Effect of Distance and Orientation between Arginine-302, Histidine-322, and Glutamate-325 on the Activity of *lac* Permease from *Escherichia coli*

Jonathan A. Lee, Irene B. Püttner, and H. Ronald Kaback*
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110
Received September 28, 1988; Revised Manuscript Received November 10, 1988

ABSTRACT: *lac* permease of *Escherichia coli* was modified by site-directed mutagenesis in order to investigate the effects of polarity, distance, and orientation between the components of a putative H^+ relay system (Arg302/His322/Glu325) postulated to be involved in lactose-coupled H^+ translocation. The importance of polarity between His322 and Glu325 was studied by interchanging the residues, and the modified permease—H322E/E325H—is inactive in all modes of translocation. The effect of distance and/or orientation between His322 and Glu325 was investigated by interchanging Glu325 with Val326, thereby moving the carboxylate one residue around putative helix X. The resulting permease molecule—E325V/V326E—is also completely inactive; control mutations, E325V [Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry* (second paper of three in this issue)], and E325A/V326E, indicate that a Glu residue at position 326 inactivates the permease. The wild-type orientation between His and Glu was then restored by further mutation of E325V/V326E to introduce a His residue into position 323 or by interchanging Met323 with His322. The resulting permease molecules—M323H/E325V/V326E and H322M/M323H/E325V/V326E—contain the wild-type His/Glu orientation, but the His/Glu ion pair is rotated about the helical axis by 100° relative to Arg302 in putative helix IX. Both mutants are inactive with respect to all modes of translocation. The results provide strong support for the contention that the polarity between His322 and Glu325 and the geometric relationship between Arg302, His322, and Glu325 are critical for permease activity. In addition, it is suggested that the region of the permease between residues 326 and 328 is particularly sensitive to structural perturbations and may therefore play an important role in protein folding or conformational changes during lactose/H^+ symport.

As outlined in the preceding two papers (Püttner et al., 1989; Carrasco et al., 1989), oligonucleotide-directed site specific mutagenesis has been utilized to focus on the role of specific amino acids in the mechanism of lactose/H^+ symport via the *lac* permease of *Escherichia coli*. By utilizing this approach, Püttner et al. (1986, 1989), Carrasco et al., 1986, 1989), and Menick et al. (1987b) have provided evidence consistent with the idea that Arg302 (putative helix IX) and His322 and Glu325 (putative helix X) may be sufficiently close to interact and form a H^+ relay system that is postulated to function as a chemical pathway for H^+ movement through *lac* permease.

Although the approach described cannot unambiguously delineate a mechanism without detailed structural information, the working model clearly suggests that alteration of the geometry between the three residues should disrupt the proposed H^+ relay and thereby cause dramatic effects on permease activity. In order to test the hypothesis, we have utilized site-directed mutagenesis to alter the polarity, distance, and/or orientation between Arg302, His322, and Glu325 and tested the effects on permease activity.

Experimental Procedures

Materials

All materials were of reagent grade and obtained from commercial sources (Sarkar et al., 1985; Püttner et al., 1989; Carrasco et al., 1989).

Methods

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1985) with one of the following modifications to improve the frequency of mutant recovery: (i) Construction of mutant H322E/E325H[1] utilized template single-stranded (ss)[2] DNA containing the wild-type *lac* permease gene isolated from phage grown in *E. coli* BW313 (*dut⁻ung⁻*) as described by Kunkel (1985). Closed-circular (cc) heteroduplex DNA with the desired mutations was synthesized in vitro as described (Sarkar et al., 1985) and transfected into *E. coli* JM109 (*ung⁺*). Phage bearing the mutation(s) were screened as described below. (ii) *lac* permease mutation E325V/V326E was constructed by utilizing ssDNA containing the wild-type *lac* permease gene; subsequent construction of *lac* permease E325A/V326E, H322M/M323H/E325V/V326E, and M323H/E325V/V326E utilized ssDNA containing the E325V/V326E permease gene. Closed-circular heteroduplex DNA encoding permease mutants E325A/V326E, E325V/V326E, H322M/M323H/E325V, and M323H/E325V/V325E was synthesized in vitro (Sarkar et al., 1985) and used to transfect *E. coli* BMH/71-18 *mutL* (Kramer et al., 1984) without further treatment. Phage harboring the mutations were identified initially by colony-blot hybridization using the appropriate ^{32}P-labeled mutagenic primer (Carter et al., 1984). Phage from positive colonies were plaque-purified, and the

* To whom correspondence should be addressed.

[1] 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 wild-type *lac* permease. The sequence is followed by a second letter denoting the amino acid replacement at this position (e.g., H322E/E325H designates that His322 is replaced with Glu and that Glu325 is replaced with His).

[2] Abbreviations: ss, single stranded; cc, closed circular; RF, replicative form; EMB, eosin–methylene blue; *p*CMBS, *p*-(chloromercuri)benzenesulfonate.

0006-2960/89/0428-2540$01.50/0

Table I: Mutations in *lac* Permease[a]

lac permease	*lac Y* DNA sequence codons 321–327	*lac* permease amino acid sequence (residues 321–327)
wild type	CTG CAT ATG TTT GAA GTA CCG	LHMFEVP
H322E/E325H	CTG *GAA* ATG TTT *CAT* GTA CCG	L*E*MF*H*VP
E325V/V326E	CTG CAT ATG TTT G*TA G*A*A* CCG	LHMF*VE*P
E325A/V326E	CTG CAT ATG TTT G*C*A G*A*A CCG	LHMF*AE*P
H322M/M323H/E325V/V326E	CTG *ATG CAT* TTT G*TA G*A*A* CCG	L*MHFVE*P
M323H/E325V/V326E	CTG *CAT* CAT TTT G*TA G*A*A* CCG	LH*HFVE*P

[a] Italics indicates nucleotide or amino acid replaced in mutants.

mutation(s) was (were) verified by dideoxyoligonucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) utilizing synthetic primers complementary to regions of *lac Y* 50–100 bases downstream from the site of the mutation. The efficiency of mutant recovery was 5–10% with method i and greater than 35% with method ii.

Double-stranded *lac Y* DNA from several independent clones of each mutant was restricted from M13mp19 replicative form (RF) DNA and ligated into the *Eco*RI site of pACYC184. The resulting plasmids (Table I) were used to transform *E. coli* T184 (Z^-Y^-) or HB101 (Z^+Y^-) as indicated. Plasmid DNA was sequenced by the dideoxynucleotide method following alkaline denaturation (Hattori & Sakaki, 1986) using synthetic primers complementary to a region of *lac Y* approximately 50 bases downstream from the site of the mutation. In addition, the entire *lac Y* gene was sequenced by using six synthetic deoxyoligonucleotide primers complementary to appropriate regions of the gene.

Other procedures and bacterial strains utilized are described in the preceding two papers (Püttner et al., 1989; Carrasco et al., 1989).

Results

Verification of Mutations by DNA Sequencing. Initially, mutations of *lac* permease were confirmed by sequencing ss phage DNA containing the mutated *lac Y* genes (Sanger et al., 1977; Sanger & Coulson, 1978) (Table I). Subsequently, *lac Y* DNA containing the mutations was restricted from the RF phage DNA and cloned into pACYC184. The *lac Y* genes encoded by these plasmids were sequenced (Hattori & Sakaki, 1986), and the mutations shown in Table I were reconfirmed. With the exception of the mutation(s) given, the remaining nucleotide sequences were identical with those reported by Büchel et al. (1980).

Effect of His322/Glu325 Polarity. In order to reverse the polarity of His322/Glu325, codon 322 encoding His and codon 325 encoding Glu in *lac Y* were interchanged by site-directed mutagenesis, yielding H322E/E325H permease (Table I). When cryptic strain *E. coli* HB101 (Z^+Y^-) is transformed with pH322E/E325H and grown on eosin–methylene blue (EMB) plates containing 25 mM lactose, the cells grow as white colonies indistinguishable from HB101/pACYC184. Thus, H322E/E325H permease appears to be unable to catalyze lactose influx to any extent whatsoever [cf. Padan et al. (1985)]. Furthermore, right-side-out (RSO) membrane vesicles containing H322E/E325H exhibit no significant $\Delta\bar{\mu}_{H^+}$-dependent lactose accumulation (Figure 1), no significant downhill lactose efflux (Figure 2), and no significant equilibrium exchange activity (Figure 3). Importantly, however, the vesicles contain a normal complement of permease, as judged by immunoblotting experiments (Figure 4), and they catalyze $\Delta\bar{\mu}_{H^+}$-dependent proline accumulation to the same extent as vesicles containing wild-type permease (data not shown).

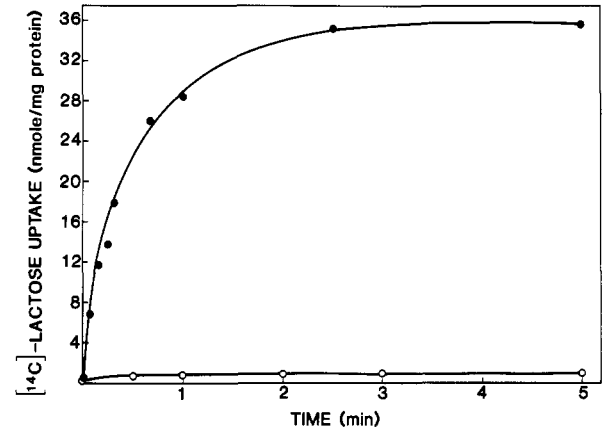

FIGURE 1: $\Delta\bar{\mu}_{H^+}$-driven transport of lactose in RSO membrane vesicles. Aliquots containing 100 μg of membrane protein (50 μL) were assayed for [1-^{14}C]lactose (10 mCi/mmol) uptake at a final concentration of 0.38 mM lactose. At indicated times, transport was terminated by dilution and rapid filtration (Kaback, 1971, 1974). (●) Wild type in the presence of ascorbate/PMS; (○) wild type in the absence of ascorbate/PMS or H322E/E325H, E325V/V326E, E325A/V326E, H322M/M323H/E325V/V326E, or M323H/E325V/V326E in the presence or absence of ascorbate/PMS. Variation between samples was less than the size of the symbol.

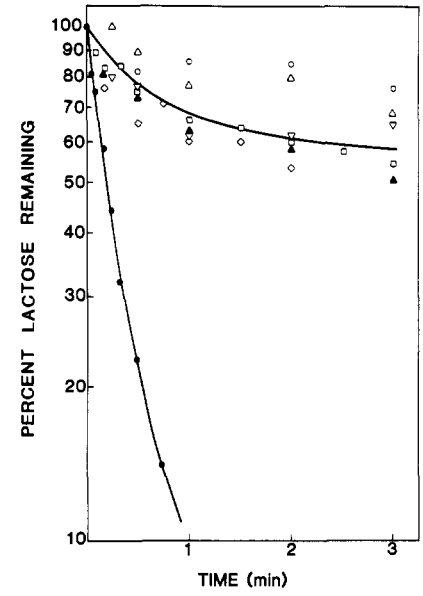

FIGURE 2: Lactose efflux in RSO membrane vesicles. Membrane vesicles (~30 mg of protein/mL) containing approximately the same amount of permease were equilibrated at 4 °C overnight with 10 mM [1-^{14}C]lactose (5.9 mCi/mmol). Aliquots (2 μL) were then rapidly diluted into media devoid of lactose. At the times indicated, the reactions were stopped with 3 mL of 0.1 M potassium phosphate (pH 5.5)/0.1 M lithium chloride/20 mM $HgCl_2$ and immediately filtered as described (Kaczorowski & Kaback, 1979). (●) Wild type; (◇) H322E/E325H; (□) E325V/V326E; (○) E325A/V326E; (▽) H322M/M323H/E325V/V326E; (△) M323H/E325V/V326E; (▲) RSO vesicle preparation treated with 1.4 mM *p*CMBS to inactivate *lac* permease. Data are expressed as a percentage of lactose retained with zero-time points for normalization.

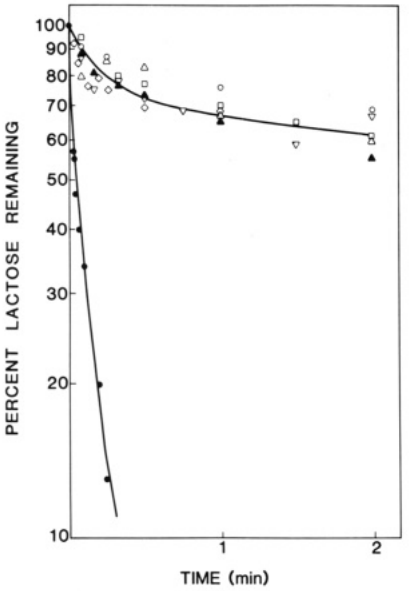

FIGURE 3: Lactose exchange in RSO membrane vesicles. Membrane vesicles were loaded with [1-^{14}C]lactose and assayed as described in the legend to Figure 2, except that the dilution media contained 10 mM unlabeled lactose. (●) Wild type; (◇) H322E/E325H; (□) E325V/V326E; (○) E325A/V326E; (▽) H322M/M323H/E325V/V326E; (△) H323M/E325V/V326E; (▲) RSO vesicle preparation treated with 1.4 mM *p*CMBS to inactivate *lac* permease. Data are expressed as percentage of lactose retained with zero-time points for normalization.

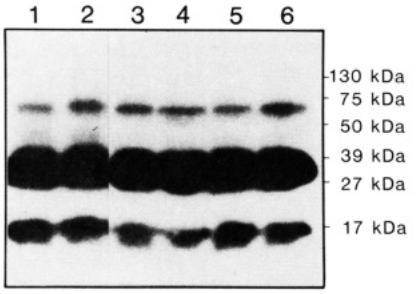

FIGURE 4: Immunoblot analyses of membranes containing wild-type and mutant forms of *lac* permease. Membrane vesicles (35 μg of protein) from *E. coli* T184 harboring wild-type *lac Y* (lane 1) or *lac Y* mutations H322E/E325H (lane 2), E325V/V326E (lane 3), E325A/V326E (lane 4), H322M/M323H/E325V/V326E (lane 5), and M323H/E325V/V326E (lane 6) were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis, electroblotted onto nitrocellulose, incubated sequentially with monoclonal antibody 4A1OR and ^{125}I-labeled protein A, and autoradiographed for 3 h as described (Herzlinger et al., 1985). *lac* permease migrates as a broad band with an apparent molecular mass of 33 kDa; the immunoreactive bands at 63 and 17 kDa correspond to aggregated forms and proteolysis products of *lac* permease, respectively.

Effect of Distance and/or Orientation between His322 and Glu325. If His322 and Glu325 interact and form a hydrogen-bonded or ion-paired network that is important for lactose/H^+ transport, then alteration of the relative distance and/or orientation between the imidazole and carboxylate is predicted to inhibit active transport without affecting equilibrium exchange (Carrasco et al., 1986, 1989). Since the region of *lac* permease between positions 322 and 325 is predicted to be a transmembrane α-helix (Foster et al., 1983; Bieseler et al., 1985; Vogel et al., 1985), the geometry between the imidazole and carboxylate groups may be altered by changing the number of intervening residues between the functional groups. The addition of an extra residue between the imidazole and the carboxylate therefore is expected to rotate the carboxylate component of the putative H^+ relay 100° about the helical axis and increase the distance between the functional groups by approximately 1.5 Å (Figure 5).

When the carboxylate–imidazole orientation is altered by interchanging Glu325 with Val326, the resulting permease is completely inactivated. Thus, HB101 bearing pE325V/V326E grow as white colonies on EMB/lactose (not shown), and RSO vesicles from T184/pE325V/V326E are inactive with respect to active transport (Figure 1), efflux (Figure 2), and equilibrium exchange (Figure 3), although the vesicles contain a normal complement of permease (Figure 4) and catalyze respiration-dependent proline transport (not shown).

Since replacement of Glu325 with various amino acid residues including Val inactivates lactose/H^+ symport without affecting exchange or counterflow (Carrasco et al., 1989), the finding that E325V/V326E does not catalyze equilibrium exchange is unexpected. In order to determine whether the defect is due to the bulk of the isopropyl group of Val at position 325 or to the presence of a carboxylate at position 326, *lac Y* encoding E325V/V326E permease was further mutated so that an Ala codon was introduced at codon 325 (Table I). The resulting permease—E325A/V326E—exhibits properties identical with those of E325V/V326E permease. That is, HB101/pE325A/V326E is white on EMB/lactose, and vesicles containing E325A/V326E permease (Figure 4) are completely defective in all modes of translocation (Figures 1–3). Clearly, therefore, the inability of these permease molecules to catalyze equilibrium exchange is due to the Glu residue at position 326.

The presence of a carboxyl function rather than an isopropyl group at position 326 may alter permease structure by at least two mechanisms. Increasing the number of intervening residues between His322 and Glu325 should alter the interaction between the imidazole and carboxyl groups (Figure 5), leaving the functional groups unable to hydrogen bond or ion pair, a

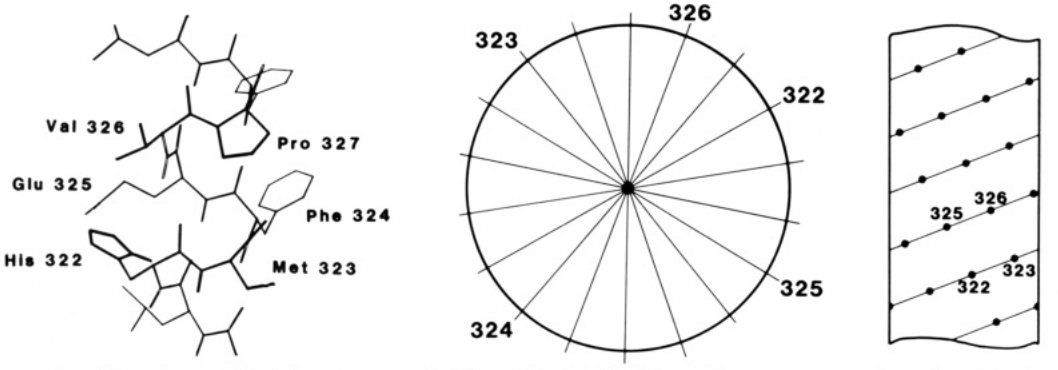

FIGURE 5: Representation of putative helix X in *lac* permease. Residues 322–329 of wild-type *lac* permease are shown in α-helical configuration (left). The relative orientations of positions 322–326 in an α-helix viewed from the end of the helix and from the side of the helix are shown in the middle and on the right, respectively.

configuration that may be energetically unfavorable. Alternatively, inactivation may be due to an unfavorable interaction between the carboxyl group at position 326 and residues of an adjacent part of the permease. In other words, the molecular factors responsible for inactivation of the E325V/V325E and E325A/V326E permeases may be due, to a first approximation, to intrahelical or nonintrahelical interactions with the carboxyl group of Glu326.

Effect of Distance and/or Orientation between the Putative His/Glu Ion Pair and Arg302. In principle, factors that are intrahelical may be differentiated from nonintrahelical factors by reconstructing the His/Glu configuration present in wild-type permease. Thus, *lac Y* encoding E325V/V326E permease was mutated so as to replace Met323 with His (M323H/E325V/V326E permease) or to interchange His322 and Met323 (H322M/M323H/E325V/V326E permease) (Table I). Both molecules contain His and Glu in the same relative orientation as found in wild-type permease (i.e., His323/Glu326 versus His322/Glu325), but the putative His/Glu ion pair is rotated about the helical axis by 100° relative to Arg302 (Figure 5). In addition, the molecules differ by the presence or absence of His at position 322. Thus, M323H/E325V/V326E permease, which retains Arg302 and His322 in the wild-type configuration, but not H322M/M323H/E325V/V326E permease is expected to catalyze equilibrium exchange if the inhibitory interactions caused by Glu326 are primarily intrahelical. However, both permease molecules are completely inactive. That is, *E. coli* HB101 harboring either pM323H/E325V/V326E or pH322M/M323H/E325V/V326E grow as white colonies on EMB/lactose, and vesicles prepared from the strains do not catalyze active transport (Figure 1), efflux (Figure 2), or equilibrium exchange (Figure 3), although they contain permease (Figure 4) and catalyze respiration-dependent proline accumulation (not shown).

Discussion

The results presented in this paper are consistent with the notion that Arg302, His322, and Glu325 in the *lac* permease of *E. coli* play a critical role in the mechanism of action of the permease, possibly as components of a H^+ relay system. Thus, each translocation reaction catalyzed by the permease is inactivated when the geometric configuration between these residues is altered by changing the polarity of His322/Glu325, by increasing the distance and/or orientation between His322 and Glu325, or by changing the orientation between the putative His322/Glu325 ion pair and Arg302.

Movement of the carboxyl group of Glu from position 325 by interchanging His322 and Glu325 or by moving Glu325 to position 326 completely inactivates $\Delta\bar{\mu}_{H^+}$-driven active transport, efflux, and equilibrium exchange. Moreover, it is unlikely that the mutants are able to catalyze facilitated diffusion based on the phenotype of *E. coli* HB101 on EMB/lactose plates after transformation with plasmids encoding the mutated *lac Y* genes (Padan et al., 1985). The cells grow as white colonies indistinguishable from HB101 transformed with the same plasmid devoid of *lac Y*, indicating that the rate of lactose entry into the cell is negligible. Importantly, on the other hand, the mutant permeases are expressed normally, as judged by immunoblotting experiments.

The inactivity of H322E/E325H permease is consistent with the proposed interaction of His322 with Arg302 (Menick et al., 1987b). The results also suggest that perturbation of the putative His322/Glu325 ion-pair alone is insufficient to account for inactivation and is consistent with the proposed role of His322 and Glu325 as components of a H^+ relay (Püttner et al., 1986; Carrasco et al., 1986).

The absence of lactose/H^+ symport activity in permease molecules with Glu at position 326 is consistent with the postulated His/Glu ion pair, since alteration of the geometry and/or distance between the imidazole and carboxyl groups would be expected to disrupt interaction between the functional groups. In contrast, loss of equilibrium exchange activity in permease molecules with Glu326 but retaining the putative Arg302/His322 interaction is unexpected (Carrasco et al., 1986, 1989). The inhibitory effect of Glu326 is not due to artifactual secondary mutations, steric hindrance of Val at position 325, or, to a first approximation, destabilization of permease structure through intrahelical interactions. However, the presence of a Glu residue at position 326 may inactivate the permease by blocking a step in protein folding as suggested for phage P22 tail spike protein or β-lactamase (Goldenberg & King, 1981; Yu & King, 1984; Craig et al., 1985). Although this possibility cannot be excluded, it is noteworthy that the inactive mutants are recognized by monoclonal antibodies and are inserted into the membrane in amounts comparable to those of wild-type permease (Figure 4). Other possibilities for inactivation by Glu326 include destabilization of the permease by unfavorable electrostatic or hydrogen-bonding interactions between the carboxylate and a neighboring portion of the permease.

Loss of permease activity when Glu is moved to position 326 stands in marked contrast to other mutations in neighboring portions of the permease. As shown in the preceding paper (Carrasco et al., 1989) permease with Asp in place of Glu325 retains about 20% of wild-type symport activity. Replacement of Ser300 or Ser306 in putative helix IX with Ala or replacement of Cys333 in putative helix X with Ser has no significant effect on lactose/H^+ symport (Menick et al., 1987a,b). Various amino acid replacements for Arg302, His322, or Glu325 do not completely inactivate the permease, although lactose/H^+ symport is abolished (Padan et al., 1985; Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987b). Collectively, the studies suggest that putative helices IX and X, as well as other putative transmembrane regions of the permease, can accept a variety of mutations without complete inactivation of permease function and presumably, therefore, without large alterations in structure.

In this context, complete inactivation of permease function by Glu326 suggests that the region around position 326 is particularly sensitive to structural perturbations. This notion is supported by several recent mutations in the permease. When the *lac Y* gene encoding E325A is further mutated to replace Phe328 with Asp, the resulting permease is apparently proteolyzed at a markedly enhanced rate (R. Zbar, J. D. Larigan, and H. R. Kaback, unpublished information). When Pro327 is replaced with Gly or Ala, the permease catalyzes lactose/H^+ symport, but replacement with Leu leads to complete inactivation (Lolkema et al., 1988). Therefore, alterations of the local structure in the 326–328 region of putative helix X by electrostatic, hydrogen-bonding, or steric mechanisms may have profound effects on the structure and function of the permease.

Elucidation of the molecular basis of these mutations obviously requires a high-resolution, three-dimensional structure of the permease; however, several points concerning the effect of amino acid replacements in proteins of known structure may be relevant. Site-directed mutagenesis of Pro86 in T4 lysozyme results in a conformational change that propagates about 20 Å across the surface of the protein (Albers et al., 1988). Although the mutations cause large conformational changes, the activity of T4 lysozyme is only marginally affected, ap-

parently because Pro86 is 24 Å distant from the scissile bond. In contrast, mutations located in the active site or in the substrate binding site of T4 lysozyme (Grütter & Matthews, 1982), dihydrofolate reductase (Howell et al., 1986), and trypsin (Craik et al., 1987; Sprang et al., 1987) result in significant alterations in the catalytic activity of the enzymes with only minor alterations in the local structure of the proteins. Although the number of examples may be insufficient for generalizations, it is interesting that a similar trend may be emerging for *lac* permease. Thus, the peculiar sensitivity of the 326–328 region of putative helix X to amino acid replacements may underline the importance of this region to conformational changes that occur during turnover of the permease.

ACKNOWLEDGMENTS

We are indebted to Vincent Madison for modeling putative helix X.

Registry No. Arg, 74-79-3; His, 71-00-1; Glu, 56-86-0; Val, 72-18-4; Met, 63-68-3; H^+, 12408-02-5; lactose, 63-42-3; lactose permease, 9068-45-5.

REFERENCES

Albers, T., Bell, J. A., Sun, D. P., Nicholson, H., Wozniak, J. A., Cook, S., & Matthews, B. W. (1988) *Science 239*, 631.

Bieseler, B., Prinz, H., & Beyreuther, K. (1985) *Ann. N.Y. Acad. Sci. 456*, 309.

Büchel, D. E., Gronenborn, B., & Müller-Hill, B. (1980) *Nature (London) 283*, 541.

Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486.

Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry* (second paper of three in this issue).

Carter, P. J., Winter, C., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835.

Craig, S., Hollecker, M., Creighton, T. E., & Pain, R. H. (1985) *J. Mol. Biol. 185*, 681.

Craik, C. S., Roczniak, S., Largmen, C., & Rutter, W. J. (1987) *Science 237*, 909.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31.

Goldenberg, D. P., & King, J. (1981) *J. Mol. Biol. 145*, 633.

Grütter, M. C., & Matthews, B. W. (1982) *J. Mol. Biol. 154*, 525.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232.

Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221.

Howell, E. E., VillaFranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science 231*, 1123.

Kaback, H. R. (1971) *Methods Enzymol. 22*, 99.

Kaback, H. R. (1974) *Methods Enzymol. 31*, 698.

Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691.

Kramer, B., Kramer, W., & Fritz, H. J. (1984) *Cell 38*, 879.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Lolkema, J. S., Püttner, I. B., & Kaback, H. R. (1988) *Biochemistry 27*, 8307.

Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H., & Kaback, H. R. (1987a) *Biochemistry 26*, 1132.

Menick, D. R., Carrasco, N., Antes, L., Patel, L., & Kaback, H. R. (1987b) *Biochemistry 26*, 6638.

Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765.

Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483.

Püttner, I. B., Sarkar, H. K., Padan, E., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry* (first paper of three in this issue).

Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.

Sarkar, H. K., Viitanen, P. V., Padan, E., Trumble, W. R., Poonian, M. S., McComas, W., & Kaback, H. R. (1985) *Methods Enzymol. 125*, 214.

Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., Finer-Moore, J., Xuong, N.-H., Hamlin, R., Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905.

Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625.

Yu, M. H., & King, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6584.

Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468.