Conformational flexibility at the substrate binding site in the lactose permease of *Escherichia coli*

(bioenergetics/transport/membrane proteins/conformational change)

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ABSTRACT   Glu-126 (helix IV) and Arg-144 (helix V) are charge paired and play a critical role in substrate binding in the lactose permease of *Escherichia coli*. When Glu-126 is replaced with Asp, the permease has relatively high activity, implying that helix V has sufficient flexibility to allow Arg-144 to accommodate the decreased length of the carboxylate-containing side chain of Asp-126. Helices IV and V contain five Gly residues at positions 115, 121, 141, 147, and 150, all of which are conserved in the oligosaccharide/H⁺ symport family of the major facilitator superfamily. To test the notion that these residues may contribute to conformational flexibility, each residue was replaced with Ala in either the wild type or the Glu-126 → Asp mutant. Although the replacements are well tolerated in the wild type, the mutations severely inactivate substrate binding and transport in the Glu-126 → Asp background, with the exception of Gly-121 → Ala, which retains significant activity. Strikingly, moreover, in two instances (Gly-150 → Ala and Gly-141 → Ala), significant activity is recovered when Ala residues at approximately parallel positions in helix IV (Ala-122 or Ala-127, respectively) are replaced with Gly. In addition to providing further evidence that the major determinants for substrate binding in the permease are at the interface between helices IV and V, the findings indicate that the region is conformationally flexible.

The lactose permease (lac permease) encoded by the *lacY* gene of *Escherichia coli* catalyzes galactoside/H⁺ symport and is an important model for secondary transport proteins from Archaea to the mammalian central nervous system that transduce free energy stored in electrochemical ion gradients into solute concentration gradients (reviewed in refs. 1 and 2). The permease has been solubilized and purified in a completely active state (reviewed in ref. 3) and functions as a monomer (see ref. 4). The protein contains 12 α-helices that traverse the membrane in zigzag fashion, connected by relatively hydrophilic loops with both the N and C termini on the cytoplasmic face (5, 6).

In a functional mutant devoid of native Cys residues, each residue has been replaced with Cys (reviewed in ref. 7). Analysis of the mutant library has led to the following developments (see ref. 5 and 6–8). (*i*) The great majority of the mutants are expressed normally in the membrane and have significant activity, and only six side chains are clearly irreplaceable for active transport: Glu-126 (helix IV) and Arg-144 (helix V), which are indispensable for substrate binding, as well as Glu-269 (helix VIII), Arg-302 (helix IX), His-322 (helix X), and Glu-325 (helix X), which are critical for H⁺ translocation and coupling with substrate translocation. (*ii*) Helix packing, tilts, and ligand-induced conformational changes have been determined by using site-directed biochemical and biophysical techniques. (*iii*) Positions that are accessible to solvent have been revealed. (*iv*) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified. (*v*) The permease has been shown to be a highly flexible molecule. (*vi*) A working model describing a mechanism for lactose/H⁺ symport has been formulated.

A variety of observations (reviewed in refs. 7 and 8) indicate that lac permease is a highly flexible molecule. In this communication, dynamic aspects of the interface between helices IV and V (Fig. 1), which contain the major determinants for substrate binding, are documented. The conservative mutation E126D† has relatively mild effects on substrate binding or transport (9, 10), suggesting that its charge partner, Arg-144 (11, 12), must be able to move to compensate for the decrease in length of the carboxylate-containing side chain at position 126. Sequence analysis of helices IV and V shows that 10 of the 36 Gly residues in the permease lie in the vicinity of helices IV and V, and several are conserved (13). When four of the five conserved Gly residues in helix V are replaced individually with Ala in the E126D mutant, binding and transport are severely impaired, and specific replacement of Ala-122 or Ala-127 in helix IV with Gly, respectively, rescues activity in the E126D/G150A or the E126D/G141A mutant.

MATERIALS AND METHODS

Materials. Deoxynucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Restriction endonucleases, T4 DNA ligase, and Vent polymerase were from New England Biolabs. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus was prepared as described (14) by Babco (Richmond, CA). *p*-Nitrophenyl-α,D-[6-³H]galactopyranoside (NPG) was kindly provided by Gérard LeBlanc (Univ. of Nice, Villefranche sur mer cedex, France). All other materials were reagent grade and obtained from commercial sources.

Growth of Bacteria. *E. coli* T184 [*lacI⁺O⁺Z⁻Y⁻(A)*, *rpsL*, *met⁻*, *thr*, *recA*, *hsdM*, *hsdR/F′*, *lacI^qO⁺Z^D118(Y⁺A⁺)*] (15), transformed with plasmid pt7-5/cassette *lacY* encoding given permease mutants, was grown aerobically at 37°C in Luria–Bertani 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 *i*-propyl 1-thio-β,D-galactopyranoside. After additional growth for 2 h at 37°C, cells were harvested by centrifugation.

Abbreviations: lac permease, lactose permease; TDG, D-galactopyranosyl-1-thio-β,D-galactopyranoside; NPG, *p*-nitrophenyl-α,D-galactopyranoside; RSO, right-side-out; KPᵢ, potassium phosphate.
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†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, which is followed by the desired mutation at the position.

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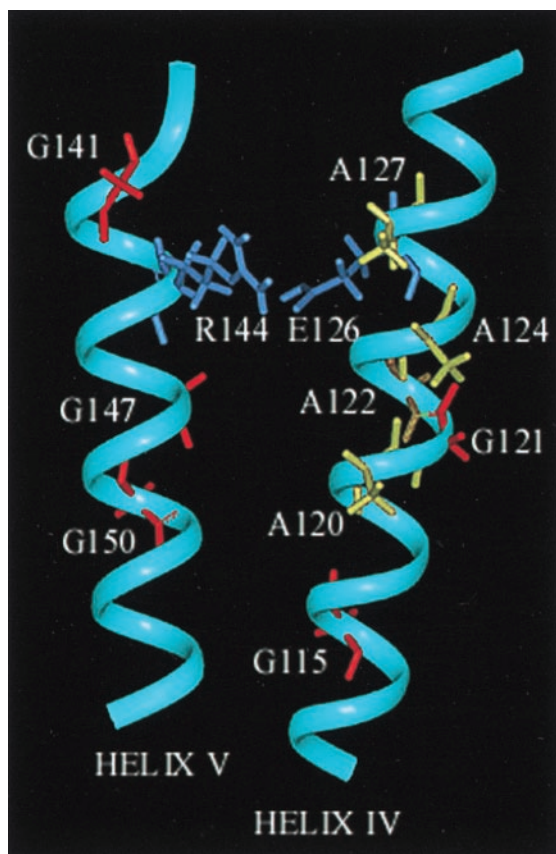*Proc. Natl. Acad. Sci. USA* 96 (1999)   11179

FIG. 1.   Model of helices IV and V in lac permease. Helices IV and V are represented as light blue ribbons. The charge pair (Glu-126 and Arg-144; dark blue), as well as the relevant Gly (G115, G121, G141, G147, and G150; red) and Ala (A120, A122, A124, and A127; yellow) residues are indicated.

Construction of Permease Mutants. By using plasmid pT7-5/cassette *lacY* encoding wild-type or mutant E126D permease, as indicated, oligonucleotide-directed site-specific mutagenesis by two-step PCR (16) was used to replace each of the five conserved Gly residues in helices IV and V with Ala mutants (G115A, G121A, G141A, G147A, and G150A). After restriction endonuclease digestion with *Pst*I and *Xho*I, the PCR products were subcloned back into the similarly treated parental vector. For replacement of Ala residues at positions 120, 122, 124, or 127 in helix IV with Gly, plasmid DNA encoding the Gly replacement mutants described above was subjected to two-step PCR, and the *Pst*I and *Xho*I restriction endonuclease fragments were cloned back into the parental vector. The *Pst*I–*Xho*I region of the *lacY* gene in all mutants described was fully sequenced through the restriction sites by using the dideoxynucleotide method (17) after alkaline denaturation (18) on an Applied Biosystems 373A automatic sequencer.

Preparation of Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared by lysozyme-EDTA (sodium salt) treatment and osmotic lysis as described (19, 20). The vesicles were suspended in 100 mM potassium phosphate (KP$_i$), pH 7.5/10 mM MgSO$_4$ at a protein concentration of 10–15 mg/ml, frozen in liquid N$_2$, and stored at $-80°C$ until use.

Transport Assays. *E. coli* T184 expressing given permease mutants 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/ml protein). Transport was initiated by addition of [1-^{14}C]lactose (5 mCi/mmol) to a final concentration of 0.4 mM. Samples were quenched at given times with 100 mM KP$_i$,

pH 5.5/100 mM LiCl and assayed by rapid filtration as described (21).

Flow Dialysis. Binding of [^{3}H]NPG was measured by flow-dialysis as described (10, 22). The upper chamber contained 250 μl of RSO vesicles at a concentration of 20–22 mg/ml protein in 0.1 M KP$_i$ (pH 7.5) and was stirred constantly. To ensure complete deenergization, 20 μM valinomycin and 0.4 μM nigericin (final concentrations) were added to the vesicles in the upper chamber. Buffer (0.1 M KP$_i$, pH 7.5) was pumped through the lower chamber at a flow rate of 0.6 ml/min, and 1.2-ml fractions were collected. Aliquots (1.0 ml) were assayed for radioactivity by addition of 5 ml of ScintiSafe Econo 2 (Fisher Scientific) scintillation mixture and liquid scintillation spectrometry.

Western Blotting. Crude membranes from the same cells used for active transport assays were prepared by osmotic lysis and sonication as described (23). Total membrane protein was assayed by a modified Lowry procedure (24). A sample containing 60 μg of membrane protein from each sample was subjected to SDS/12% PAGE (25). Proteins were electroblotted on to poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with site-directed polyclonal antibody against the C terminus of lac permease (14), followed by treatment with protein A-conjugated horse radish peroxidase.

RESULTS

Lactose Transport by Mutants with Ala in Place of Gly in Helices IV and V. As shown previously (10), E126D permease catalyzes lactose accumulation at a significantly slower rate than the wild-type permease to a comparable steady-state level of accumulation (Fig. 2 *A–E*). When the three conserved Gly residues at positions 141, 147, and 150, which are predicted to lie on the same face of helix V as Arg-144 (Fig. 1), are replaced with Ala in wild-type permease, rates of lactose accumulation are reduced, but steady-state levels of accumulation are comparable to those of wild type (Fig. 2 *C–E*). However, the same replacements in the E126D mutant virtually abolish transport activity. Similarly, Ala replacement for the two conserved Gly residues in helix IV in wild type (G115A or G121A) decreases the rate of transport with little or no effect on the steady-state level of accumulation (Fig. 2 *A* and *B*). In the E126D background, introduction of the G115A mutant almost completely abrogates activity, whereas mutant E126D/G121A accumulates lactose to about half of the steady-state level of the wild type at a significantly impaired rate. Immunoblots with membrane fractions from cells expressing each of the mutants show that the mutant proteins are expressed at levels comparable to those of wild type (data not shown). Thus, the alterations observed with respect to transport activity cannot be attributed to differences in expression levels.

NPG Binding. RSO membrane vesicles containing each Ala replacement mutant in the wild-type or E126D background were also assayed for binding of the high-affinity ligand NPG (22) by flow dialysis under nonenergized conditions (Fig. 2 *A*–E**). At the inception of the experiments, [^{3}H]NPG is added to the upper chamber containing membrane vesicles, and radioactivity in the dialysate increases rapidly to a maximum (fraction 4) and then decreases at a slow rate. When excess TDG is added to the upper chamber, bound NPG is displaced, and the concentration of radioactivity in the dialysate increases. As shown clearly in Fig. 2 *A** and *C*–E**, mutations G115A (helix IV) as well as G141A, G147A, and G150A (helix V) virtually abolish the ability of TDG to displace NPG in the E126D background, as opposed to the wild type where a significant increase in dialyzable NPG is observed on addition of TDG. On the other hand, mutant G121A (helix IV) seems to bind NPG normally in both the wild-type or E126D background (Fig. 2*B**), which is consistent
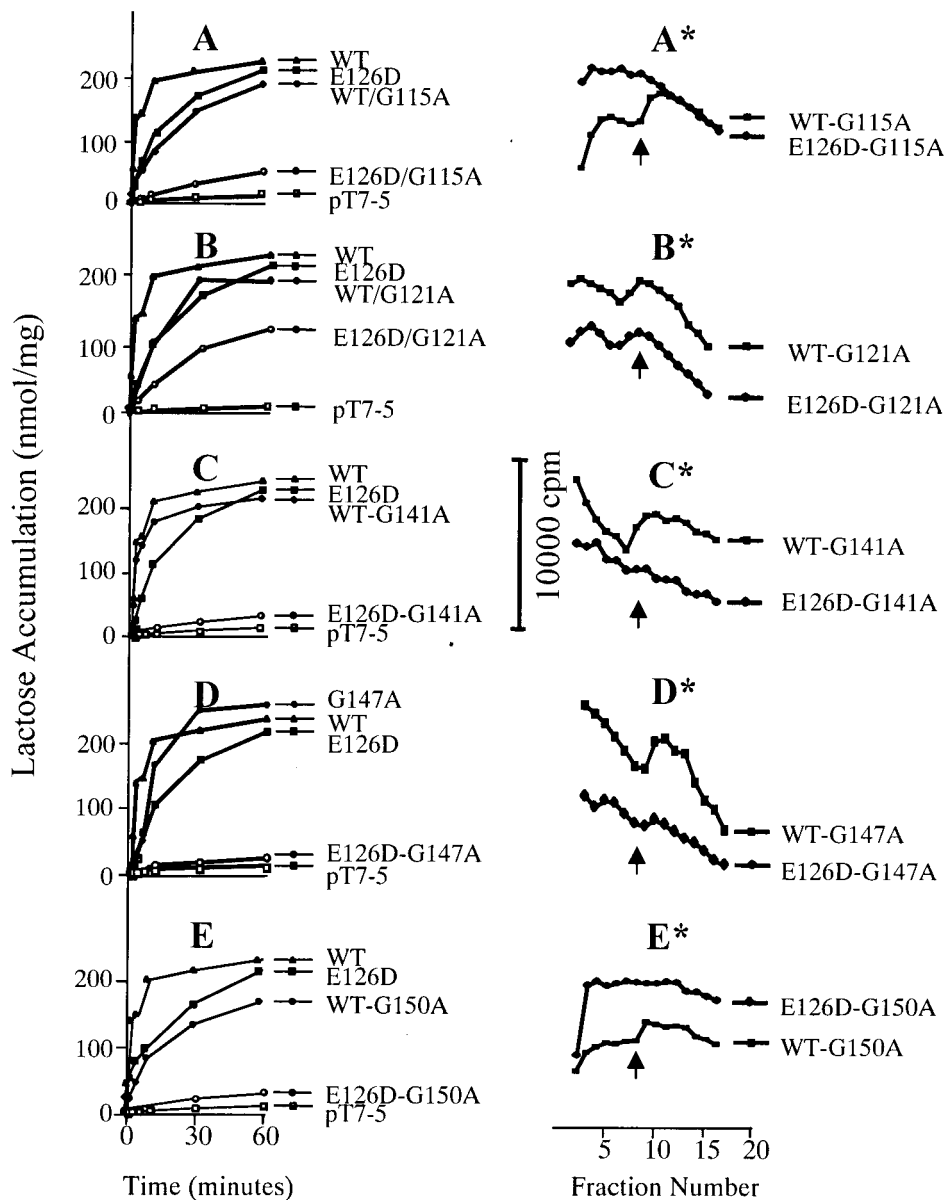
FIG. 2.   Characterization of mutants with Ala → Gly mutations in helices IV and V. (*A–E*) Time courses of lactose transport by *E. coli* T184 expressing wild-type (WT) permease or mutants with Ala in place of Gly at positions 115 (*A*), 121 (*B*), 141 (*C*), 147 (*D*), or 150 (*E*) in the wild-type or E126D background, as indicated. Experiments were carried out as described in *Materials and Methods*. (*A*–E**) Flow dialysis profiles comparing binding of NPG to RSO vesicles containing mutants G115A (*A**), G121A (*B**), G141A (*C**), G147A (*D**), or G150A (*E**) in the wild-type or E126D background, as indicated. An aliquot of RSO vesicles (250 μl) in 0.1 M KP$_i$ (pH 7.5) at a concentration of 25 mg/ml protein was placed in the upper chamber, and 5 μl of [^{3}H]NPG (0.95 mCi/mmol) was added to a final concentration of 20 μM at fraction 1. Where indicated by the arrow, 2.5 μl of D-galactopyranosyl-1-thio-β,D-galactopyranoside (TDG) was added to the upper chamber to yield a final concentration of 10 mM.

with the observation that the mutants have significant transport activity.

Rescue of Double Mutants E126D/G150A and E126D/G141A. Helix IV contains four conserved Ala residues (Ala-120, Ala-122, Ala-124, and Ala-127) in the vicinity of Glu-126 (Fig. 1; ref. 13). Although data are not shown, individual replacement of these Ala residues with Gly does not cause significant reduction in the rate and steady-state level of lactose accumulation, apart from position 127 where activity is reduced by about 50%. Furthermore, these effects are observed in both the wild-type and E126D backgrounds.

By using mutants E126D/G141A, E126D/G147A, and E126D/G150A, which have minimal transport activity, Ala residues at positions 120, 122, 124, or 127 in helix IV were replaced individually with Gly. Remarkably, by replacing Ala-122 with Gly in mutant E126D/G150A or Ala-127 with

Gly in mutant E126D/G141A, transport activity is restored significantly, whereas replacement of the other Ala residues elicits no rescue of activity (Fig. 3 *A* and *B*). In contrast, the activity of mutant E126D/G147A is not rescued significantly when each Ala → Gly replacement is introduced (data not shown).

DISCUSSION

Glu-126 and Arg-144 are irreplaceable residues (9) positioned in the cytoplasmic loop between helices IV and V, as determined by hydropathy analysis (26). However, studies with single amino acid deletions (27), as well as nitroxide-scanning electron paramagnetic resonance and accessibility measurements (M. Zhao, K.-C. Zen, J. Hernandez Borrell, C. Altenbach, W. L. Hubbell, and H.R.K., submitted for publication),
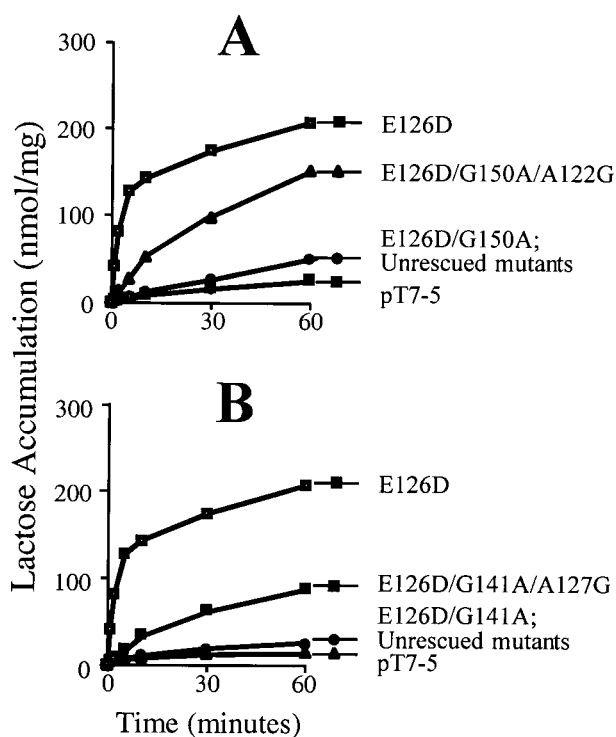
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FIG. 3. Rescue of transport activity in permease mutants E126D/G150A or E126D/G141A. (*A*) Time courses of lactose transport by *E. coli* T184 expressing mutant permeases E126D, E126D/G150A, E126D/G150A/A122G, or no permease (i.e., cells transformed with pT7-5 with no insert). "Unrescued mutants" refers to mutant E126D/G150A containing, in addition, mutations A120G, A124G, or A127G, which do not vary from the line shown by more than 20%. (*B*) Time courses of lactose transport by *E. coli* T184 expressing mutant permeases E126D, E126D/G141A, E126D/G141A/A127G, or no permease (i.e., cells transformed with pT7-5 with no insert). "Unrescued" refers to E126D/G141A containing mutations A120G, A122G, or A124G, which do not vary from the line shown by more than 20%.

indicate that both residues are within helices IV and V, rather than in the loop (Fig. 1). Other experiments (11) provide evidence that Glu-126 and Arg-144 are charge paired, and most recently (12), it has been shown that E126H/R144H permease binds Mn(II) with a K_d of about 40 μM at pH 7.5, and the appropriately labeled double-Cys mutant has spin–spin interactions and excimer fluorescence. Thus, positions 126 and 144 are in close proximity.

Replacement of Glu-126 or Arg-144 with neutral amino acids completely abolishes binding (11) and transport (9), and activity is not observed with double-neutral substitutions or when the residues are interchanged. The only mutants that have significant transport and/or binding are E126D, which has wild-type steady-state levels of accumulation but a K_m that is about 6-fold higher than that of wild type with a similar V_{max}, and R144K, which transports lactose at a minimal rate to a low steady-state and has no detectable ligand binding (9–11). Taken with observations regarding the role of Cys-148 in substrate binding (28, 29), the observations are consistent with a model for the substrate binding site in which Arg-144 is held in place by charge pairing with Glu-126 in such a fashion as to enable H bonding of one guanido NH$_2$ with the OH groups at the C4 and/or C3 positions of the galactosyl moiety of the substrate. Given this scenario and the observation that replacement of Glu-126 with Asp has relatively minor effects on binding and transport, it is apparent that to form an effective charge pair with an Asp residue at position 126, Arg-144 must be able to move to compensate for the decrease in the length of the carboxylate-containing side chain.

An obvious target for such flexibility is Gly residues, and interestingly, five conserved Gly residues are present in helices IV and V (13). Previous studies (30) show that individual replacement of these conserved Gly residues with Cys is tolerated at position 121, 141, or 150, whereas mutant G115C or G150C have decreased activity. As shown here, however, replacement of Gly-115 or Gly-150 with Ala is tolerated in wild-type permease. In any case, replacement of Gly-141, Gly-147, or Gly-150 in helix V with Ala is tolerated in the wild type, albeit with significant reduction in the rate of transport, suggesting that these residues are not essential with respect to packing constraints. In marked contrast, the same replacements in mutant E126D almost completely abolish activity. Furthermore, in each of the Gly → Ala replacement mutants, ligand binding is severely reduced or abolished, indicating that loss of transport activity results primarily from a defect in binding *per se* rather than a subsequent translocation step or steps. As such, the results are consistent with the argument that the major determinants for substrate binding in lac permease are located at the interface between helices IV and V.

Assuming an α-helical configuration of helix V, Gly-141, Gly-147, and Gly-150 lie on the same face as Arg-144 (Fig. 1). Therefore, reducing conformational flexibility on this face by Gly → Ala replacement may prevent motions that allow interaction of Arg-144 with an Asp residue in place of Glu at position 126. The properties of mutants G115A and G121A in helix IV indicate that Gly-115 may also be important for effective charge-pair formation between an Asp at position 126 and Arg-144. In contrast, Gly-121 seems to be unimportant, because its replacement with Ala is tolerated reasonably well in both the wild-type and E126D backgrounds. Although Gly-115 presumably lies approximately four α-helical turns from Glu-126 on the same face of helix IV, Gly-121 lies on the opposite face of the helix, and replacement with Ala may not cause a significant conformational alteration. In addition, direct binding measurements with these mutants are consistent with the conclusion that replacement of Gly-115 with Ala alters substrate binding rather than translocation.

Based on the notion that the Gly → Ala replacements in helix V limit conformational flexibility at the substrate binding site, thereby preventing effective charge pairing between an Asp residue at position 126 and Arg-144 with elimination of substrate binding and transport, Ala-120, Ala-122, Ala-124, or Ala-127 in helix IV were replaced with Gly in an effort to reintroduce flexibility. Remarkably, when introduced into the E126D/G141A mutant, activity is significantly recovered by replacement of Ala-127 with Gly, and with mutant E126D/G150A, activity is rescued significantly by replacement of Ala-122 with Gly. In both instances, the effect is specific for the given Ala replacement. As shown in Fig. 1, Ala-122 and Ala-127 in helix IV lie at approximately the same level as Gly-150 and Gly-141 in helix V, respectively. Thus, the findings also provide support for structure postulated for this region of the permease.

Nominally, the results with respect to rescue of activity may be interpreted either in terms of changes in conformational flexibility or direct effects on interhelical packing (see ref. 31). Regarding conformational flexibility, Gly → Ala replacements should be unfavorable, whereas Ala → Gly mutations should increase flexibility. In contrast, Gly → Ala replacements should be unfavorable with respect to helix packing, whereas Ala → Gly replacements should be favorable. The results presented here are more consistent with the interpretation that the region is conformationally flexible, because, although Gly → Ala replacements in helix V reduce activity, in only two instances (E126D/G141A and E126D/G150A) is rescue observed when Ala → Gly replacements are made in helix IV (A127G and A122G, respectively).

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