatory site will have a similar effect on the exchange activity. Table III, which is routinely assayed at 10 mM lactose, a concentration sufficient to saturate the regulatory site in the wild-type enzyme. The relative insensitivity of active transport and downhill efflux to replacement of Cys148 with Ser suggests that the changes in the molecule are restricted to the regulatory site.

The binding studies with the Cys154 mutants indicate that the regulatory site is altered in a manner similar to that described for C148S (Table III). These mutants also show diminished equilibrium exchange activity (Table I). However, in contrast to C148S permease, all the Cys154 replacements catalyze \( \Delta \nu \)-driven transport at reduced rates (Kaback, 1987; van Iwaarden et al., 1991). Clearly, in addition to the putative regulatory site, these mutations affect the catalytic cycle. The residual activity of C154V permease was high enough to allow kinetic analysis of the exchange reaction under nonequilibrium conditions catalyzed by this mutant. The biphasic behavior was clearly retained, which correlates with the presence of a high-affinity NPG binding site. The somewhat lower activity of the low-affinity pathway relative to the high-affinity pathway may reflect changes at the regulatory site.

Since the affinities of C154V and wild-type permease are not very different (Table II), the decreased rate of equilibrium exchange and active transport seems to be mainly a consequence of the slowing down of intrinsic steps of the catalytic cycle.

REFERENCES


