Functional Conservation in the Putative Substrate Binding Site of the Sucrose Permease from *Escherichia coli*†

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Received January 20, 2000; Revised Manuscript Received March 24, 2000

ABSTRACT: The sucrose (CscB) permease is the only member of the oligosaccharide:H^+ symporter family in the Major Facilitator Superfamily that transports sucrose but not lactose or other galactosides. In lactose permease (lac permease), the most studied member of the family, three residues have been shown to participate in galactoside binding: Cys148 hydrophobically interacts with the galactosyl ring, while Glu126 and Arg144 are charge paired and form H-bonds with specific galactosyl OH groups. In the present study, the role of the corresponding residues in sucrose permease, Asp126, Arg144, and Ser148, is investigated using a functional Cys-less mutant (see preceding paper). Replacement of Ser148 with Cys has no significant effect on transport activity or expression, but transport becomes highly sensitive to the sulfhydryl reagent *N*-ethylmaleimide (NEM) in a manner similar to that of lac permease. However, in contrast to lac permease, substrate affords no protection whatsoever against NEM inactivation of transport or alkylation with [^{14}C]-NEM. Neutral (Ala, Cys) mutations of Asp126 and Arg144 abolish sucrose transport, while membrane expression is not affected. Similarly, combination of two Ala mutations within the same molecule (Asp126→Ala/Arg144→Ala) yields normally expressed, but completely inactive permease. Conservative replacements result in highly active molecules: Asp126→Glu permease catalyzes sucrose transport comparable to Cys-less permease, while mutant Arg144→Lys exhibits decreased but significant activity. The observations demonstrate that charge pair Asp126-Arg144 plays an essential role in sucrose transport and suggest that the overall architecture of the substrate binding sites is conserved between sucrose and lac permeases.

The sucrose (CscB) permease is the only transporter in the six-member oligosaccharide:H^+ symporter (OHS)[1] family of the Major Facilitator Superfamily (MFS) that transports sucrose but not lactose and/or other galactosides (*1*−*3*). This remarkable substrate specificity difference makes it an ideal candidate for comparative analysis of sugar binding. In the preceding paper (*4*) construction of a fully functional Cys-less sucrose permease was described, and in the present study this molecule is utilized in order to investigate the role of amino acid residues in the putative substrate binding site.

Lactose permease (lac permease) of *Escherichia coli* is the best characterized member of the OHS family, and it exhibits 30% homology with the sucrose permease. On the basis of sugar specificity studies (*5*−*7*), as well as detailed analysis of mutants[2] at Cys148 (*8*, *9*), Glu126, and Arg144 (*10*−*13*), the following model for galactoside binding was recently postulated (*7*, *11*): (i) Arg144 forms H-bonds with the OH groups at the C-3 and C-4 positions of the galactosyl moiety of the substrate, an interaction that plays a key role in substrate specificity. (ii) In addition, Arg144 forms a salt bridge with Glu126, and the interaction holds Arg144 and Cys148 in an orientation that allows specific interaction with the galactosyl moiety. (iii) One of the oxygen atoms of the carboxylate at position 126 acts as an H-bond acceptor from the C-6 OH of the galactosyl moiety. (iv) Cys148, which is protected by substrate against alkylation by *N*-ethylmaleimide (NEM), interacts hydrophobically with the galactosyl end of lactose and other galactosides. Although interactions with the non-galactosyl moiety are not clearly understood, Met145, which is on the same face of helix V as Cys148, is thought to be important in this respect, particularly with respect to α anomers (*7*).

† This work was supported in part by NIH Grant DK51131 to H.R.K. Part of this work was carried out by M.S.-T. at the Department of Physiology, Semmelweis University, Budapest, Hungary, supported by Hungarian Research Fund (OTKA) Grant T-019021.

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[1] Abbreviations: lac permease, lactose permease; NEM, *N*-ethylmaleimide; KP_i, potassium phosphate; DTT, dithiothreitol; PMS, phenazine methosulfate; OHS family, oligosaccharide:H^+ symporter family; MFS, Major Facilitator Superfamily; DDM, dodecyl β-D-maltopyranoside; RSO vesicles, right-side-out membrane vesicles; BAD, biotin acceptor domain.

[2] Site-directed mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in the wild-type sucrose permease, followed by a second letter indicating the amino acid replacement. For easier comparison, numbering of residues in the lac permease is used to designate residues Asp126, Arg144, and Ser148 in sucrose permease throughout the paper; the corresponding amino acid positions in the CscB sequence are Asp129, Arg147, and Ser151, respectively.

10.1021/bi000125g CCC: $19.00
Published on Web 04/27/2000

Charge pair Glu126 and Arg144 is present in each galactoside transporter of the OHS family, while sucrose permease contains an Asp side chain at position 126 with Arg144 conserved. Similarly, Cys148 is present in all five galactoside transporters, but it is replaced by a Ser residue in sucrose permease [see Figure 1 of the preceding paper (*4*)]. It seems reasonable to speculate that the Glu126→Asp and Cys148→Ser changes may play a role in the unique substrate specificity of sucrose permease among the members of the OHS family. In this context, it is noteworthy that both Glu126→Asp (*12*) and Cys148→Ser (*8*, *14*−*17*) mutations in lac permease have been shown to decrease binding affinity, although lactose transport activity is not impaired significantly.

In the present study, the function of the putative binding site residues, Asp126, Arg144, and Ser148, in sucrose permease was investigated by site-directed mutagenesis. The results indicate that the Asp126-Arg144 charge pair plays an essential role in sucrose transport, while a direct interaction between Ser148 and sucrose cannot be demonstrated.

EXPERIMENTAL PROCEDURES

Materials. [^{14}C(U)]Sucrose was purchased from DuPont NEN (Boston, MA). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C-terminus of lac permease was prepared by BabCo (Richmond, CA). All other materials were of reagent grade and were obtained from commercial sources.

Construction of Affinity Tags in Cys-less Permease. Insertion of six His residues before the C-terminal LacY epitope sequence was carried out by linker mutagenesis. A linker containing codons for six His and the C-terminal dodecapeptide from lac permease was synthesized and ligated into the pSP72/*cscB*/Cys-less plasmid between the *Xho*I and *Hin*dIII restriction sites [see Figure 1 in the preceding paper (*4*)]. A minimal recognition sequence for biotin ligase (*18*) (Biotag; see exact sequence in ref *19*) was engineered using a similar strategy. Due to a fortuitous frame shift, in addition to the 13-residue Biotag, this construct contains an additional 14 amino acids at its C-terminus. The 95 amino acid biotin acceptor domain (BAD) of an oxaloacetate decarboxylase from *Klebsiella pneumoniae* (*20*) was PCR amplified from a plasmid carrying the *lacY* gene and a BAD sequence at its 3′ end (*21*). The PCR product was digested with *Xho*I and *Hin*dIII and ligated into the similarly treated pSP72/*cscB*/Cys-less vector.

Construction of Mutants. Mutations D126A, D126C, D126E, and S148C were introduced into Cys-less sucrose permease by oligonucleotide-directed site-specific mutagenesis using the overlap−extension PCR method (*22*). Mutations R144A, R144C, and R144K were constructed by one-step PCR mutagenesis. The PCR products carrying the mutations were digested with *Pst*I−*Bam*HI (D126 and R144 mutants) and *Pst*I−*Kpn*I [S148C; in this mutant the mutation destroys the unique *Bam*HI site; see Figure 1 in the preceding paper (*4*)] and ligated into the similarly treated pSP72/*cscB*/Cys-less expression vector. The entire PCR products were DNA sequenced through the ligation junctions. Double mutant D126A/R144A was constructed by introducing the R144A mutation into the D126A mutant as described above.

Growth of Bacteria, Sucrose Transport Assays, NEM Inhibition and Substrate Protection Experiments, Western Blot Analysis, and Protein Determinations. These experimental procedures were carried out exactly as described in the preceding paper (*4*).

Preparation of Right-Side-Out (RSO) Membrane Vesicles. *E. coli* T184 expressing S148C permease with a BAD at the C-terminus were grown in Luria−Bertani broth, and RSO membrane vesicles were prepared as described previously *(23)* with the following modification: to prevent Cys oxidation during vesicle preparation, 5 mM dithiothreitol (DTT) was included in all buffers. At the end of the preparation, vesicles were washed with 100 mM potassium phosphate (KP_i; pH 7.5) to remove the DTT, resuspended in the same buffer at a protein concentration of 15 mg/mL, frozen in liquid N_2, and stored at −80 °C until use.

[^{14}C]NEM Labeling. Reactivity of Cys148 with [^{14}C]NEM in situ was determined in the absence and presence of sucrose as described in (*7*, *12*, *24*). The S148C permease mutant used for the assays contains a BAD at the C-terminus and is biotinylated in vivo. RSO membrane vesicles were preequilibrated in a final volume of 50 μL (containing 0.75 mg of protein) with given sugar concentrations for 10 min at room temperature. Labeling was initiated by addition of [^{14}C]NEM to the indicated final concentrations, and the vesicles were incubated for 5 min at 25 °C or the temperature indicated. Reactions were quenched by addition of 10 mM DTT (final concentration). The vesicles were solubilized with 2% dodecyl β-D-maltopyranoside (DDM, final concentration), and the samples were mixed with immobilized monomeric avidin (avidin−Sepharose) equilibrated with 50 mM NaP_i (pH 7.5)/0.1 M NaCl/0.02% DDM (w/v). After a 15 min incubation at 4 °C, the resin was washed with 5 mL of equilibration buffer, and biotinylated permease was then eluted with 2 × 50 μL of equilibration buffer containing 5 mM *d*-biotin. After addition of 25 μL of electrophoresis sample buffer (concentrated 5×), an 100 μL aliquot was analyzed electrophoretically on a sodium dodecyl sulfate ($NaDodSO_4$)−12% polyacrylamide gel. The gel was dried and exposed to a PhosphoImager screen for 5−8 days. Incorporation of [^{14}C]NEM was visualized and quantitated by a Storm 860 PhosphoImager (Molecular Dynamics). An aliquot (10−20 μL) of purified [^{14}C]NEM-labeled permease was electrophoresed on a $NaDodSO_4$−12% polyacrylamide gel, electroblotted to poly(vinylidene diflouride) membranes (Immobilon-PVDF, Millipore), and treated with horseradish peroxidase conjugated avidin. The PVDF membrane was subsequently developed with fluorescent substrate (Renaissance, DuPont NEN) and exposed to film.

RESULTS

Engineering Cys-less Permease for Affinity Purification. To facilitate site-directed [^{14}C]NEM labeling of Cys mutants, various affinity tags were engineered onto the C-terminus of Cys-less permease. Sucrose permease encoded by the pSP72/*cscB* expression vector contains a 12-residue epitope at the C-terminus, corresponding to the C-terminal dodecapeptide of lac permease against which antibodies are available (*3*). To construct a permease suitable for metal chelation chromatography, a six His sequence was inserted prior to the LacY epitope. Unexpectedly, sucrose permease carrying a 6His−LacY epitope extension at its C-terminus exhibits diminished transport activity compared to the parent

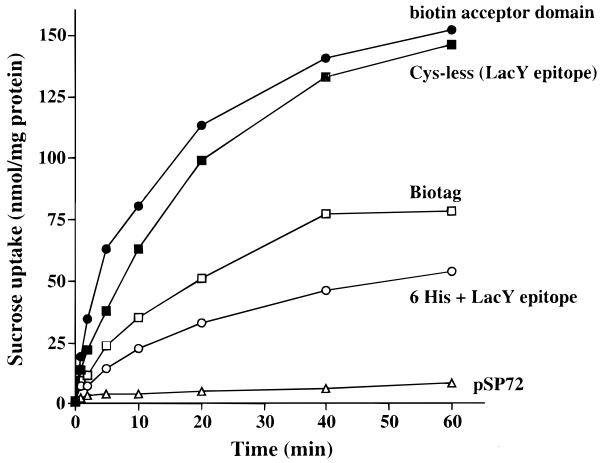

FIGURE 1: Transport activity of Cys-less sucrose permease carrying various affinity tags. Time courses of active sucrose transport by *E. coli* T184 expressing Cys-less sucrose permease with the LacY epitope, no permease (pSP72 vector only), or given mutants. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL, containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at 0.4 mM final external sucrose concentration as described in Experimental Procedures. Biotin acceptor domain: permease with a 95-residue biotination domain from a *K. pneumoniae* oxaloacetate decarboxylase at the C-terminus. Biotag: permease containing a 13-residue biotination sequence. 6His + LacY epitope: permease with a six His tag inserted before the LacY epitope at the C-terminus. See text for details.

construct, Cys-less with LacY epitope (Figure 1). Western blot analysis of membrane fractions reveals significantly decreased permease expression, suggesting increased proteolytic breakdown of this construct (not shown). Similar instability and decreased activity are observed with a fortuitous construct, where the LacY epitope was replaced with a 27 amino acid sequence containing a 13-residue minimal recognition sequence for biotin ligase (*18*, *19*). Finally, a 95 amino acid long biotin acceptor domain (BAD) of an oxaloacetate decarboxylase from *K. pneumoniae* (*20*, *21*) was engineered in place of the LacY epitope. Permease carrying BAD at its C-terminus exhibits high transport activity, and the in vivo biotinated species is easily detectable in the membrane using horseradish peroxidase conjugated avidin (see Figure 3 inset).

Mutant Ser148→Cys Is Highly Sensitive to NEM. Replacement of Ser148 with Cys in sucrose permease has no significant effect on transport activity or membrane expression. Thus, S148C permease accumulates sucrose essentially as well as Cys-less permease (Figure 2A), and permease levels in the membrane are indistinguishable from those of Cys-less (not shown). Comparison of transport−kinetic parameters reveals an essentially unchanged K_m [5.9 ± 1 mM (SE, $n = 4$)] and a somewhat decreased V_{max} [126 ± 6 nmol min^{-1} (mg of protein)$^{-1}$ (SE, $n = 4$)] relative to Cys-less permease [K_m 4.8 mM, V_{max} 192 nmol min^{-1} (mg of protein)$^{-1}$; see preceding paper (*4*)]. In sharp contrast to Cys-less permease, which is resistant to inhibition by NEM, mutant S148C is completely inactivated by the alkylating reagent (Figure 2B). As indicated, at 1 mM NEM concentration the reaction rate is too rapid to determine an accurate $t_{1/2}$ value. For comparison, NEM inhibition of wild-type sucrose permease is also shown, where the $t_{1/2}$ is approximately 1.5 min [see also Figure 3A in the preceding paper (*4*)]. When inhibition is tested with 0.1 mM NEM,

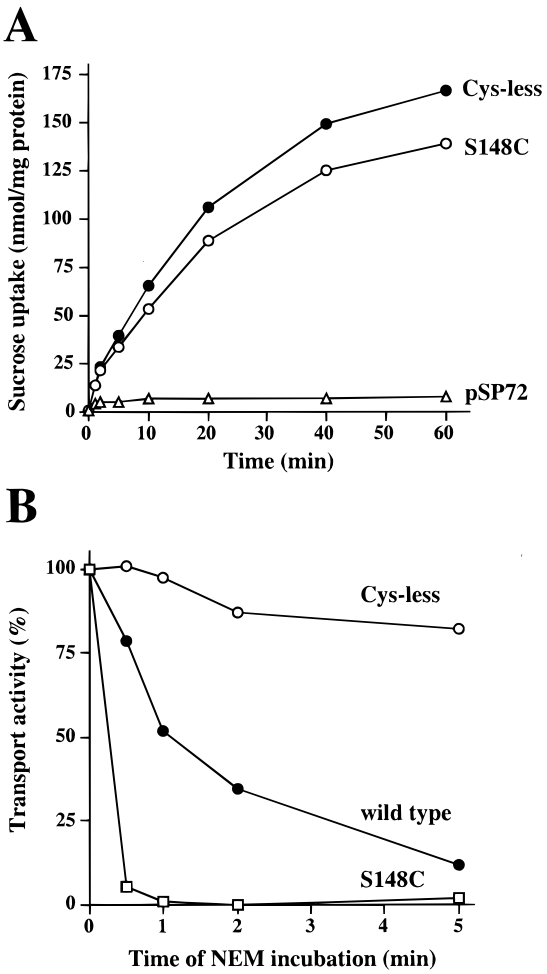

FIGURE 2: Transport activity and NEM inhibition of S148C permease. (A) Time courses of active sucrose transport by *E. coli* T184 expressing Cys-less sucrose permease, no permease (pSP72 vector only), or mutant S148C. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL, containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at 0.4 mM final external sucrose concentration as described in Experimental Procedures. (B) Effect of NEM on sucrose transport by *E. coli* T184 harboring plasmids encoding wild-type sucrose permease, Cys-less sucrose permease, or mutant S148C. Cells were incubated with 1 mM NEM at room temperature for the indicated times, the reaction was stopped with DTT, and cells were assayed for initial rates of sucrose uptake in the presence of reduced phenazine methosulfate (PMS) as described in Experimental Procedures.

half-maximal inactivation is observed around 20−25 s incubation time (Figure 3).

Sucrose Does Not Protect S148C Permease against NEM Alkylation. Sucrose, the only known substrate of sucrose permease, affords no protection whatsoever against NEM inhibition over a concentration range from 50 to 200 mM. In contradistinction, at 200 mM sucrose slight stimulation of inactivation is observed (Figure 3). In an effort to directly visualize the reaction of Cys148 with NEM and study the effect of sucrose, mutation S148C was subcloned into the expression vector containing a BAD at the C-terminus (see Figure 1), and labeling with [^{14}C]NEM was studied. In lac permease, at 25 °C, labeling with 0.5 mM [^{14}C]NEM is linear for up to 10 min at pH 7.5, and the reaction saturates at 10−15 min (*12*, *24*). Surprisingly, reaction of Cys148 in sucrose permease with NEM is much more rapid and

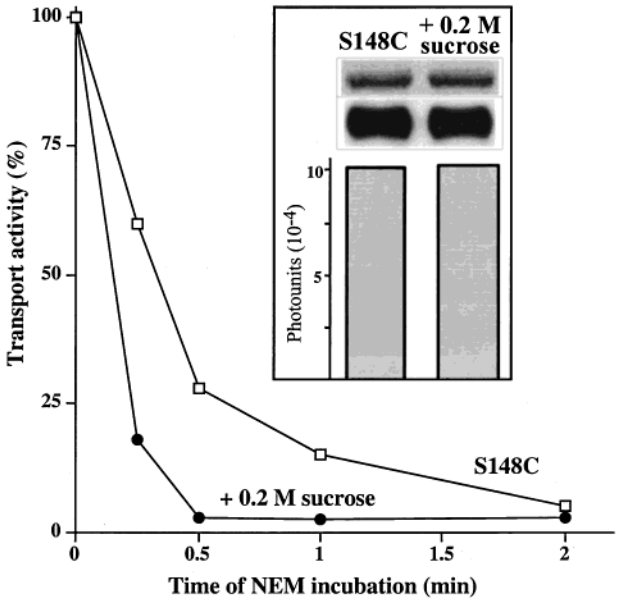

FIGURE 3: Effect of sucrose on reaction of S148C permease with NEM. Cells expressing S148C permease were incubated with 0.1 mM NEM at room temperature for the indicated times in the absence or presence of 200 mM sucrose. The reaction was stopped with DTT, and cells were washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and assayed for initial rates of sucrose uptake in the presence of reduced PMS as described in Experimental Procedures. (Inset) Effect of sucrose on [^{14}C]NEM labeling of S148C permease. RSO vesicles containing S148C permease with a biotin acceptor domain at the C-terminus were incubated in 100 mM KP_i (pH 7.5) with 0.1 mM [^{14}C]NEM at 25 °C for 5 min in the absence or presence of 200 mM sucrose. Reactions were quenched by DTT, and biotinylated permease was solubilized and purified by affinity chromatography on monomeric avidin. Aliquots of protein were separated on a 12% $NaDodSO_4$−polyacrylamide gel, and ^{14}C-labeled protein was visualized by autoradiography (upper panel). A fraction of the protein was analyzed by Western blotting using horseradish peroxidase conjugated avidin in order to determine the amount of permease in each sample (lower panel). Incorporation of [^{14}C]NEM was quantitated by a Storm 860 PhosphoImager, and labeling was expressed in arbitrary photounits.

saturates within 1 min, even at NEM concentrations as low as 50 μM. Sucrose (50−200 mM) has no appreciable effect on labeling with 0.05−0.5 mM [^{14}C]NEM at pH 7.5, 25 °C (Figure 3 inset). To slow the labeling reaction, experiments were carried out also at 4 °C and at pH 5.5 with identical results.

Asp126 and Arg144 Are Essential for Sucrose Transport. Replacement of Asp126 or Arg144 with Ala or Cys (mutants D126A, D126C, R144A, and R144C) completely inactivates sucrose permease (Figure 4). Similarly, the D126A/R144A double mutant is inactive. In contrast, conservative replacements at these positions yield highly active transporters; D126E permease accumulates sucrose slightly better than Cys-less permease, while mutant R144K exhibits ca. 50% activity of Cys-less. Analysis of kinetic parameters of D126E permease indicates a moderately lower K_m for sucrose [2.7 ± 0.5 mM (SE, $n = 4$)] and a slightly decreased V_{max} [160 ± 8 nmol min^{-1} (mg of protein)$^{-1}$ (SE, $n = 4$)] relative to Cys-less permease [see preceding paper (*4*)]. Mutant R144K exhibits a K_m similar to that of D126E permease [3.6 ± 0.5 mM (SE, $n = 3$)], while the V_{max} value is more significantly decreased [92 ± 4 nmol min^{-1} (mg of protein)$^{-1}$ (SE, $n = 3$)]. Immunoblot analysis of membrane fractions reveals that levels of mutants R144A and R144C are slightly elevated,

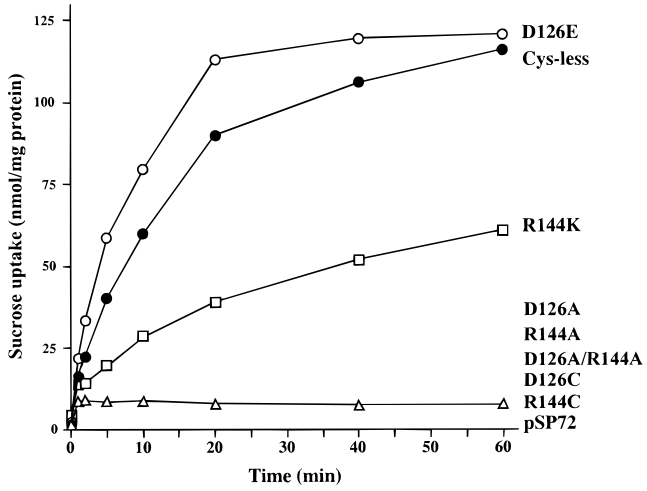

FIGURE 4: Transport activity of sucrose permease mutants at Asp126 and Arg144. Time courses of active sucrose transport by *E. coli* T184 expressing Cys-less sucrose permease, no permease (pSP72 vector only), or given mutants were assayed as described in Figure 1 and Experimental Procedures.

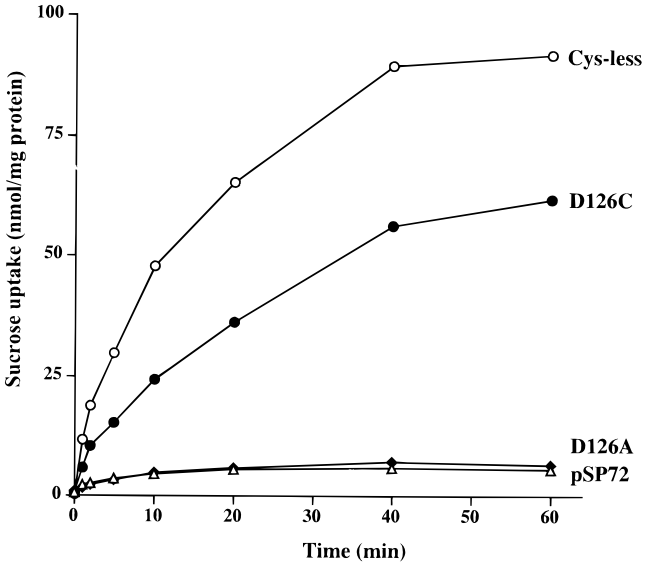

FIGURE 5: Restoration of transport activity in D126C permease by iodoacetic acid. *E. coli* T184 expressing Cys-less sucrose permease, no permease (pSP72 vector only), or mutants D126C and D126A were incubated with 1 mM iodoacetic acid (final concentration) at room temperature for 30 min. The reaction was quenched with 10 mM DTT (final concentration), and time courses of active sucrose transport were assayed in the presence of reduced phenazine methosulfate (PMS) as described in Experimental Procedures.

while expression of mutants D126E, D126A, D126C, R144K, and D126A/R144A is comparable to Cys-less permease (not shown).

Iodoacetic Acid Restores Transport Activity of D126C Permease. Incubation of T184 cells expressing D126C permease with 1 mM iodoacetic acid results in significant reactivation of sucrose transport (Figure 5). Thus, carboxymethylated D126C permease transports at a rate of about 50% and to a steady state of approximately 65% of the Cys-less control. No reactivation is observed with the D126A mutant, indicating that the effect of iodoacetic acid is Cys-specific. In contrast, attempts to restore a functional negative charge at position 126 by reacting D126C permease with methanethiosulfonate ethylsulfonate (MTS-ES) (*25*) yield no

measurable sucrose transport. Similarly, incubation of R144C permease with the positively charged MTS analogue methanethiosulfonate ethylammonium (MTS-EA) does not restore transport activity.

DISCUSSION

The present study investigates the role of putative binding site residues Asp126, Arg144, and Ser148 in the sucrose permease. The corresponding residues in lac permease (Glu126, Arg144, and Cys148) participate in ligand binding and are conserved in all other members of the OHS family. In lac permease, Glu126 and Arg144 are thought to form a neutralizing charge pair (salt bridge) and provide essential H-bonding interactions for galactoside binding (*10*−*13*). Neutral mutations at these positions abolish transport and binding, while conservative mutations have drastically different phenotypes. Mutant E126D binds ligand with slightly decreased affinity but catalyzes lactose transport to normal levels, while R144K permease is only marginally active and ligand binding is not detectable. Cys148, on the other hand, interacts hydrophobically with the galactosyl moiety of lactose and other galactosides (*8*, *9*). Alkylation of this residue with NEM completely inhibits binding and transport, and substrates of the permease fully protect against sulfhydryl inactivation (*7*, *9*, *12*, *24*, *26*). The C148S mutation markedly decreases binding affinity and diminishes galactose transport, although the mutant accumulates lactose to wild-type levels (*8*, *14*−*17*).

Replacement of Ser148 with Cys in sucrose permease results in an interesting phenotype. Transport activity of S148C permease is only slightly decreased relative to Cys-less permease, and it is completely inactivated by NEM. The rate of NEM inhibition is more than 20 times faster than NEM-dependent inactivation of lac permease. However, sucrose affords no protection whatsoever against NEM alkylation. The observations suggest that (i) Ser148 in sucrose permease is found near or at the binding site but (ii) it does not participate directly in sucrose binding and (iii) it is located in a different, presumably more water-accessible environment than Cys148 of lac permease. The unusually high reactivity of Cys148 in sucrose permease may also be related to altered interactions with neighboring charged residues, which may modify its pK_a. In this context, Asp126 with the shortened side chain (Glu126 in lac permease) and Glu272 with its anomalous position (Glu269 in lac permease) are likely candidates. On the other hand, neither mutation E126D (*12*) nor mutations of Glu269 appear to alter markedly the reactivity of Cys148 in lac permease ((*27*).

Replacements with neutral side chains (Ala or Cys) of Asp126 or Arg144 result in normally expressed, but completely inactive proteins. In addition, the double mutant D126A/R144A is also inactive, indicating that restoration of electroneutrality per se between 126 and 144 does not restore activity. The observed properties are identical to those of the corresponding neutral mutants in lac permease (*10*, *12*), indicating that charge pair Asp126-Arg144 plays an essential role in sucrose transport, in all likelihood as binding site residues. Carboxymethylation of the Cys residue in D126C permease with iodoacetic acid restores transport activity, confirming that a negatively charged side chain is essential at this position. Interestingly, the analogous mutant E126C in lac permease is not reactivated by iodoacetic acid, and in situ labeling experiments indicate that the Cys residue is not accessible to this thiol reagent (I. Kwaw and H. R. Kaback, unpublished observations). Taken together with the higher reactivity of Cys148, the observations provide a clear indication that the interface of helices IV and V in sucrose permease is more accessible to solvent from the periplasmic side of the membrane than the same region in lac permease. Conservative mutants at positions 126 and 144 exhibit characteristics that differ significantly from their lac permease counterparts. Thus, mutant D126E transports as well as Cys-less permease, and R144K permease exhibits high activity, indicating that details of sucrose binding in sucrose permease are different from those of galactoside binding in lac permease. The results are not entirely surprising, in view of the significantly different chemical structures and properties of sucrose and lactose. In addition, sucrose in crystalline form and bound to sugar binding proteins has a globular conformation with the glucosyl and fructosyl moieties bent toward each other, while lactose has a more planar arrangement (*28*).

Taken together, the observations indicate that location (helices IV and V) and chemical nature of the residues (amino acids 126 and 144) involved in substrate binding are conserved between the sucrose and lac permeases, while specific aspects of ligand binding differ. Direct binding assays with sucrose permease mutants will be invaluable to confirm and extend these conclusions. In this respect, the extremely low affinity of sucrose for the permease hinders further studies, and experiments are currently underway to identify high-affinity ligands of sucrose permease.

ACKNOWLEDGMENT

Thanks are due to Stathis Frillingos for help with some of the experiments and stimulating discussions. Christopher D. Wolin is acknowledged for his help in the initial characterization of the S148C mutant. Special thanks to Mary C. Lawrence and Joseph Runner for technical assistance.

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BI000125G