Helix Packing in the Sucrose Permease of *Escherichia coli*: Properties of Engineered Charge Pairs between Helices VII and XI

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ABSTRACT: Of four putative intramembrane charge pairs in lactose permease, only three are conserved in the homologous sucrose permease of *Escherichia coli* [Bockmann, J., Heuel, H., & Lengeler, J. W. (1992) *Mol. Gen. Genet. 235*, 22−32]. The missing charge pair was introduced into wild-type sucrose permease by site-directed mutagenesis of Asn234 (helix VII) and Ser356 (helix XI). Individual replacement of either residue with a charged amino acid abolishes active sucrose transport with the exception of the Asn234→Asp mutant. However, simultaneous replacement of Asn234 with Asp or Glu and Ser356 with Arg or Lys results in high activity. Thus, an acidic residue at position 234 rescues the activity of the Ser356→Arg or Ser356→Lys mutant, and a basic residue at position 356 rescues the activity of the Asn234→Glu mutant. Furthermore, when expressed at a relatively low rate, the double mutant Asn234→Asp/Ser356→Arg is present in the membrane in a significantly greater amount than wild-type, suggesting that the charge pair improves insertion of sucrose permease into the membrane. The results indicate that helices VII and XI of sucrose permease are in close proximity and that a charge pair interaction can be established between residues 234 (helix VII) and 356 (helix XI). However, interchange of the acidic residue at position 234 with the basic residue at position 356 abolishes sucrose transport. Clearly, therefore, the interaction between the engineered residues in sucrose permease is more complex than the corresponding Asp237−Lys358 interaction in lactose permease where reversal of the charge pair has little or no effect on activity [Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547−10551].

Cluster 5 in the major facilitator superfamily (MFS;[1] Marger & Saier, 1993) includes four oligosaccharide/H^+ symporters from enteric bacteria: the lactose (lac) permease of *Escherichia coli* (Büchel et al., 1980: Kaback, 1983), the lac permease of *Klebsiella pneumoniae* (McMorrow et al., 1988), the raffinose permease of *E. coli* (Aslanidis et al., 1989), and the sucrose permease of *E. coli* (Bockmann et al., 1992; Lengeler et al., 1992). Recently, a fifth gene encoding a lac permease in *Citrobacter freundii* which exhibits 70% identity to the lac permease of *E. coli* has been identified (Lee et al., 1994). On the basis of alignment of the primary sequences (Bockmann et al., 1992) and the detailed secondary structure analysis of the *E. coli* lac permease [reviewed in Kaback (1989, 1992) and Calamia and Manoil (1990)], all 5 molecules are likely to consist of 12 transmembrane α-helices that traverse the membrane in a zig-zag fashion connected by hydrophilic loops with both N and C termini on the cytoplasmic face.

A common feature among the members of cluster 5, as well as the *C. freundii* lac permease (Lee et al., 1994), is conservation of three intramembrane charged residues that are essential for active transport, as judged by extensive site-directed mutagenesis studies on *E. coli* lac permease [reviewed in Kaback et al. (1993, 1994) and Kaback (1995)]. The conserved residues are Arg302 (helix IX), His322 (helix X), and Glu325 (helix X), and they represent 3 of the 4 residues out of over 320 mutated that are obligatory for active lactose transport. Furthermore, the properties of mutants with various amino acid replacements at these positions indicate that the side chains are involved in H^+ translocation and/or substrate binding (Kaback, 1989, 1992). With the exception of sucrose permease, each member of the family also has a Glu residue at position 269 which has been shown to be essential for activity in the lac permease of *E. coli*. In the sucrose permease, a Glu residue is present four residues removed toward the C terminus from the corresponding position in lac permease (i.e., approximately one turn of a helix). In addition to these residues, Asp240 (helix VII) and Lys319 (helix X), which are presumably charge-paired but not essential for activity (Sahin-Tóth et al., 1992; Lee et al., 1992; Sahin-Tóth & Kaback, 1993), are conserved in all members of the subfamily. On the other hand, Asp237 (helix VII) and Lys358 (helix XI), which are likely to form a salt bridge that is important for membrane insertion but not for activity in *E. coli* lac permease (King et al., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993), are conserved in all members except the sucrose permease where neutral residues are found at the corresponding positions (i.e., Asn234 and Ser356, respectively).

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[1] Abbreviations: MFS, major facilitator superfamily; lac, lactose; *csc*, chromosomally encoded sucrose operon; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

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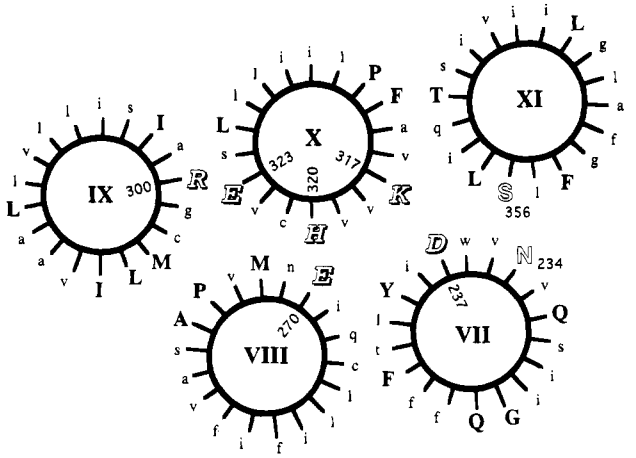

FIGURE 1: Postulated arrangement of helices VII through XI of sucrose permease, based on the model for lac permease (Jung et al., 1993; Kaback et al., 1993, 1994). Eighteen residues of each putative transmembrane helix are shown on helical wheels viewed from the periplasmic surface of the membrane. Helices include residues Val223–Leu240 (helix VII), Leu262–Phe279 (helix VIII), Ala289–Leu306 (helix IX), Leu315–Phe332 (helix X), and Thr346–Leu363 (helix XI). Putative charge pair interactions between Asp237 and Lys317, between Glu270 and His320, and between Arg300 and Glu323 are highlighted (shadowed open capitals). Residues Asn234 and Ser356, which are the targets of site-directed mutagenesis in the present study, are shown as open capital letters. Also shown (filled capital letters) are noncharged residues identical in all members of cluster 5 of MFS (Bockmann et al., 1992; Marger & Saier, 1993).

Recently (Jung et al., 1993), site-directed pyrene excimer fluorescence was used to study proximity relationships between the intramembrane charged residues in the lac permease of *E. coli*. The results demonstrate that helices VIII (Glu269) and IX (Arg302) are in close proximity to helix X (His322 and Glu325, respectively). These and other findings showing that helix VII (Asp237 and Asp240, respectively) is close to helices XI (Lys358) and X (Lys319) form the basis of a model describing helix packing in the C-terminal half of *E. coli* lac permease (Jung et al., 1993; Kaback et al., 1993, 1994). A hypothetical application of the model to the sucrose permease is shown in Figure 1. Clearly, sucrose permease exhibits only three putative charge pairs out of the four found in lac permease. The three putative charge pairs are between Asp237 (helix VII) and Lys317 (helix X), between Glu270 (helix VIII) and His320 (helix X), and between Arg300 (helix IX) and Glu323 (helix X). However, no charge pair is found between helices VII and XI in sucrose permease, where the positions corresponding to Asp237 and Lys358 in lac permease are Asn234 and Ser356, respectively. In order to examine whether helices VII and XI in sucrose permease are in close proximity, Asn234 and Ser356 were replaced either individually or in pairs with different charged residues. The results demonstrate that placement of a negatively charged residue at position 234 and a positively charged residue at position 356 yields functional sucrose permease. Moreover, when expressed at a relatively low rate, the mutant with the engineered charge pair is inserted into the membrane more efficiently than wild-type sucrose permease. The results indicate that the lac and sucrose permeases are structurally homologous. However, as opposed to lac permease, the polarity of the engineered charge pair in sucrose permease appears to be important.

MATERIALS AND METHODS

Materials. [U-^{14}C]Sucrose was purchased from DuPont NEN, Boston, MA. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984) was prepared by BabCo, Richmond, CA. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli HB101 [*hsdS20* (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacYa*, *galK2*, *rpsL20* (*Str*^{r}), *xyl-t*, *mtl-1*, *supE44*, λ^-/F^-] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*), *rspL*, *Met*$^-$, *Thr*$^-$, *recA*, *hsdM*, *hsdR/F'*. *lacI*^{q}*O*$^+$*Z*^{D118}(*Y*$^+$*A*$^+$)] (Teather et al., 1980) harboring plasmid pT7-5/*Bam*HI/*cscB* or pSP72/*cscB* (Sahin-Tóth et al., 1995) with given mutations was used for expression of sucrose permease from the *lacZ* promoter/operator by induction with isopropyl 1-thio-β-D-galactopyranoside (IPTG). *E. coli* HB101 transformed with plasmid pJBL137 carrying a constitutively expressed invertase gene (*cscA*; Bockmann et al., 1992) was used for detection of the sucrose permease activity on MacConkey-agar indicator plates (Difco Laboratories) containing 30 mM sucrose and 1 mM IPTG.

Oligonucleotide-Directed, Site-Specific Mutagenesis. Plasmid pT7-5/*Bam*HI/*cscB* or pSP72/*cscB* (Sahin-Tóth et al., 1995) was used as a template for mutagenesis with synthetic mutagenic primers (Table 1). To facilitate mutagenesis of Asn234, a unique *Kpn*I site was engineered into *cscB* by introducing silent mutations at codons 228 (GGG→GGT) and 229 (ACG→ACC). Mutant N234D[2] and the Ser356-replacement mutants were constructed by two-stage PCR (overlap–extension; Ho et al., 1989), using restriction sites *Kpn*I/*Eco47*III (Asn234-replacement mutants) or *Acc*I/*Xho*I (Ser356-replacement mutants). Mutants N234E, N234K, and N234R were constructed by one-step PCR using mutagenic oligonucleotides containing the *Kpn*I site (Table 1). For construction of mutants containing pairs of amino acid replacements, *Eco*RI–*Acc*I restriction fragments of Asn234-replacement mutants were isolated and ligated to similarly treated vectors of Ser356-replacement mutants.

DNA Sequencing. Double-stranded plasmid DNA was sequenced using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulsen, 1978) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Active Sucrose Transport. E. coli T184 transformed with a given plasmid was grown aerobically at 37 °C in Luria–Bertani medium containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Dense cultures were diluted 10-fold and allowed to grow for another 2 h before induction with 0.5 mM IPTG, and growth was continued for 2 h. Cells were harvested, washed with 100 mM potassium phosphate (pH 7.5)/10 mM magnesium sulfate, and adjusted to an OD_{420} of 10 (approximately 0.7 mg of protein/mL). Transport of [U-^{14}C]sucrose (5 mCi/mmol; 1 mCi = 37 MBq) at a final

[2] 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 sucrose permease and a second letter denoting the amino acid replacement at this position.

Table 1: DNA Sequence Analysis of Single Charge-Replacement Mutants in the *cscB* Gene

mutant[a]		mutagenic oligonucleotide[b]	codon change
N234D	sense	TCTTTCTAT**gac**ATTTTTGATCAA	AAC→GAC
	antisense	ATCAAAAAT**gtc**ATAGAAAGACC	
N234E	sense	TTATGTGGGTACCTGGTCTTTCTAT**gag**ATTTTTGATCAA	AAC→GAG
N234K	sense	TTTATTGTGGGTACCTGGTCTTTCTA**Taaa**ATTTTTGATCAA	AAC→AAA
N234R	sense	TTTATTGTGGGTACCTGGTCTTTCTAT**cgc**ATTTTTGATCAA	AAC→CGC
S356D	sense	CAAATTGCC**gat**TCGCTTGGGATT	AGT→GAT
	antisense	CCCAAGCCA**atc**GGCAATTTGAAA	
S356E	sense	CAAATTGCC**gaa**TCGCTTGGGATT	AGT→GAA
	antisense	CCCAAGCGA**ttc**GGCAATTTGAAA	
S356K	sense	CAAATTGCC**aaa**TCGCTTGGGATT	AGT→AAA
	antisense	CCCAAGCGA**ttt**GGCAATTTGAAA	
S356R	sense	CAAATTGCC**agg**TCGCTTGGG	AGT→AGG
	antisense	CCCAAGCGA**cct**GGCAATTTG	

[a] Double-replacement mutants were constructed by subcloning from the single mutants, as described in the text. [b] Sequences of mutagenic primers are presented in the 5′→3′ order with altered codons in lowercase, boldface type. The sequence of the unique *Kpn*I site is underlined.

concentration of 0.4 mM was assayed by rapid filtration (Consler et al., 1991).

Membrane Preparation. Crude membrane fractions from *E. coli* T184 were prepared by osmotic shock and sonication (Frillingos et al., 1994).

Immunological Analyses. In constructs pT7-5/*Bam*HI/*cscB* and pSP72/*cscB*, an epitope containing the C-terminal dodecapeptide of lac permease was engineered onto the C terminus of the sucrose permease so that the *cscB* gene product could be identified by Western blot analysis (Sahin-Tóth et al., 1995). Membrane fractions were subjected to 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Newman et al., 1981). Proteins were electroblotted on poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C-terminal dodecapeptide of lac permease (Carrasco et al., 1984; Herzlinger et al., 1985).

Protein Determinations. Protein was assayed in the presence of sodium dodecyl sulfate (Peterson et al., 1977).

RESULTS

Construction of Charge Pair Mutants. Sequencing of the *cscB* gene (Bockmann et al., 1992) reveals an Asn codon (AAC) at position 234 (corresponding to Asp237 in *E. coli* lac permease) and a Ser codon (AGT) at position 356 (corresponding to Lys358 in *E. coli* lac permease). To test the hypothesis that a charge pair interaction can be established between residues 234 and 356, mutants N234D, S356R, and N234D/S356R were constructed by introducing an Asp codon (GAC) at position 234 and/or an Arg codon (AGG) at position 356.[3] The mutated *cscB* genes were cloned into either a medium (pT7-5/*Bam*HI) or a high copy number (pSP72) plasmid which was transformed into either *E. coli* T184 or *E. coli* HB101, as indicated. Subsequently, to test the effect of length, charge, and orientation of the side chains, the following groups of mutants were constructed in pSP72/*cscB*: single-charge replacements—N234E, N234K, N234R, S356D, S356E, S356K; double replacements with opposite charges—N234D/S356K, N234E/S356K, N234E/S356R, N234K/S356D, N234K/S356E, N234R/S356D, N234R/S356E; double replacements with homonymous charges—N234D/S356D, N234D/S356E, N234E/S356D, N234E/S356E, N234K/S356K, N234K/S356R, N234R/S356K, N234R/S356R. All mutants were verified by sequencing the entire length of the PCR fragments through the ligation junctions and contained only the base changes introduced (Table 1).

Transport by Single Mutants. Since the levels of expression and transport activity achieved with pSP72/*cscB* are significantly higher than those achieved with pT7-5/*Bam*HI/*cscB* (Sahin-Tóth et al., 1995), sucrose permease mutants in the pSP72 background were used to analyze the effect of the mutations on sucrose transport. Initially, *E. coli* HB101 harboring both pJBL137 (which expresses *cscA* which encodes invertase constitutively) and pSP72/*cscB* with given mutations was plated on MacConkey indicator plates containing 30 mM sucrose and 1 mM IPTG. Cells expressing functional sucrose permease import sucrose which is cleaved by cytoplasmic invertase, and metabolism of the monosaccharides released causes acidification with the appearance of red colonies. Cells impermeable to sucrose grow as white colonies, while cells with low activity grow as pink colonies. With the exception of N234D which grows as red colonies, cells expressing single-charge replacement mutants for either Asn234 or Ser356 grow as white or pink colonies (Table 2).

In agreement with the qualitative observations, all of the single mutants with the exception of N234D are unable to catalyze significant sucrose accumulation (Figure 2). Surprisingly, a mutant N234D accumulates sucrose up to 45% as well as wild-type sucrose permease, a level corresponding to about a 16-fold concentration gradient.

Transport by Double Mutants. A series of double-charge replacement mutants at positions 234 and 356 was constructed in order to examine whether introduction of a neutralizing charge at the alternative position rescues sucrose transport activity in the inactive single mutants. Cells expressing double mutants with a negative charge at position 234 and a positive charge at position 356, regardless of whether the replacement at 234 is Asp or Glu and the replacement at 356 is Lys or Arg, grow as red colonies, indicating a good ability to translocate sucrose (Table 2). Cells expressing each of the other double mutants grow as white colonies, indicating that sucrose is impermeant.

[3] The Asp234−Arg356 pair was selected because this pair reflects the most conservative alteration of the mutated codons relative to wild-type (see Table 1). In lac permease, replacement of Lys358 with Arg significantly increases the steady-state level of accumulation relative to wild-type permease which contains Asp-Lys [see Figure 3 in Sahin-Tóth and Kaback (1993)].

Table 2: Phenotype of Sucrose Permease Mutants on Sucrose−MacConkey Plates[a]

single mutants	phenotype	double mutants	phenotype
wild-type	+++	wild-type	+++
pSP72	−	pSP72	−
N234D	++	N234D/S356K	++
N234E	+	N234D/S356R	++
N234K	−	N234E/S356K	++
S356D	+	N234K/S356D	−
S356E	+	N234K/S356E	−
S356K	+	N234R/S356D	−
S356R	−	N234R/S356E	−
		N234D/S356D[b]	−

[a] *E. coli* HB101 harboring both pJBL137 and pSP72 (vector with no *cscB* gene), pSP72 encoding wild-type sucrose permease, or given mutants was plated on MacConkey−agar indicator plates containing 30 mM sucrose and 1 mM IPTG. The plates were incubated at 37 °C for 14 h. Phenotype code: +++, deep red; ++, red; +, pink; −, white. [b] Mutants N234E/S356E, N234K/S356K, and N234R/S356R also yielded white colonies.

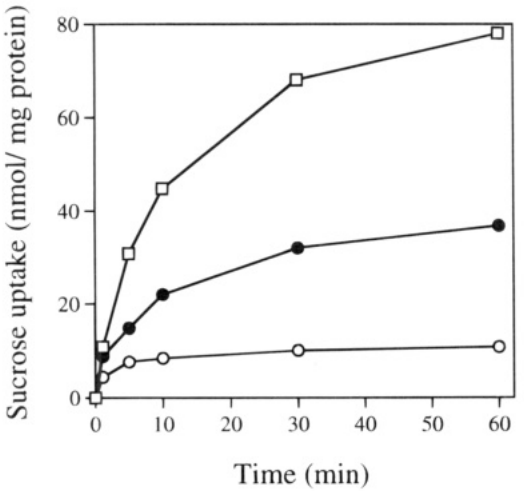

FIGURE 2: Active transport of sucrose by single-charge replacement mutants. *E. coli* T184 harboring pSP72 (vector with no *cscB* gene) or pSP72 encoding wild-type sucrose permease or given mutants was grown and assayed as described under Materials and Methods. (□) Wild-type; (●) N234D; (○) pSP72 with no *cscB* insert or mutants N234E, N234K, N234R, S356D, S356E, S356K, and S356R, where no significant differences were observed.

Time courses of active sucrose transport for the double mutants are shown in Figure 3. Clearly, the charge pair mutants N234D/S356R, N234D/S356K, N234E/S356K, and N234E/S356R catalyze significant sucrose accumulation at rates of about 40−60% of wild-type and to steady-state levels of 30−55% of wild-type (corresponding to concentration gradients of 11−20-fold). However, interchanging the positions of the charged residues in each of the pairs leads to complete inactivation, as mutants N234K/S356D, N234K/S356E, N234R/S356D, or N234R/S356E exhibit transport activities similar to those observed with pSP72 devoid of a *cscB* gene. Negligible levels of sucrose accumulation are also observed with mutants containing homonymous charges at both positions (i.e., N234D/S356D, N234D/S356E, N234E/S356D, N234E/S356E, N234K/S356K, N234K/S356R, N234R/S356K, or N234R/S356R).

Expression of Sucrose Permease Mutants. Western blot analysis of the sucrose permease mutants was carried out on membrane fractions with antibody against the C-terminal dodecapeptide of lac permease which was fused to the C terminus of sucrose permease (Sahin-Tóth et al., 1995). When

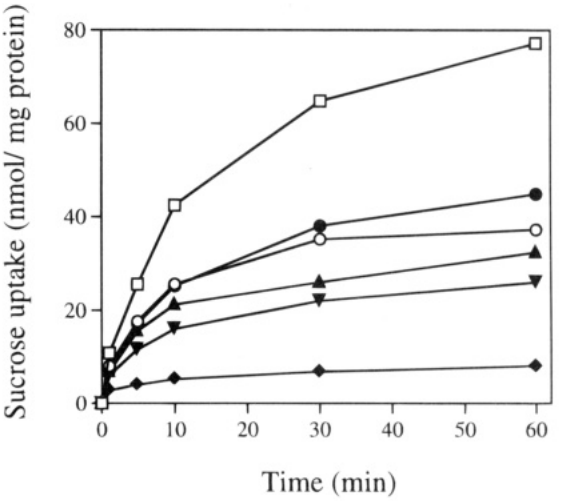

FIGURE 3: Active transport of sucrose by double-charge replacement mutants. *E. coli* T184 harboring pSP72 (vector with no *cscB* gene) or pSP72 encoding wild-type sucrose permease or given mutants was grown and assayed as described under Materials and Methods. (□) Wild-type; (●) N234D/S356R; (○) N234D/S356K; (▲) N234E/S356K; (▼) N234E/S356R; (♦) pSP72 with no *cscB* insert or N234K/S356D, N234K/S356E, N234R/S356D, N234R/S356E, N234D/S356D, N234D/S356E, N234E/S356E, N234E/S356E, N234K/S356K, N234K/S356R, N234R/S356K, and N234R/S356R, where no significant differences were observed.

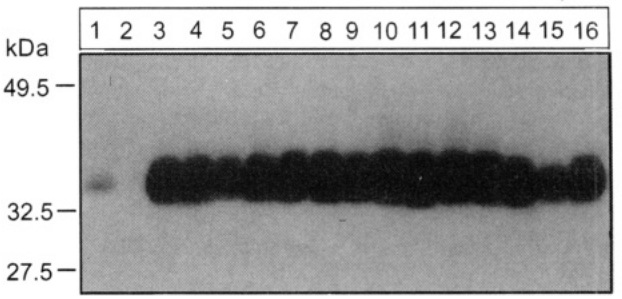

FIGURE 4: Western blot analysis of single- and double-replacement mutants expressed from plasmid pSP72. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids, and 50 μg of membrane protein was subjected to electrophoresis and electroblotting, as described under Materials and Methods. The blot was incubated with anti-C-terminal antibody, followed by incubation with horseradish peroxidase-conjugated protein A, and finally with fluorescent substrate before exposure to film. Lane 1, pT7-5/*Bam*HI with wild-type sucrose permease (shown for comparison); lanes 2−16, pSP72 with no *cscB* insert (lane 2), wild-type (lane 3), N234D (lane 4), S356R (lane 5), N234D/S356R (lane 6), N234D/S356K (lane 7), N234E/S356K (lane 8), N234E/S356R (lane 9), N234K/S356D (lane 10), N234E (lane 11), N234K (lane 12), N234R (lane 13), S356D (lane 14), S356E (lane 15), or S356K (lane 16) sucrose permease. Although not shown, membranes prepared from cells harboring pSP72 with N234K/S356E, N234R/S356D, N234R/S356E, N234D/S356D, N234E/S356E, N234K/S356K, or N234R/S356R also exhibit wild-type levels of immunoreactive material at 33−34 kDa. Migration positions for the marker proteins ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), and soybean trypsin inhibitor (27.5 kDa) are shown on the left.

expressed from the high copy plasmid pSP72, the level of the single or double mutants in the membrane is comparable to that of wild-type sucrose permease (Figure 4). Thus, the differences in transport activity cannot be attributed to variations in expression or insertion of the mutant permeases relative to wild-type.

Although lac permease mutants with Cys and/or Ala in place of Asp237 and Lys358 exhibit high activity, mutants

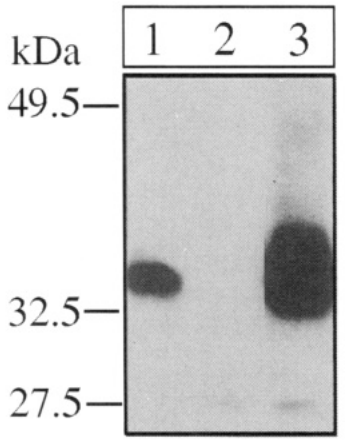

FIGURE 5: Western blot analysis of wild-type sucrose permease (lane 1) or mutant N234D/S356R (lane 3) expressed from plasmid pT7-5/*Bam*HI. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids, and 100 μg of membrane protein was subjected to electrophoresis and Western blotting as described in Figure 4. Membranes prepared from cells harboring pT7-5/*Bam*HI with no *cscB* insert exhibit no immunoreactive material (lane 2). Migration positions for the marker proteins are shown on the left.

devoid of the putative salt bridge are expressed at relatively low levels when synthesized at low rates from the *lacZ* promoter/operator (Dunten et al., 1993). When synthesized at high rates from the T7 promoter, however, the mutants are expressed normally and are stable once inserted into the membrane. The observations indicate that the charge interaction between Asp237 and Lys358 is important for insertion of lac permease into the membrane and suggest that the C terminus of lac permease may be inserted posttranslationally. In order to examine whether the engineered charge residues in sucrose permease play a similar role in membrane insertion, the wild-type or mutant N234D/S356R was expressed at a relatively low rate from the *lacZ* promoter/operator in pT7-5/*Bam*HI [Figure 4; see Sahin-Tóth et al. (1995) in addition]. After induction with 0.5 mM IPTG, the level of expression of N234D/S356R sucrose permease (Figure 5, lane 3) is clearly greater than that of the wild-type (lane 1). However, as observed with lac permease [see Figure 6 in Dunten et al. (1993)], when wild-type or N234D/S356R sucrose permease is expressed at a high rate from a high copy plasmid (Figure 4) or from the T7 promoter by using the system of Tabor and Richardson (1985), no significant difference in expression is observed (data not shown).

DISCUSSION

The results presented here provide an initial indication that the sucrose and lac permeases may be similar with respect to helix packing in the C-terminal halves (see Figure 1). Although the two permeases are homologous in sequence (ca. 31% identical residues), direct evidence for similarity in structure and/or mechanism is lacking, due partially to poor expression of the *cscB* gene from the native promoter which makes analysis of the gene product difficult (Bockmann et al., 1992). We (Sahin-Tóth et al., 1995) have demonstrated recently that overexpression of the *cscB* gene from a high copy number plasmid results in high sucrose transport activity, and in this study, the expression system has been exploited to study the effect of introducing a charge pair found between helices VII and XI in the lac permease of *E. coli* into the sucrose permease.

Individual replacement of Asn234 with Glu or Ser356 with Lys or Arg in sucrose permease abolishes active sucrose transport, while simultaneous replacement with Glu and Lys or Glu and Arg yields active permease. Furthermore, the inactivation effect of Arg or Lys replacement for Ser356 is suppressed with Asp in place of Asn234 (Figures 2 and 3). The simplest interpretation of these findings is that positions 234 and 356 are in close proximity in the tertiary structure of sucrose permease (Figure 1) and interact functionally. Thus, the single-charge substitutions introduce an uncompensated charge in the low dielectric of the membrane which leads to inactivation, while double replacements with a negatively charged residue at position 234 and a positively charged residue at position 356, respectively, create a charge-neutralizing pair that is more favorable energetically (Honig & Hubbell, 1984) and does not severely compromise activity. It is also apparent that neither Asn234 nor Ser356 is important for active sucrose transport, since replacement of both residues with appropriately charged amino acids yields mutants with highly significant transport activity.

Analogous observations have been made for Asp237 and Lys358 in the *E. coli* lac permease (King et al., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993) which corresponds to Asn234 and Ser356 in sucrose permease. However, many properties of the interactions in the two permeases are significantly different. In lac permease, the charge interaction can be reversed without a significant effect on transport activity (Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993), and replacement of Asp237 with Glu and/or Lys358 with Arg or extension of the native side chains in the charge pair with chemical modification by up to five bond lengths results in fully functional permease (Sahin-Tóth & Kaback, 1993). In contrast, the interaction between Asp234−Lys356 in the mutant sucrose permease is more constrained, as the polarity of the charged residues is critical for sucrose transport. Thus, reversing the positions of the engineered charged residues inactivates the sucrose permease. Furthermore, although the double mutants clearly catalyze sucrose accumulation, the activity of the double mutants is only one-third to one-half of wild-type, and N234E/S356R exhibits lower activity than that of N234D/S356K or N234D/S356R (Figure 3). Unexpectedly, these properties are similar to those of the Asp240−Lys319 pair in lac permease (Sahin-Tóth & Kaback, 1993). It is also noteworthy that the single-replacement mutant N234D catalyzes significant sucrose accumulation (Figure 2), suggesting that the "unpaired" Asp234 may have a perturbed pK_a value and exist as an uncharged species at neutral pH. In comparison, none of the lac permease mutants with either Asp237 or Lys358 and a neutral substitution at the opposite position catalyzes significant accumulation (King et al., 1991; Sahin-Tóth et al., 1992), and only Asp237→Cys exhibits measurable activity (Frillingos et al., 1994). On the other hand, all of the single-Cys or single-Ala mutants at these positions grow as red colonies, indicating that they retain at least some ability to translocate substrate "downhill" (Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993). Similarly, all of the single mutants in sucrose permease containing an unpaired acidic residue at either position 234 or position 356 also grow as red colonies (Table 2).

The Asp237−Lys358 charge pair in lac permease is not mandatory for active transport but appears to be important for insertion of the permease into the membrane (Dunten et al., 1993). Functional permeases lacking the charge pair are defective in a step between translation and insertion into the membrane, but the mutants are stable once inserted into the membrane (Dunten et al., 1993). Therefore, we reasoned that the low activity of native sucrose permease (Bockmann et al., 1992) may be due to poor insertion into the membrane secondary to the absence of the charge pair between helices VII (Asn234) and XI (Ser356). To test this possibility, the functional mutant N234D/S356R[3] was expressed from a medium copy number plasmid which affords a low level of wild-type expression [Figure 4; see Sahin-Tóth et al. (1995) in addition], and it is apparent that increased amounts of the mutant protein appear in the membrane (Figure 5). Thus, the charge pair interaction between helices VII and XI apparently enhances folding and insertion of sucrose permease in a manner similar to that described for lac permease. However, increased expression is not accompanied by an increase in transport activity (compare Figures 3 and 5), indicating that the catalytic efficiency of sucrose permease function is compromised relative to wild-type. Finally, in agreement with observations made with lac permease (Dunten et al., 1993), the differences in expression are not observed when the wild-type and mutant N234D/S356R sucrose permeases are expressed at a high rate either from a high copy number plasmid or from the T7 promoter (Figures 4 and 5; data not shown).

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