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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 slow 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 *Km* 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 *Km* component. Moreover, the absence of the low *Km* pathway in the C148S permease is correlated with the absence of a high-affinity binding site for *p*-nitrophenyl α-D-galactopyranoside (NPG). Interestingly, the affinity of the permease for NPG appears to increase with the hydrophobicy 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 *lac* permease of *Escherichia coli* is a hydrophobic polytopic cytoplasmic membrane protein that catalyzes concomitant translocation of β-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 *lac* gene, has been solubilized from the membrane, purified to homogeneity, and reconstituted into proteoliposomes. It has been demonstrated to be solely responsible for β-galactoside transport probably as a monomer. On the basis of circular dichroism with purified permease and hydrophobic analysis of the primary amino acid sequence, a secondary structure has been proposed in which the protein consists of 12 hydrophobic domains in an α-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; Püttnere, 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–sulfhydryl interchange is probably not involved in regulation of permease activity (Konings & Robillard, 1982). 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 Δψ-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 α-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<sub>in</sub>/lactose<sub>out</sub> 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

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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).
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[6-2H]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-β-D-galactopyranoside (IPTG) as described 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.

**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 sonicated 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 t½ 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.
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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>&lt;5</td>
<td>1</td>
</tr>
<tr>
<td>C17S</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>

* Exchange and efflux were measured as described in the legend to Figure 2.

RESULTS

Efflux and Equilibrium Exchange. When proteoliposomes reconstituted with the purified wild-type permease were loaded with 10 mM [1-14C]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, (Garcia et al., 1983)]. The purified C148S permease catalyzes exchange at a significantly slower rate, both at pH 5.5 (Figure 2A, O) and at pH 7.5 (Table I). At pH 7.5 the $t_{1/2}$ of exchange of the C148S permease is 37 s as compared to less than 6 s for the wild-type permease. At pH 5.5 the $t_{1/2}$ of exchange is 60 s for the C148S permease and <5 s for the wild-type permease. The lower activity of the C148S mutant in the exchange reaction is in marked contrast to the rate of A-$\Delta$-driven active transport with this mutant, which was shown to be comparable to the activity of wild-type permease (Sarkar et al., 1986; Van Iwaarden et al., 1991). The rate of efflux at pH 7.5 (Figure 2B) with C148S permease is reduced only 2-fold (O) relative to wild-type permease (O). The rate of efflux with both C148S and wild-type permease is very slow at pH 5.5 (Figure 2A, $\Delta$ and $\Delta$, respectively), as shown previously for the wild-type permease (Garcia et al., 1983).

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 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,
vesicles. In spite of the higher sensitivity of the method, we
affinity binding site of 16 pM in purified RSO membrane
reliable measurement of a few micromolar bound NPG at
that was used to demonstrate two NPG binding sites on
permease (Lolkema et al., 1991). The data on active
transport represent initial rates of ΔV-driven lactose uptake taken from

Table II: Affinities, \( K_d \), 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_d ) (mM)</th>
<th>( V_{max} ) (μM/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.4 and 3</td>
<td>0.6 and 2.6</td>
</tr>
<tr>
<td>C148S permease</td>
<td>0.5 and 1.9</td>
<td>0.4 and 0.9</td>
</tr>
<tr>
<td>C154V permease</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The experiments are described in the legend to Figure 3. \(^1\) Internal
lactose concentration was 3 mM. \(^2\) Internal lactose concentration was 10
mM.

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

<table>
<thead>
<tr>
<th>permease</th>
<th>( K_d ) (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>

\(^*\) Binding of NPG to RSO vesicles was measured by an improved
centrifugation method (Lolkema & Walz, 1990). The data on active
transport represent initial rates of ΔV-driven lactose uptake taken from
van Iwaarden et al. (1991). \(^\ast\) Undetectable.

with apparent affinity constants, \( K_d \), of 0.6 mM and 2.6 mM
(Figure 3, A; Table II) at an internal concentration of 10 mM
[\(^1\)\(^4\)C]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 \(^3\)H[NPG results in an increase in the
dialyzable concentration of the ligand. The increase reflects
the amount of \(^3\)H[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 (\( K_d = 5 \) μM 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
affinity for NPG in C148S (Table III) is correlated with the loss of one kinetic phase in the lactose$_{in}$/lactose$_{out}$ 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 protonmotive force (Robertson et al., 1980). Both binding of lactose to the regulatory site and the protonmotive 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 protonmotive 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 has no effect on active transport for reasons discussed above. The loss of the regulatory site is also in line with the loss of a kinetic phase in lactose$_{in}$/lactose$_{out}$ exchange with respect to the external lactose concentration (Figure 3). The remaining phase has the characteristics of exchange catalyzed by wild-type permease with an unoccupied regulatory site (low $K_a$ and low $V_{max}$; Table II). Apparently, C148S permease cannot be shifted to the high-activity exchange pathway by external substrate. This explains the decreased activity of the mutant with respect to equilibrium exchange (Table I), 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 $\Delta$V-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