The lactose (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) (cf. Refs. 1-4 for reviews). Encoded by the lacY gene that has been cloned and sequenced, this prototype membrane transmembrane, purified, and reconstituted into proteoliposomes. The transport activity of proteoliposomes reconstituted with each mutant permease relative to the wild-type is virtually identical with that reported for intact cells and/or right-side-out membrane vesicles. Moreover, a double mutant containing Ser in place of both Cys¹⁴⁸ and Cys¹⁵⁴ exhibits significant ability to catalyze active lactose transport. The results provide strong confirmation for the contention that cysteinyl residues in lac permease do not play an important role in the transport mechanism. The effect of sulfhydryl oxidant 5-hydroxy-2-methyl-1,4-naphthoquinone on lactose transport in proteoliposomes reconstituted with wild-type or mutant permeases was also investigated, and the results indicate that inactivation is probably due to formation of a covalent adduct with Cys¹⁴⁸ and/or Cys¹⁵⁴ rather than disulfide formation. Thus, it seems unlikely that sulfhydryl-disulfide interconversion functions to regulate permease activity.

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1 In addition to circular dichroism and laser Raman spectroscopy, Fourier transform infrared studies also show that purified lac permease is largely helical (P. D. Roepke, H. R. Kaback, and K. J. Rothschild, unpublished work).

2 The abbreviations used are: plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone; DTT, dithiothreitol.
or replacement of Cys$^{148}$ with Gly leads to inactivation. Furthermore, Cys$^{148}$ and Cys$^{154}$ are both located in putative transmembrane helix V (Fig. 1) and are predicted to be on the same face. Therefore, an attractive explanation for inactivation by sulfhydryl oxidants is catalysis of disulfide formation between these 2 residues. In order to test this possibility, we have solubilized and purified each site-directed Cys mutant in the permease, as well as a double mutant containing Ser in place of both Cys$^{148}$ and Cys$^{154}$, and studied transport activity and inactivation by plumbagin in reconstituted proteoliposomes. The results indicate that permease inactivation by the sulfhydryl oxidant results from formation of a covalent adduct with either Cys$^{148}$ or Cys$^{154}$ and not from disulfide formation.

**EXPERIMENTAL PROCEDURES**

**Materials**

[1-$^1$C]Lactose (57 mCi/mmol) was obtained from Amersham (Buckinghamshire, U. K.). All other reagents were obtained as described (32, 33) and were reagent grade.

**Methods**

Bacterial Strains and Plasmids—E. coli T206, E. coli T184, and plasmid pGM21 encoding wild-type lac-Y (34) or lac-Y with site-directed mutants in single Cys codons (25-31) have been described.

Construction of C148S/C154S Double Mutant—Starting with the M13 mp18 (lac-Y) DNA containing a Ser codon at position 148 (25), the double mutant containing C148S/C154S was constructed as described by Menick et al. (29). Subsequently, mutations were confirmed by sequencing the entire lac-Y gene in single-stranded M13 mp18 DNA and the region containing the mutations in plasmid pGM21. In both cases, with the exception of the mutations described, the remainder of the lac-Y sequence was identical with that reported by Bichel et al. (35).

Cell Growth—E. coli T184 transformed with a given plasmid was grown and induced with isopropyl-1-thio-β-D-galactopyranoside as described by Teather et al. (34), with the exception that 0.2-0.5% of the residue in the wild-type lac permease. This is followed by a second letter denoting the amino acid replacement at this position (e.g. C148S designates that Cys$^{148}$ is replaced by Ser).

**RESULTS**

**ΔΨ-Driven Lactose Accumulation**—As demonstrated previously (39-42), proteoliposomes reconstituted with purified lac permease rapidly accumulate lactose against a concentration gradient in the presence of the potassium diffusion gradient ($K_{\text{in}} \rightarrow K_{\text{out}}$) and the ionophore valinomycin (Fig. 2). Under identical conditions, proteoliposomes reconstituted with purified C117S, C148S, C176S, C234S, C333S, or C353S/C355S transport lactose at initial rates to steady-state levels of accumulation that are comparable with proteoliposomes reconstituted with wild-type permease (cf. Table I for quantitation of initial rates relative to wild-type). In contrast, proteoliposomes reconstituted with C148G, C154S, or C154V transport lactose at initial rates that are about 15, 10, and 35% respectively, of wild-type proteoliposomes (Fig. 2 and Table I). In each instance, the level of accumulation continues to increase as the time course of the experiments is extended (Fig. 2; data not shown). Finally, proteoliposomes reconstituted with C148S/C154S permease transport at about 5% the rate of wild-type but continue to accumulate the disaccharide with time, whereas proteoliposomes reconstituted with C154G...
Cysteine Mutants of lac Permease

FIG. 2. Membrane potential (ΔΨ)-driven lactose transport in proteoliposomes reconstituted with purified wild-type (○), C148G (◆), C154G (●), C154S (□), or C148S/C154S (▲) lac permease. lac permease was purified from membranes of E. coli T206 or T184 transformed with the appropriate plasmid and reconstituted into proteoliposomes at a protein concentration of 85 μg/ml (wild-type), 75 μg/ml (C148G), 45 μg/ml (C154G), 70 μg/ml (C154S), 65 μg/ml (C154V), or 40 μg/ml (C148S/C154S). Lactose transport was measured as described (32) by diluting 1 μl of proteoliposomes containing 20 μM valinomycin into 200 μl of 50 mM sodium phosphate (pH 7.5) containing 35 μM [1-14C]lactose (57 mCi/mmol). Control experiments (△) were performed by diluting 1 μl of proteoliposomes into 50 mM potassium phosphate (pH 7.5).

TABLE I

Initial rate of ΔΨ-driven lactose transport in proteoliposomes reconstituted with wild-type or mutant lac permease

<table>
<thead>
<tr>
<th>Permease</th>
<th>Initial rate of transport</th>
<th>% of wild-type activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C117S</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>C148S</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>C148G</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>C154S</td>
<td>10</td>
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</tr>
<tr>
<td>C154G</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C154V</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>C148S/C154S</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>C176S</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C234S</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>C333S</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>C353S/C355S</td>
<td>61</td>
<td>61</td>
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</tbody>
</table>

* Initial rates of transport were calculated from the initial linear portion of transport curves (cf. Fig. 2).

The turnover number for wild-type permease in this preparation was 16 s⁻¹.

peptidase are essentially devoid of transport activity (Fig. 2; Table I).

Effect of Plumbagin—Treatment of right-side-out membrane vesicles with the sulfhydryl oxidant plumbagin at a concentration of 0.5 mM leads to complete inactivation of lactose transport in a manner that can be restored by 10 mM DTT (22). Similar phenomena are observed in proteoliposomes reconstituted with purified lac permease. Thus, ΔΨ-driven lactose transport is progressively inactivated by relatively low concentrations of plumbagin, and maximal inactivation is observed at about 0.5 mM (Figs. 3 and 4A). Moreover, when 10 mM DTT is added after treatment of the proteoliposomes with plumbagin, transport is restored to a great extent (Fig. 4A; Table II). Parenthetically, when the proteoliposomes are washed and resuspended in buffer without addition of DTT, lac permease is apparently slightly inactivated by air oxidation, as evidenced by the observation that addition of DTT by itself causes a small stimulation of transport activity.

Results of similar experiments carried out on proteoliposomes reconstituted with each mutant permease are summarized in Table II. The initial rate of ΔΨ-driven lactose transport in proteoliposomes reconstituted with C117S, C148S, C154V, C176S, C234S, C333S, or C353S/C355S is essentially completely inactivated by treatment with 0.5 mM plumbagin, and activity is restored by addition of 10 mM DTT in each instance. In contrast, C148G or C154S permease is inactivated by only about 50%, and strikingly, C148S/C154S permease...
port by proteoliposomes reconstituted with each site-directed modification of either CYS\textsubscript{117} or CYS\textsubscript{148} alone can lead to inactivation. The sulfhydryl oxidant catalyzes the formation of disulfide bonds, sulfhydryl-disulfide interconversion probably does not have a regulatory function (22). Since alkylation of CYS\textsubscript{117} or any of the other cysteinyl residues in the permease molecule containing mutations at each individual cysteinyl residue exhibit transport activities relative to wild-type proteoliposomes that are virtually identical with those reported for intact cells and/or right-side-out membrane vesicles. Specifically, purified permease with C117S, C148S, C176S, C234S, C333S, or C353S/C355S exhibits any small if any defects in \(\Delta\Psi\)-driven lactose accumulation, permease with C148G, C154S, or C154V exhibits reduced rates of lactose uptake, and C154G peptide is devoid of transport activity. Importantly, the double mutant C148S/C154S exhibits low but significant activity. Clearly, therefore, although Cys\textsuperscript{148} is important for substrate protection against N-ethylmaleimide inactivation (18, 20), Cys\textsuperscript{154} is the only cysteinyl residue in the permease that is important for activity; but even this residue is not obligatory.

Although cysteinyl residues do not play a direct role in the mechanism of lac permease, based on the observation that sulfhydryl oxidants reversibly inactivate lactose/H\textsuperscript{+} symport, it was suggested that sulfhydryl-disulfide interconversion may have a regulatory function (22). Since alkylation of Cys\textsuperscript{148} or replacement of Cys\textsuperscript{154} with Gly leads to inactivation and these residues should be on the same face of putative helix V, the effect of the sulfhydryl oxidant plumbagin on lactose transport by proteoliposomes reconstituted with each site-directed Cys mutant was investigated. As discussed, one possible mechanism of inactivation is that the sulfhydryl oxidant catalyzes disulfide formation between Cys\textsuperscript{148} and Cys\textsuperscript{154}. Alternatively, since modification of either Cys\textsuperscript{148} or Cys\textsuperscript{154} alone can lead to inactivation, it is possible that disulfide formation between either of these residues and another cysteinyl residue in the permease causes inactivation. Finally, sulfhydryl oxidants may undergo “half-reactions” with cysteinyl residues (21, 43, 44), thereby forming sulfhydryl adducts without catalyzing disulfide formation. Our results favor the last possibility for the following reasons. (i) Plumbagin treatment leads to a marked inactivation of C117S, C176S, C234S, C333S, or C353S/C355S permease, suggesting that none of these cysteinyl residues is involved in the phenomenon. Consequently, it is unlikely that mixed disulfide formation between Cys\textsuperscript{148} and Cys\textsuperscript{154} and any of the other cysteinyl residues in the permease is responsible for inactivation. (ii) The double mutant C148S/C154S, which has a low but significant activity, is resistant to inactivation by plumbagin, thereby demonstrating directly that Cys\textsuperscript{148} and/or Cys\textsuperscript{154} are the residues involved. (iii) The single mutants C148S and C154V are essentially completely inactivated by the sulfhydryl oxidant, whereas C148G or C154S permease are partially inactivated. Thus, a single cysteinyl residue at either of these positions is sufficient for inactivation by plumbagin, a result not expected if disulfide formation between the residues is required for inactivation.

In conclusion, since it is unlikely that permease inactivation by sulfhydryl oxidants involves the formation of disulfide bonds, sulfhydryl-disulfide interconversion probably does not have a regulatory function in the activity of this transport protein.

Acknowledgments—We wish to thank Dr. J. S. Lolkema and Dr. M. G. P. Page for helpful discussions and M. Y. D. van Iwaarden for typing the manuscript.

REFERENCES

Table II

<table>
<thead>
<tr>
<th>Permease</th>
<th>Residual activity after plumbagin inhibition</th>
<th>Restoration of activity by DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>C117S</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>C148S</td>
<td>8</td>
<td>85</td>
</tr>
<tr>
<td>C148G</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>C154S</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>C154V</td>
<td>7</td>
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</tr>
<tr>
<td>C148S/C154S</td>
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<td>100</td>
</tr>
<tr>
<td>C176S</td>
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<td>C234S</td>
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<td>C333S</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>C353S/C355S</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

The data presented in this paper provide strong confirmation of the conclusion that cysteinyl residues in lac permease do not play a direct role in the mechanism of lactose/H\textsuperscript{+} symport (2, 25-31). Proteoliposomes reconstituted with purified permease molecules containing mutations at each individual cysteinyl residue exhibit transport activities relative to wild-type proteoliposomes that are virtually identical with those reported for intact cells and/or right-side-out membrane vesicles.

DISCUSSION

The inhibition of the initial rate of \(\Delta\Psi\)-driven lactose transport with 0.5 mM plumbagin in proteoliposomes reconstituted with mutant permeases and the restoration of activity by DTT

Initial rates of lactose transport were measured as in Table I. Proteoliposomes were treated with plumbagin and DTT as described in the legend to Fig. 4.

(Fig. 4B) is only marginally inactivated by the sulfhydryl oxidant. Although the transport activity of C148G, C154S, or C148S/C154S permease is low, accumulation of lactose in proteoliposomes reconstituted with these permeases clearly increases with time before and after treatment with plumbagin (Fig. 4B). In contrast, proteoliposomes reconstituted with wild-type permease or any of the other mutants exhibit no accumulation of lactose after treatment with plumbagin (Fig. 4A).
Cysteine Mutants of lac Permease