The Sucrose Permease of *Escherichia coli*: Functional Significance of Cysteine Residues and Properties of a Cysteine-less Transporter†

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ABSTRACT: The sucrose (CscB) permease belongs to the oligosaccharide:H^+ symporter family of the Major Facilitator Superfamily and is homologous to the lactose permease from *Escherichia coli*. Sucrose transport in cells expressing sucrose permease is completely inhibited by *N*-ethylmaleimide (NEM), suggesting that one or more of the seven native Cys residues may be important for transport. In this paper, each Cys residue was individually replaced with Ser, and transport activity, membrane expression, and NEM sensitivity are documented. All seven single Cys→Ser mutants are expressed normally in the membrane and catalyze sucrose transport with activities ranging from 80% to 180% of wild type. Six of the seven Ser mutants are completely inactivated by NEM, while Cys122→Ser permease is insensitive to the sulfhydryl reagent, indicating that NEM inhibition results from alkylation of Cys122. Subsequently, a sucrose permease devoid of Cys residues (Cys-less) was constructed in which all Cys residues were replaced with Ser simultaneously by using a series of overlap−extension PCRs. Membrane expression and kinetic parameters for Cys-less [K_m 4.8 mM, V_{max} 192 nmol min^{-1} (mg of protein)$^{-1}$] are essentially identical to those of wild type [K_m 5.4 mM, V_{max} 196 nmol min^{-1} (mg of protein)$^{-1}$]. However, Cys-less permease catalyzes sucrose accumulation to steady-state levels that are approximately 2-fold higher than those of wild type. As anticipated, Cys-less permease is completely resistant to NEM inhibition. The observations demonstrate that Cys residues play no functional role in sucrose permease. Furthermore, the approach described to create the Cys-less transporter is generally applicable to other proteins. An application of Cys-less permease in the study of the substrate binding site is presented in the accompanying paper.

The oligosaccharide:H^+ symporter (OHS)[1] family in the Major Facilitator Superfamily (MFS) (*1*) includes six sugar transporters from enteric bacteria: the lactose permease (lac permease, LacY) of *Escherichia coli* (*2*), the raffinose permease (RafB) of *E. coli* (*3*), the lac permease (LacY) from *Klebsiella pneumoniae* (*4*), the lac permease (LacY) from *Citrobacter freundii* (*5*), the melibiose permease (MelY) from *Enterobacter cloacae* (*6*), and the sucrose permease (CscB) from *E. coli* (*7*). *CscB* was first described as one of the structural genes of a chromosomally encoded sucrose metabolic pathway (the *csc* regulon) from a wild-type isolate of *E. coli* EC3132 (*7*). Independently, the same regulon was identified in a clinical isolate of *E. coli* B-26 (*8*, *9*). Sequencing of the *cscB* gene revealed a high degree of similarity with lac permease (30% identity), and it was postulated that *cscB* encodes a sucrose transporter (*7*). Subsequently, it was demonstrated that overexpression of the *cscB* gene from a high copy-number plasmid under the control of the *lacZ* promoter/operator results in high sucrose transport activity, providing direct evidence that the *cscB* gene product is a sucrose permease (*10*).

On the basis of alignment of the primary sequences and the detailed structural analysis of lac permease from *E. coli* (*11*, *12*), all members of the OHS family are likely to consist of 12 transmembrane α-helices that traverse the membrane in a zigzag fashion connected by hydrophilic loops with both C- and N-termini on the cytoplasmic face (Figure 1). In addition, with the exception of the sucrose permease, the symporters have significantly overlapping substrate specificities, too, inasmuch as they all transport lactose and/or other galactosides. In contrast, sucrose permease appears to transport sucrose only (*7*, *10*). Another common feature among members of the family is conservation of six intramembrane charged residues essential for ligand binding and transport, as judged by extensive mutagenesis studies on *E. coli* lac permease (*12*, *13*). Residues[2] essential for H^+ translocation and coupling (Arg302 in helix IX, His322 in helix X, and Glu325 in helix X) are strictly conserved (Figure 1). Glu126 and Arg144, a charge pair mandatory for sugar binding (*14*−*17*), are present in each galactoside transporter, while sucrose permease contains an Asp side chain at position 126. With the exception of sucrose permease, each member of the family also has a Glu residue at position 269 which is thought to play an essential role in substrate binding and/

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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.

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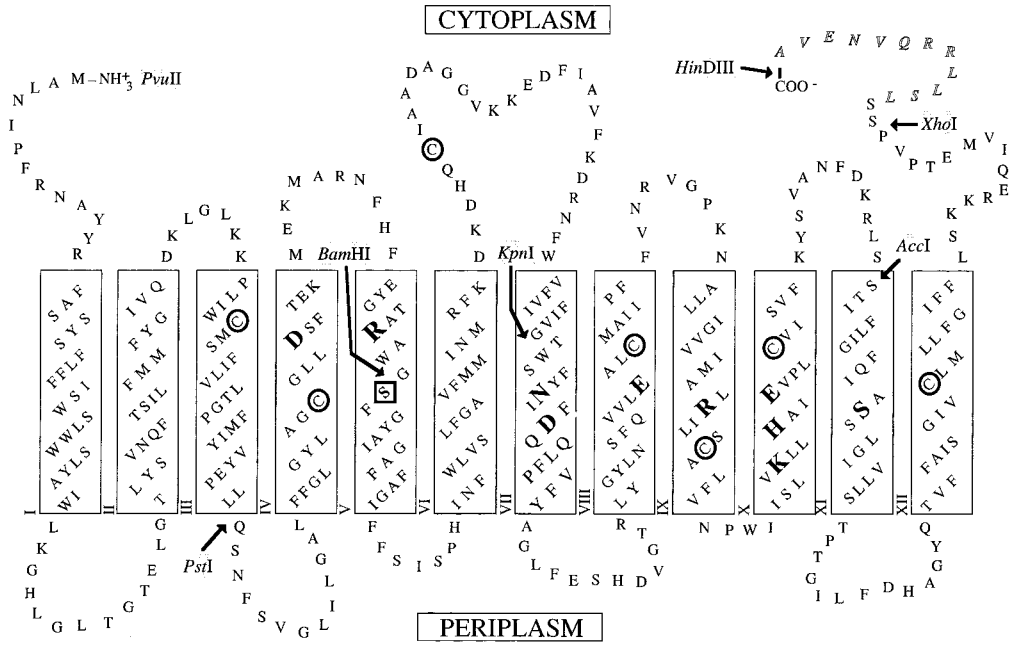

FIGURE 1: Putative secondary structure of sucrose permease, based on the secondary structure model of lac permease. Transmembrane helices are shown in boxes. The positions of Asp126, Arg144, Asn237, Asp240, Glu272, Arg302, Lys319, His322, Glu325, and Ser358 are emboldened (see text for details). Native Cys residues are circled, and Ser148 in helix V, which is studied in the accompanying paper (*33*), is boxed. Also indicated are the restriction endonuclease sites used for construction of the Cys→Ser mutants.

or coupling (*18*−*20*). Interestingly, sucrose permease carries a Val at the corresponding position (269), and on the same face of helix VIII as Val269 there is a Glu at position 272. It is tempting to speculate that the repositioning of the Glu in helix VIII might be responsible for the unique sugar specificity of sucrose permease among members of the OHS family. However, when the whole helix VIII in sucrose permease is replaced with the corresponding segment of lac permease, the resulting chimera is completely inactive with respect to both lactose or sucrose transport, although it is expressed normally in the membrane (*10*).

In addition to the residues described above, Asp240 (helix VII) and Lys319 (helix X), which are presumably charge paired but not essential for activity (*21*−*24*), are conserved in all members of the family. On the other hand, Asp237 (helix VII) and Lys358 (helix XI), which form a salt bridge that is important for membrane insertion but not for activity in *E. coli* lac permease (*21*, *22*, *25*, *26*), are conserved in all members except the sucrose permease where neutral residues are found at the corresponding positions (Asn237 and Ser358). Recently, charged residues at positions 237 and 358 were reintroduced into sucrose permease, and the mutants were shown to exhibit significant transport activity and improved insertion into the membrane, indicating that a functional charge pair interaction can be established between these positions (*27*). In addition, the observations indicate that helices VII and XI are in close proximity in sucrose permease and provide the first evidence for similar helix packing in the lactose and sucrose permeases.

Cys scanning mutagenesis combined with site-directed sulfhydryl labeling and various biochemical and spectroscopic techniques is a powerful approach to study structural and dynamic aspects of membrane protein structure and function (*13*). To subject the sucrose permease to Cys mutagenesis, construction of a functional Cys-less permease is mandatory. None of the seven native Cys residues of sucrose permease is conserved in lac permease, indicating that they might not be functionally important. On the other hand, *N*-ethylmaleimide (NEM) completely inhibits sucrose transport, suggesting that one or more Cys may play a role in the transport mechanism. In the present study, the role of the Cys residues was investigated by replacement with Ser, and a functional Cys-less sucrose permease was constructed and characterized.

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 Single Cys→Ser Mutants. The seven native Cys residues were individually replaced with Ser in plasmid pSP72/*cscB* (*10*) by oligonucleotide-directed site-specific mutagenesis using the overlap−extension PCR method (*28*). To facilitate subcloning of the amplified PCR fragments, unique *Pst*I and *Bam*HI restriction sites were engineered into *cscB* by introducing silent mutations at codons 103 (CAA→CAG) and 151 (TCT→TCC, *cscB* numbering). The PCR products carrying the mutations were digested with

[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 Cys residues in sucrose permease (Cys79, Cys122, Cys195, Cys275, Cys306, Cys329, and Cys390) throughout the paper. The corresponding amino acid positions in the CscB sequence are Cys82, Cys125, Cys197, Cys273, Cys304, Cys327, and Cys388, respectively. Similarly, Val269, Glu272, Asn237, and Ser358 are used to designate Val267, Glu270, Asn234, and Ser356, respectively.

*Pvu*II−*Pst*I (C79S), *Pst*I−*Bam*HI (C122S), *Bam*HI−*Kpn*I (C195S), *Kpn*I−*Acc*I (C275S, C306S, C329S), or *Acc*I−*Xho*I (C390S) and ligated into the similarly treated pSP72/*cscB* expression vector (see Figure 1). The entire PCR products were DNA sequenced through the ligation junctions.

Construction of a Cys-less cscB Gene. Due to the relatively few unique restriction endonuclease sites in the *cscB* gene, combination of the single Cys→Ser mutations in one gene by "cut-and-paste" subcloning techniques was not feasible. Therefore, a *cscB* gene devoid of Cys codons was constructed by simultaneous replacement of the seven native Cys residues with Ser codons, using a series of overlap−extension PCRs (Figure 4). First, using primers carrying Cys→Ser mutations, the whole *cscB* gene was amplified in eight overlapping, 100−200 nucleotide long fragments. As indicated, the eight PCR products were then combined in four pairs and reamplified, generating four fragments, each 300−400 nucleotides long. Subsequently, the four products were combined in two pairs and amplified to yield two fragments. Finally, these two overlapping fragments were amplified, and a full-length *cscB* gene was generated. The Cys-less gene was digested with *Pvu*II and *Hin*dIII and subcloned into pSP72 under the control of the *lacZ* promoter/operator. The nucleotide sequence of the entire gene was verified by DNA sequencing.

Growth of Bacteria. *E. coli* T184 [$lacI^+O^+Z^-Y^-(A)$,*rpsL*,-*met*$^-$,*thr*$^-$,*recA*,*hsdM*,*hsdR*/*F*′,$lacI^qO^+Z^{D118}(Y^+A^+)$] expressing given permease mutants was grown aerobically at 37 °C in LB broth with ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2 h at 37 °C, cells were harvested by centrifugation.

Sucrose Transport Assays. For active transport, *E. coli* T184 was washed once with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10.0 at 600 nm (0.7 mg of protein/mL). Transport was initiated by addition of [^{14}C(U)]sucrose (10 mCi/mmol specific activity, 0.4 mM final concentration) to 50 μL cells, and samples were quenched at given times by 100 mM KP_i (pH 5.5)/100 mM LiCl and assayed by rapid filtration. To determine kinetic parameters (K_m, V_{max}), cells were concentrated to an optical density of 20, and 50 μL cells were mixed with 50 μL of [^{14}C(U)]sucrose (1.5−40 mM final concentrations). Uptake values were corrected for sucrose uptake by cells carrying the pSP72 vector only.

NEM Inactivation and Substrate Protection Experiments. *E. coli* T184 cells expressing given permease mutants were preincubated with freshly prepared NEM at a final concentration of 1 mM for the indicated times at room temperature. The reactions were quenched with 10 mM dithiothreitol (DTT), and sucrose transport was assayed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) (*29*, *30*). To determine the effect of substrate on NEM inactivation, cells were incubated with 1 mM NEM in the presence of 200 mM sucrose (final concentrations) for the times indicated. Reactions were quenched with DTT, cells were washed three times with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ to remove sucrose, and transport was assayed in the presence of reduced PMS.

Western Blot Analysis. In the pSP72/*cscB* expression vector, an epitope corresponding to the C-terminal dodecapeptide of lac permease was engineered onto the C-terminus of sucrose permease, so that the *cscB* gene product could be identified on Western blots (*10*). Crude membrane fractions from *E. coli* T184 expressing given mutants were prepared by osmotic shock and sonication (*30*). Membrane fractions were electrophoresed on sodium dodecyl sulfate−12% polyacrylamide gels, electroblotted to poly(vinylidene difluoride) membranes (Immobilon P, Millipore), and probed with a site-directed polyclonal antibody against the C-terminus of lac permease (*31*).

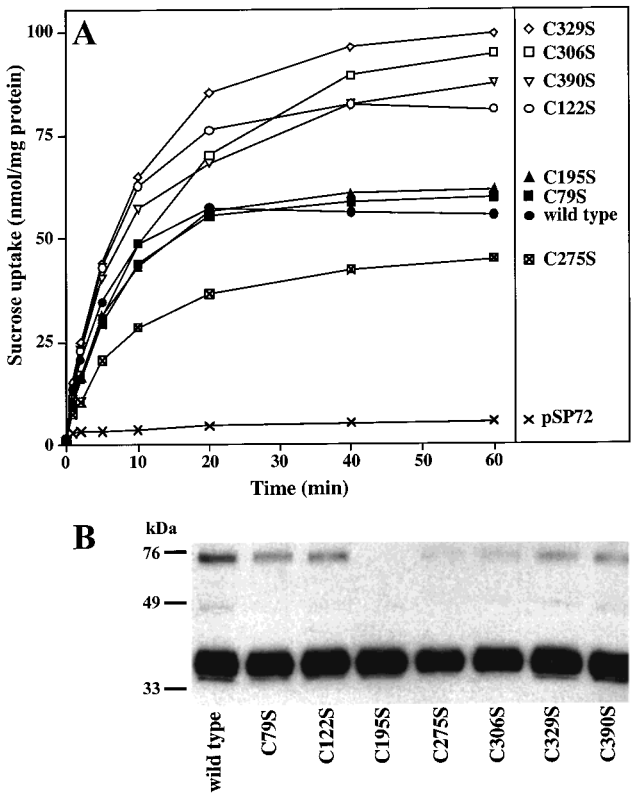

FIGURE 2: Transport activity and membrane expression of single Cys→Ser sucrose permease mutants. (A) Time courses of active sucrose transport by *E. coli* T184 expressing wild-type sucrose permease, no permease (pSP72 vector only), or given single Cys→Ser mutants. Cells were grown at 37 °C, and aliquots 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. (B) Immunological analysis of membrane fractions from cells expressing wild-type permease or single Cys→Ser mutants. Membrane preparations containing approximately 25 μg of protein per sample were subjected to $NaDodSO_4$−polyacrylamide gel electrophoresis, and Western blot analysis using an antibody directed against the C-terminus was carried out as described in Experimental Procedures.

Protein Determinations. Protein was assayed in the presence of sodium dodecyl sulfate by a modified Lowry procedure (*32*).

RESULTS

Single Cys→Ser Mutants Exhibit High Sucrose Transport Activity. Each Cys residue in sucrose permease was individually replaced with Ser using overlap−extension PCR mutagenesis as described in Experimental Procedures. The ability of mutants to catalyze H^+-driven sucrose accumulation was tested in *E. coli* T184 cells (Figure 2A). Two mutants, C195S and C79S, catalyze sucrose transport at 75% of the

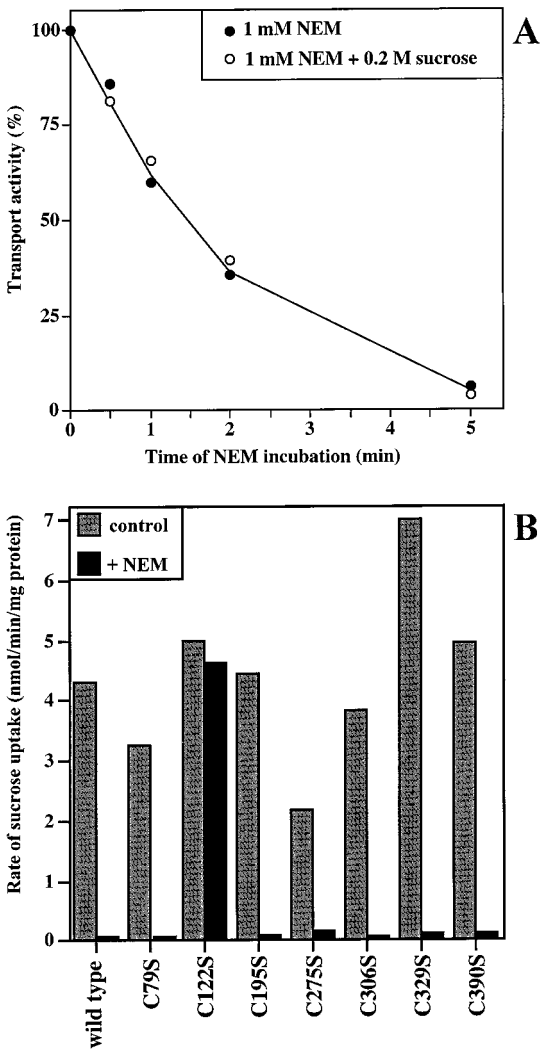

FIGURE 3: Effect of NEM on sucrose transport by *E. coli* T184 harboring plasmids encoding wild-type sucrose permease (A) or single Cys→Ser mutants (B). (A) Cells expressing wild-type permease were incubated with 1 mM NEM at room temperature for the indicated times in the absence or presence of 200 mM 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. (B) Cells expressing indicated single Cys→Ser mutants were incubated with 1 mM NEM for 20 min, the reaction was quenched with DTT, and sucrose uptake was assayed as described in Experimental Procedures.

wild-type rate to wild-type steady-state levels. Mutant C275S accumulates sucrose at approximately 55% of the wild-type rate to ca. the 80% steady-state level. Interestingly, four mutants, C122S, C306S, C329S, and C390S, catalyze sucrose transport at approximately the wild-type rates (85−115%), but to significantly higher steady-state levels (145−180%). Western blot analysis of membrane fractions demonstrates that mutants and wild-type permease are expressed to identical levels in the membrane (Figure 2B).

NEM Inhibits Transport through Alkylation of Cys122. Incubation of cells expressing wild-type sucrose permease with 1 mM NEM results in complete inhibition of sucrose uptake, and the time required to reach half-maximal inhibition ($t_{1/2}$) is approximately 1.5 min. The presence of 200 mM sucrose affords no protection whatsoever against NEM inactivation (Figure 3A). To identify the Cys residue(s)

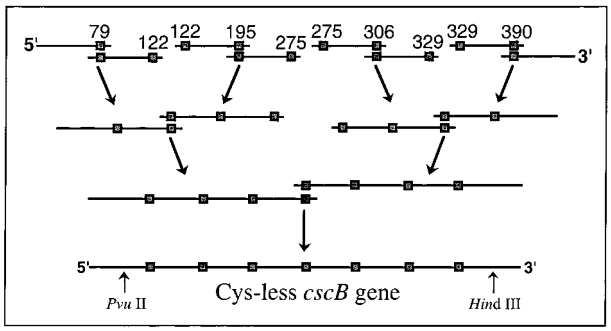

FIGURE 4: Construction of a Cys-less *cscB* gene. The *cscB* gene was amplified in eight fragments with mutagenic primers carrying indicated Cys→Ser mutations. Subsequently, the Cys-less gene was assembled by a series of overlap−extension PCR ampifications, as described in Experimental Procedures.

responsible for NEM inhibition, each Cys→Ser mutant was preincubated with 1 mM NEM for 20 min, and the initial rate of sucrose uptake was determined. As shown in Figure 3B, with the exception of Cys122→Ser, all mutants are completely inactivated by the sulfhydryl reagent, indicating that alkylation of Cys122 is solely responsible for NEM inhibition of sucrose transport.

Construction and Properties of a Cys-less Sucrose Permease. A *cscB* gene devoid of Cys codons was constructed by simultaneous replacement of the seven native Cys residues with Ser codons, using a series of overlap−extension PCRs (Figure 4), as described in Experimental Procedures. First, using primers carrying Cys→Ser mutations, the whole *cscB* gene was amplified in eight overlapping fragments. As indicated in Figure 4, the eight PCR products were then combined in overlapping pairs and assembled by sequential amplification until a full-length *cscB* gene was generated. The Cys-less gene was subcloned into pSP72, and the entire gene was verified by DNA sequencing.

T184 cells expressing Cys-less sucrose permease accumulate sucrose at a rate comparable to that of wild type, while steady-state levels are ca. 2-fold higher (Figure 5). Determination of transport kinetic parameters reveals no significant differences between wild-type [K_m 5.4 ± 1 mM (SE, $n = 4$), V_{max} 196 ± 11 nmol min^{-1} (mg of protein)$^{-1}$ (SE, $n = 4$)] and Cys-less permease [K_m 4.8 ± 0.4 mM (SE, $n = 4$), V_{max} 192 ± 5 nmol min^{-1} (mg of protein)$^{-1}$ (SE, $n = 4$)]. Levels of Cys-less or wild-type permease in the membrane are comparable (Figure 5 inset), and as anticipated, the Cys-less transporter is completely resistant to NEM inactivation [not shown; see Figure 2B in ref *33*].

DISCUSSION

The present study documents the role of Cys residues in the transport mechanism of the sucrose permease. Transport activity is completely inhibited by NEM, suggesting that one or more of the seven native Cys residues might be important. However, all seven single Cys→Ser mutants exhibit high transport activity, ranging from 55% to 130% and from 80% to 180% relative to wild type with respect to rates and steady-state levels of sucrose uptake, respectively. Interestingly, four of the seven mutants catalyze significantly better sucrose accumulation than wild-type permease for reasons that are not apparent. The observations clearly indicate that none of the Cys residues plays a direct role in the transport mech-

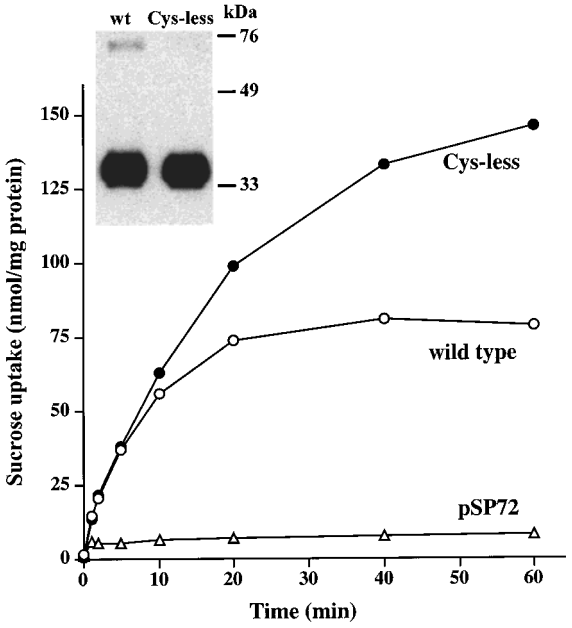

FIGURE 5: Transport activity and membrane expression of Cys-less sucrose permease. Time courses of active sucrose transport by *E. coli* T184 expressing wild-type sucrose permease, Cys-less permease, or no permease (pSP72 vector only) were assayed at 0.4 mM final external sucrose concentration as described in Figure 2 and in Experimental Procedures. (Inset) Western blot analysis of membrane fractions (approximately 25 μg of protein per sample) was carried out using an antibody directed against the C-terminus, as described in Experimental Procedures.

anism. Subsequently, a sucrose permease devoid of Cys residues was constructed. Similarly to four of the single Cys→Ser mutants, Cys-less permease catalyzes sucrose accumulation to steady-state levels that are significantly higher than those of wild type. Since no appreciable differences are observed in kinetic parameters or membrane expression between the two permeases, the enhanced activity is presumably due to more efficient coupling between H^+ and sugar transport. Alternatively, reduced sucrose efflux in Cys-less permease might lead to higher levels of accumulation. In any event, the high transport activity of Cys-less permease makes it an ideal candidate for detailed molecular analysis by Cys scanning mutagenesis and biochemical/biophysical techniques using site-directed sulfhydryl labeling.

None of the seven native Cys residues in sucrose permease is conserved in lac permease, the most studied member of the OHS family of MFS. The corresponding positions, with the exception of Ser306, are occupied by hydrophobic residues (Ile79, Ala122, Ala195, Ile275, Leu329, Leu390), and Cys replacements at these positions yield highly active transporters (*14*, *30*, *34*−*37*). Investigations into the functional role of the native Cys residues in lac permease (*38*−*45*) reveals that none of the Cys side chains is mandatory for the transport mechanism, and a permease devoid of Cys residues (C-less permease) exhibits high lactose transport activity (*46*). In this respect, the two permeases appear to be highly analogous. On the other hand, Cys-less lac permease is only about 50% active relative to wild type, and at least two Cys residues seem to play a nonessential, but significant role in transport. (i) Cys148 in helix V interacts hydrophobically with the galactosyl moiety of the substrate (*47*, *48*), and reaction of this residue with a variety of sulfhydryl reagents completely inhibits transport. Moreover, substrates of lac permease afford protection against sulfhydryl inactivation (*16*, *48*−*50*). (ii) Replacement of Cys154 in helix V with Gly or Ser markedly compromises transport, and only the Val mutant exhibits significant activity (*39*, *45*, *51*). Remarkably, the Cys154→Gly mutant binds substrate on the periplasmic surface considerably better than wild-type permease, indicating that this mutant might be locked in an outward oriented conformation (*51*).

In recent years Cys scanning mutagenesis has become an important method for investigating structure−function relationships in membrane proteins (*52*−*58*). The extraordinary power of this technique for obtaining structural and dynamic information at the molecular level was first demonstrated with lac permease (*13*). A prerequisite for these studies is the availability of a functional Cys-less protein. A significant number of studies indicate that Cys residues in membrane proteins are generally found in transmembrane regions and are functionally replaceable with other small hydrophobic residues (Ala in particular) or even with the more hydrophilic Ser. Therefore, as convincingly demonstrated by the present study, it is usually not necessary to individually investigate the role of Cys residues before construction of a Cys-less protein. The sequential use of overlap−extension PCRs (Figure 4) allows relatively rapid creation of a Cys-less construct, and this strategy is particularly useful with genes containing multiple Cys codons and few or no unique restriction sites.

Successful construction of a highly active sucrose permease opens up new horizons in comparative structure−function analysis of lac and sucrose permeases. The highly similar structure and the radically different substrate specificity present a unique opportunity for identification of common and protein-specific functional elements that play a role in substrate binding and transport, H^+ translocation, and coupling. An application of Cys-less sucrose permease in the study of the putative substrate binding site is presented in the accompanying paper.

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