Chemical Rescue of Asp237→Ala and Lys358→Ala Mutants in the Lactose Permease of *Escherichia coli*

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Received June 18, 1996; Revised Manuscript Received August 12, 1996[⊗]

ABSTRACT: Asp237 (helix VII) and Lys358 (helix XI) form a salt bridge in the lactose permease, and neutral replacement of either residue inactivates. Remarkably, noncovalent neutralization of the unpaired Asp or Lys residue, respectively, with *n*-alkylsulfonates or *n*-alkylamines of appropriate size restores active transport to high levels in the mutants. Saturation with respect to the concentration of the alkylamines and different size preferences suggest that the alkylamines bind sterically at position 358. Rescue of Asp237→Ala by alkylsulfonates is apparently more indiscriminate, since methane-, ethane-, or propanesulfonate have comparable effects. Sodium and chloride, respectively, are also effective in rescuing the Lys358→Ala and Asp237→Ala mutants, while various other compounds are ineffective. In marked contrast to Asp237→Ala or Lys358→Ala permease, alkylsulfonates or alkylamines have no effect whatsoever on the activity of mutants with neutral replacements for Asp240, Glu269, Arg302, Lys319, His322, or Glu325. The results support the conclusion that neutral replacement of one member of the charge pair between Asp237 and Lys358 leads to inactivation because of an unpaired charge in the low dielectric of the membrane. In addition, the findings are consistent with the idea that interactions between Arg302 and Glu325, His 322 and Glu269, and Asp240 and Lys319 play important roles in the mechanism of the permease, which is not the case for either Asp237 or Lys358 or the salt bridge between the two residues.

Noncovalent chemical rescue (i.e., restoration of the activity of mutant enzymes by exogenous organic compounds that mimic the side chain of an active-site residue) was demonstrated originally for the inactive K258A[1] mutant of *Escherichia coli* aspartate aminotransferase by use of primary amines (Toney & Kirch, 1989, 1992). Subsequent demonstrations of chemical rescue with primary amines include reactivation of Lys191 (Smith & Hartman, 1991) and Lys329 mutants in ribulose-bisphosphate carboxylase/oxygenase (Harpel & Hartman, 1994), a Lys296 mutant in rhodopsin (Zhukovsky et al., 1991), and a Lys80 mutant in leucine dehydrogenase (Sekimoto et al., 1993). In addition, a Tyr14 mutant in Δ^5-3-ketosteroid isomerase can be reactivated with exogenous phenols (Brooks & Benisek, 1992), and an Arg127 mutant in carboxypeptidase A has been rescued with alkylguanidines (Phillips et al., 1992).

In the present paper, we describe chemical rescue of mutants that disrupt the charge pair between Asp237 (helix VII) and Lys358 (helix XI) in the lactose (lac)[2] permease of *E. coli*, a polytopic membrane transport protein that catalyzes the coupled stoichiometric translocation of a β-galactoside and a H^+ [reviewed in Kaback et al. (1994) and Kaback (1996)]. Interaction between Asp237 and Lys358 (Figure 1) was demonstrated initially by King et al. (1991) on the basis of second-site suppressor analysis. Permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective in lactose transport, and second-site suppressor mutations of K358T exhibit neutral amino acid substitutions for Asp237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys358. Subsequently, as part of an

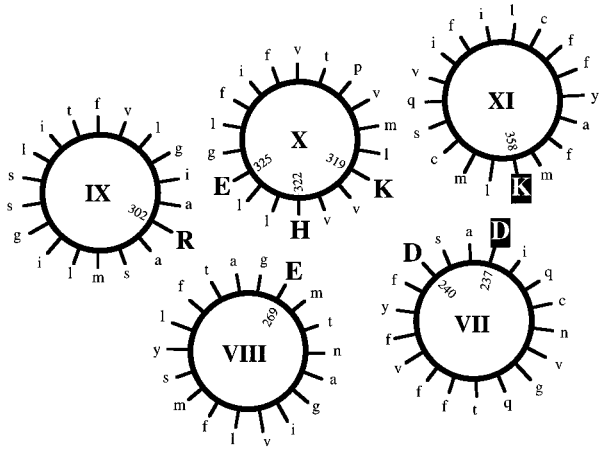

FIGURE 1: Helical wheel model of helices VII−XI of lac permease showing the relationship between Asp237 (helix VII) and Lys358 (helix XI), between Asp240 (helix VII) and Lys319 (helix X), between Glu269 (helix VIII) and His322 (helix X), and between Arg302 (helix IX) and Glu325 (helix X). The model for the arrangement of the helices in the C-terminal half of the permease is according to Jung et al. [1993; see He et al. (1995a,b) and Wu et al. (1995) in addition]. Helices are viewed from the periplasmic surface of the membrane.

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[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1996.

[1] For designation of mutants, the first letter denotes the amino acid present in the wild-type protein at the numbered position, and the final letter denotes the amino acid present at the corresponding position in the mutant protein.

[2] Abbreviations: lac, lactose; C-less permease, a functional lac permease mutant devoid of Cys residues; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; PMS, phenazine methosulfate.

S0006-2960(96)01453-5 CCC: $12.00

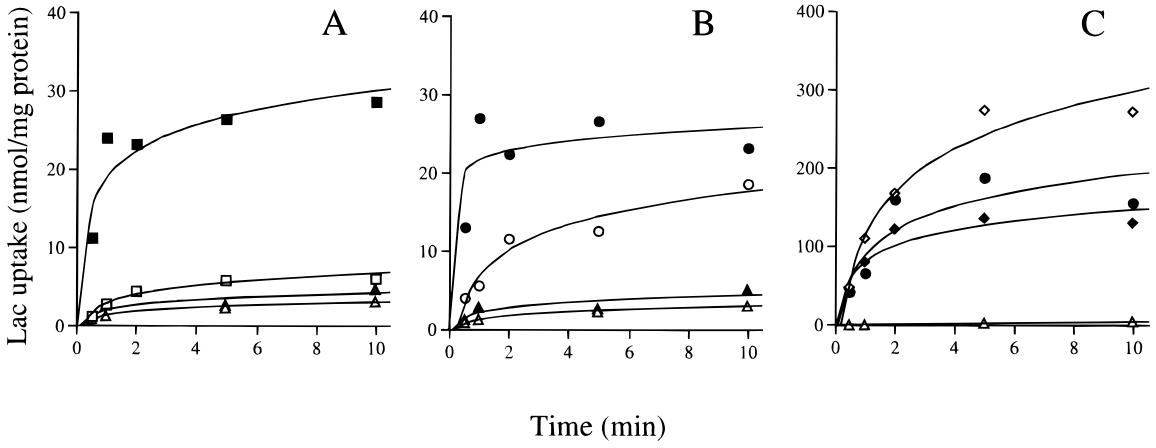

FIGURE 2: Lactose transport by *E. coli* T184 expressing D237A, K358A, or wild-type permease in the absence (open symbols) or presence of 0.5 M propanesulfonate or 0.5 M propylamine (closed symbols). Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described under Materials and Methods. (A) Time courses of lactose transport by T184 transformed with plasmid pT7-5 (vector with no *lacY* gene) in the absence (△) or presence of 0.5 M propanesulfonate (▲) or pT7-5 encoding D237A permease in the absence (□) or presence of 0.5 M propanesulfonate (■). (B) Time courses of lactose transport by T184 transformed with plasmid pT7-5 in the absence (△) or presence of 0.5 M propylamine (▲) or pT7-5 encoding K358A permease in the absence (○) or presence of 0.5 M propylamine (●). (C) Time courses of lactose transport by T184 transformed with plasmid pT7-5 (△) or pT7-5 encoding wild-type lac permease in the absence (◇) or presence of 0.5 M propanesulfonate (◆) or 0.5 M propylamine (●).

extensive Cys-scanning mutagenesis study [reviewed in Kaback et al. (1994) and Kaback (1996)] on a functional permease mutant devoid of Cys residues (C-less permease), intramembrane charged residues were systematically replaced with Cys (Sahin-Tóth et al., 1992). Individual replacement of Asp237 or Lys358 in C-less permease with Cys or Ala abolishes active lactose transport, while simultaneous replacement of both residues with Cys and/or Ala or reversal of the residues leads to active permease. Remarkably, mutant D237C is restored to full activity by carboxymethylation (Dunten et al., 1993), which reintroduces a negative charge at position 237, and mutant K358C is restored to full activity by treatment with ethylammonium methanethiosulfonate, which reintroduces a positive charge at position 358 (Sahin-Tóth & Kaback, 1993). Taken together, the findings clearly suggest that Asp237 and Lys358 neutralize each other via a salt bridge. Other observations indicate that although the salt bridge is not involved in activity, it plays an important role in the insertion of the permease into the membrane (Dunten et al., 1993). Finally, by using engineered divalent metal-binding sites (*bis*-His residues), He et al. (1995a) have demonstrated directly that position 237 is in close proximity to position 358.

As shown here, the inactive permease mutants D237A or K358A can be rescued by *n*-alkylsulfonates or *n*-alkylamines, respectively. Rescue is also observed with chloride or sodium, respectively, but a number of other charged compounds fail to restore activity. The results support the conclusion that neutral replacement of either Asp237 or Lys358 leads to inactivation due to the presence of an unpaired charge within the membrane and are consistent with the hypothesis that positions 237 and 358 are in an amphipathic environment.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose was purchased from Amersham, Arlington Heights, IL. *n*-Alkylsulfonates (sodium salts) and *n*-alkylamines (hydrochloride salts) were purchased from Sigma or Aldrich at the highest purity available (≥98%). All other compounds were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. *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/*lacY* with given mutations was used for expression of lac permease from the *lacZ* promoter/operator by induction with isopropyl 1-thio-β,D-galactopyranoside (IPTG). Construction and verification of mutants, D237A, D237K, K358A, K358D, D240A, K319A, E269C (Sahin-Tóth et al., 1992), H322N (Puttner et al., 1986), R302L (Menick et al., 1987), and E325A (Carrasco et al., 1986) have been described previously.

Growth of Bacteria. *E. coli* T184 (*lacZ*$^-$*lacY*$^-$) transformed with each plasmid was grown aerobically at 37 °C in Luria−Bertani medium containing appropriate antibiotics. Fully grown cultures were diluted 10-fold and allowed to grow for another 2 h before induction with 0.5 mM IPTG. After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

Transport Assay. Cells were washed with 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10.0 at 420 nm (approximately 0.7 mg of protein/mL). Transport of [1-^{14}C]-lactose (2.5 mCi/mmol; 1 mCi = 37 MBq) at a final concentration of 0.4 mM was assayed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS), by rapid filtration (Sahin-Tóth & Kaback, 1993).

RESULTS

Asp237 and Lys358 Mutants. *(A) Rescue of D237A Permease with n-Alkylsulfonates*. D237A permease is severely defective with respect to active lactose transport, exhibiting a rate that is only marginally higher than that observed with plasmid containing no *lacY* insert (Figure 2A; Sahin-Tóth et al., 1992; Dunten et al., 1993). Strikingly, in the presence of 0.5 M propanesulfonate, D237A permease transports lactose at a highly significant rate [ca. 20 nmol

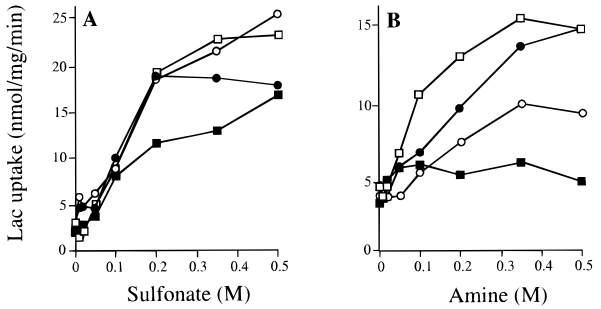

FIGURE 3: Effect of *n*-alkylsulfonates on lactose transport by D237A permease (A) or *n*-alkylamines on lactose transport by K358A permease (B). (A) *E. coli* T184 transformed with plasmid pT7-5 encoding D237A permease was grown, induced, and assayed for lactose transport as described in the legend to Figure 2 and under Materials and Methods. Initial rates of lactose uptake were determined at 0.5 and 1 min after initiation of the transport reaction, in the presence of the given final concentrations of methanesulfonate (○), ethanesulfonate (●), propanesulfonate (□), or butanesulfonate (■). (B) *E. coli* T184 transformed with plasmid pT7-5 encoding K358A permease was grown, induced, and assayed for initial rates of active lactose transport as in (A), in the presence of given final concentrations of methylamine (○), ethylamine (●), propylamine (□), or butylamine (■).

(mg of protein)$^{-1}$ min^{-1}] to a significant steady-state level of accumulation [30 nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$], while very little transport is observed in the absence of the alkylsulfonate (Figure 2A). With respect to the initial rate of transport, activity is about 30% or 20% of that observed with C-less or wild-type lac permease, respectively. However, it should be emphasized that disruption of the salt bridge between Asp237 and Lys358 causes marked reduction in the amount of permease in the membrane (i.e., 10−30% of wild-type). Thus, the specific activity of the D237A mutant in the presence of 0.5 M propanesulfonate is probably close to that of wild-type permease. In contrast to D237A permease, 0.5 M propanesulfonate causes mild inhibition of activity with C-less or wild-type permease (Figure 2C and data not shown). Analysis of the effect of methane-, ethane-, propane-, or butanesulfonate over concentrations ranging from 0.005 M to 0.5 M shows that activity is restored in all cases, and with the exception of butanesulfonate, the sulfonates exhibit saturation behavior (Figure 3A). D237A permease exhibits comparable apparent K_ds for the alkylsulfonates (170−200 mM) and similar V_{max}s [30−35 nmol (mg of protein)$^{-1}$ min^{-1}].

(B) Rescue of K358A Permease with n-Alkylamines. K358A permease also exhibits a low rate of transport (compare Figure 2B and Figure 2C) and is inserted into the membrane poorly [see Dunten et al. (1993) in addition]. In the presence of 0.5 M propylamine, the activity of K358A permease is increased at least 4-fold, attaining a rate of 15−20 nmol (mg of protein)$^{-1}$ min^{-1} (Figure 2B). Under the same conditions, 0.5 M propylamine has little effect on the initial transport rate of C-less or wild-type permease (Figure 2C; data not shown). Analysis of the effect of alkylamines at concentrations ranging from 0.005 M to 0.5 M demonstrates that significant activity is restored by propyl-, ethyl-, or methylamine in that order of efficacy, and saturation behavior is observed with respect to concentration (Figure 3B). However, no significant effect is observed with butylamine over the same concentration range (Figure 3B). Methyl-, ethyl-, or propylamine have virtually identical pK_a values (Table 1), and stimulation of transport correlates

Table 1: Amine Rescue of Lactose Transport Activity for K358A Permease

amine	pK_a[a]	molecular volume[a] (Å^3)	K_d(amine)[b] (mM)	V_{max}[b] (nmol mg^{-1} min^{-1})
(none)				(4.1)
methyl-	10.6	42.1	120	12.8
ethyl-	10.6	60.9	100	15.9
propyl-	10.5	79.8	80	20.0
butyl-	10.6	98.7	ND[c]	ND[c]
(wild-type)		(79.8)[d]		

[a] Values from Toney and Kirsch (1989). [b] Calculated from Lineweaver−Burk plots of the data in Figure 3B as described by Harpel and Hartmann (1994). [c] Insufficient rescue to determine kinetic parameters. [d] Difference in the average molecular volume of a lysyl relative to alanyl side chain (Clothia, 1975).

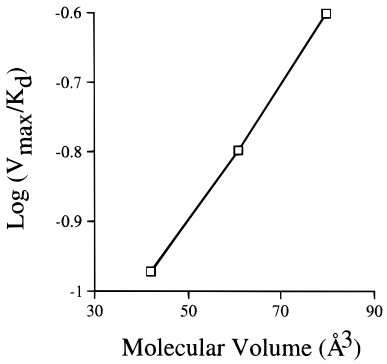

FIGURE 4: Dependence of chemical rescue of K358A permease on the molecular volume of the *n*-alkylamine. Data are from Figure 3B and Table 1.

linearly with the molecular volume of the *n*-alkylamine (Figure 4). Optimal rescue is observed with propylamine which has the closest fit to the difference in molecular volume between Lys and Ala (79.8 Å^3; Table 1).

(C) Effect of Other Ions. The possibility that rescue reflects ionic strength effects was examined by testing of a number of other salts. With D237A permease, no effect is found with sodium phosphate or glutamate; however, rescue is observed with sodium chloride, and the maximum effect is observed at 0.2−0.4 M (Figure 5A). Similarly, in the case of K358A permease, no effect of triethanolamine or lysine hydrochloride is observed, but lesser though significant rescue is observed with sodium chloride (Figure 5B). Kinetic analysis (Harpel & Hartman, 1994) shows that D237A has an apparent K_d(Cl^-) of 254 mM and a V_{max}(Cl^-) of 35 nmol (mg of protein)$^{-1}$ min^{-1}. Thus, Cl^- appears to have an effect comparable to that of the alkylsulfonates, while K358A has an apparent K_d(Na^+) of 110 mM and a V_{max}(Na^+) of 12.5 nmol mg^{-1} min^{-1}, values that are comparable to those observed for methylamine (Table 1). No sodium chloride effect is observed with wild-type permease under the same conditions (data not shown), while tetraphenylboron$^-$ (0.5 mM − 0.1 M) or tetraphenylphosphonium$^+$ (0.5 mM − 0.02 M) has no effect at low concentrations and inactivates at high concentrations (Figure 5 and data not shown).

Asp240 and Lys319 Mutants. As opposed to Asp237−Lys358, simultaneous neutral replacement of both Asp240 and Lys319 yields only partially active permease (Sahin-Tóth et al., 1992), and reversal of the residues inactivates (Dunten et al., 1993). Moreover, D240E permease is inactive, carboxymethylation of D240C permease fails to

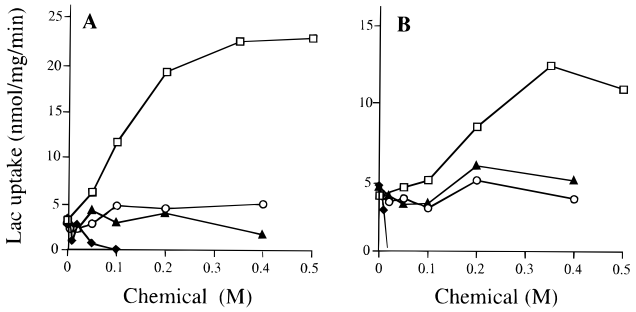

FIGURE 5: (A) Effect of sodium chloride (□), sodium phosphate (○), sodium glutamate (▲), or sodium tetraphenylboron (◆) on active lactose transport by D237A permease. (B) Effect of sodium chloride (□), triethanolamine (○), lysine hydrochloride (▲), or tetraphenylphosphonium bromide (◆) on active lactose transport by K358A permease. Assays were performed at pH 7.5. All conditions were as described in the legend to Figure 3. Tetraphenylboron$^-$ or tetraphenylphosphonium$^+$ cannot be tested at concentrations above 100 mM or 20 mM, respectively, as they completely inhibit transport by wild-type lac permease at these concentrations; however, no stimulation is observed at concentrations of 0.5 mM which do not inhibit transport by wild-type permease (not shown).

increase activity (Sahin-Tóth et al., 1992; Sahin-Tóth & Kaback, 1993), and replacement of both Asp240 and Lys319 with His does not generate a divalent metal binding site (He et al., 1995a). Thus, Asp240 and Lys319 appear to interact over a longer distance. Neither D240A nor K319A permease is rescued by alkylsulfonates or alkylamines, respectively, at concentrations ranging from 0.01 to 0.5 M (data not shown).

Glu269, His332, Arg302, and Glu325 Mutants. It is clear from both site-directed excimer fluorescence (Jung et al., 1993) and the use of engineered divalent metal binding sites (i.e., *bis*-His residues; He et al., 1995a,b) that Glu269 is paired with His322 and Arg302 is paired with Glu325. However, unlike the Asp237−Lys358 and Asp240−Lys319 pairs, double neutral replacement of Glu269 and His322 or Arg302 and Glu325 does not lead to functional permease (Sahin-Tóth et al., 1992), and these four residues are the only known irreplaceable residues in the permease with respect to active lactose transport [see Kaback et al. (1994) and Kaback (1996)]. Consistently, mutants E269C, R302L, H322N, or E325A exhibit no stimulation whatsoever by the appropriate alkylsulfonates or alkylamines at concentrations ranging from 0.01 to 0.5 M (data not shown).

DISCUSSION

Lac permease with neutral amino acid replacements for Asp237 (helix VII) or Lys358 (XI) exhibits low activity presumably because disruption of the charge pair between these residues leaves an uncompensated charge in the low dielectric of the membrane that is energetically unfavorable (Honig & Hubbell, 1984). Consistently, compensation of the unpaired residue by genetic manipulation or genetic manipulation combined with chemical modification leads to almost complete recovery of activity (King et a., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993; He et al., 1995a). Strikingly, as demonstrated here, exogenous compounds that mimic the missing charged side chain in mutants D237A or K358A also restore activity, thereby demonstrating that neutralization of the unpaired charge within the membrane can be accomplished without covalent modification. In contradistinction, disruption of the charge pair between Asp240 and Lys319, Glu269 and His322, or Arg302 and Glu325 by introduction of a neutral residue does not result in chemical rescue in all likelihood because these pairs of residues play important (Asp240−Lys319) or essential (Glu269−His322 and Arg302−Glu325) roles in the transport mechanism [reviewed in Kaback et al. (1994) and Kaback (1996)].

Neither Asp237 nor Lys358 *per se* nor the salt bridge formed between these residues is important for activity, but the interaction is important for insertion of the permease into the membrane (Dunten et al., 1993; Frillingos et al., 1995). Functional permease devoid of the charge pair is defective in a step between translation and insertion into the membrane, thereby indicating that the C-terminal half of lac permease (helices VII−XI; Figure 1) is inserted posttranslationally (Dunten et al., 1993; Frillingos et al., 1995). The possibility that Asp237 and Lys358 interact transiently as a folding intermediate during insertion into the membrane but are not in close proximity in the mature, folded state is unlikely, as chemical modification of Cys residues introduced at these positions with appropriately charged thiol reagents restores activity (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993). Moreover, replacement of both Asp237 and Lys358 with His residues generates a divalent metal binding site (He et al., 1995a). As shown here, D237A or K358A permease, respectively, is rescued by alkylsulfonates or alkylamines after insertion into the membrane, even though expression levels are low (Dunten et al., 1993). As such, the observations support the contention that positions 237 and 358 remain in close proximity even when the charge pair is disrupted. Attempts to repair the insertion defect *in vivo* by inclusion of alkylsulfonates or alkylamines in the culture medium during induction of D237A or K358A permease, respectively, were negative (S. Frillingos and H. R. Kaback, unpublished data).

The efficiency of rescue with D237A or K358A permease is highly significant and varies with the compounds tested (Figures 2−5). Importantly, rates of transport are restored to about 35% or 20% of the wild-type for D237A or K358A permease, respectively, which reflects almost complete recovery of activity if the amounts of the mutant proteins in the membrane relative to the wild-type (i.e., 10−30%) are taken into account. With K358A permease, rescue is clearly correlated with the size of the amine (Figure 4), and optimal reactivation is achieved with propylamine which restores completely both the mass and charge difference between the lysyl and alanyl side chains (Table 1), while butylamine fails to restore activity. Noncovalent rescue of D237A permease appears to be more flexible, since all for sulfonates used, as well as chloride, restore transport activity, and there is no correlation between the efficiency of rescue and the size of the alkylsulfonate. Interestingly, an analogous observation has been made in covalent modification studies (Sahin-Tóth & Kaback, 1993), where derivatization of Cys237 in mutants D237C or D237C/K358R leads to fully functional permease molecules with an overall increase of 2, 3, 4, or 5 bond lengths between the original Asp and Lys residues at positions 237 and 358, respectively.

Finally, it is noteworthy that chloride and sodium, as well as alkylsulfonates and alkylamines, respectively, are able to rescue mutants D237A and K358A. The observations are consistent with the placement of positions 237 and 358 in

an amphipathic environment (Ujwal et al., 1995) and with the observation that the double mutant D237H/K358H binds manganese with a micromolar K_d and a pK_a of about 6.3 (He et al., 1995a).

ACKNOWLEDGMENT

We are indebted to Fred C. Hartman whose studies on the chemical rescue of rubulose-bisphospate carboxylase/oxygenase (Smith & Hartman, 1991; Harpel & Hartmann, 1994) led directly to these experiments.

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BI961453C