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Characterization of Site-Directed Mutants in the *lac* Permease of *Escherichia coli*. 1. Replacement of Histidine Residues†

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ABSTRACT: Wild-type *lac* permease from *Escherichia coli* and two site-directed mutant permeases containing Arg in place of His35 and His39 or His322 were purified and reconstituted into proteoliposomes. H35-39R permease is indistinguishable from wild type with regard to all modes of translocation. In contrast, purified, reconstituted permease with Arg in place of His322 is defective in active transport, efflux, equilibrium exchange, and counterflow but catalyzes downhill influx of lactose without concomitant H^+ translocation. Although permease with Arg in place of His205 was thought to be devoid of activity [Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765], sequencing of *lac Y* in pH205R reveals the presence of two additional mutations in the 5′ end of the gene, and replacement of this portion of *lac Y* with a restriction fragment from the wild-type gene yields permease with normal activity. Permeases with Asn, Gln, or Lys in place of His322, like H322R permease, catalyze downhill influx of lactose without H^+ translocation but are unable to catalyze active transport, equilibrium exchange, or counterflow. Unlike H322R permease, however, the latter mutants catalyze efflux at rates comparable to that of wild-type permease, although the reaction does not occur in symport with H^+. Finally, as evidenced by flow dialysis and photoaffinity labeling experiments, replacement of His322 appears to cause a marked decrease in the affinity of the permease for substrate. The results confirm and extend the contention that His322 is the only His residue in the permease involved in lactose/H^+ symport and that an imidazole moiety at position 322 is obligatory. In addition, the observations are consistent with the idea that His322 functions as a component of a catalytic triad that is important for lactose/H^+ symport. In the following paper [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)], the role of Glu325 is examined in detail, and in the third paper [Lee, J. A., Püttner, I. B., & Kaback, H. R. (1989) *Biochemistry* (third paper of three in this issue)], evidence is presented supporting the hypothesis that His322 and Glu325 are ion-paired.

lac permease of *Escherichia coli* is a hydrophobic, transmembrane protein encoded by the *lac Y* gene that catalyzes symport of a single β-galactoside molecule with a single H^+ [cf. Kaback (1983, 1986a,b) for reviews]. Thus, in the presence of an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$, interior negative and/or alkaline),[1] *lac* permease utilizes free energy released from downhill translocation of H^+ with $\Delta\bar{\mu}_{H^+}$ to drive uphill accumulation of lactose against a concentration gradient. Conversely, in the absence of $\Delta\bar{\mu}_{H^+}$, movement of lactose down a concentration gradient drives uphill movement of H^+ with

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; $\Delta\Psi$, membrane potential; RSO, right side out; NPG, *p*-nitrophenyl α-D-galactopyranoside; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *p*CMBS, *p*-(chloromercuri)benzenesulfonate; EMB, eosin–methylene blue; Mab, monoclonal antibody; kDa, kilodalton(s); $HgCl_2$, mercuric chloride; DTT, dithiothreitol.

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generation of $\Delta\bar{\mu}_{H^+}$, the polarity of which depends on the direction of the substrate concentration gradient.

lac Y has been cloned and sequenced; the permease has been purified to a single polypeptide species and shown to be the product of *lac Y*. Proteoliposomes containing purified permease catalyze each translocation reaction characteristic of the β-galactoside transport system with turnover numbers and apparent K_ms comparable to those observed in right-side-out (RSO) membrane vesicles, thereby demonstrating that the *lac Y* gene product is solely responsible for β-galactoside/H^+ symport. In addition, recent evidence suggests strongly that *lac* permease is completely functional as a monomer (Costello et al., 1987).

Secondary structure models for the permease based on the hydropathy of the amino acid sequence suggest that the polypeptide is organized in 12 hydrophobic α-helical segments that traverse the membrane in zigzag fashion, connected by more hydrophilic, charged regions. Evidence supporting certain general aspects of the models has been obtained from circular dichroic, laser Raman, and Fourier transform infrared (P. D. Roepe, K. J. Rothschild, and H. R. Kaback, unpublished information) spectroscopy, from limited proteolysis studies, and from binding studies with monoclonal and site-directed polyclonal antibodies.

Chemical modification of amino acid residues in proteins can provide important information, and with this approach, initial evidence was obtained suggesting that Cys (Fox & Kennedy, 1965) and His residues (Padan et al., 1979; Garcia et al., 1982) may be important for lactose/H^+ symport. However, there are drawbacks to chemical modification which include the specificity of the reagents and the bulk of the modified residues. Thus, oligonucleotide-directed, site-specific mutagenesis using bacteriophage M13 single-stranded DNA has been introduced to produce single amino acid changes in proteins (Zoller & Smith, 1983), and during the past few years, the approach has been applied to *lac* permease [cf. Sarkar et al. (1986) and Kaback (1987, 1988) for reviews]. By use of site-directed mutagenesis, it has been demonstrated that out of a total of eight Cys residues only Cys154 is important, although it is not directly involved in either substrate binding or H^+ translocation [cf. Menick et al. (1987a)]. In addition, each of the four His residues in the permease was replaced with Arg (Padan et al., 1985). The studies indicate that permease with Arg in place of His35 and His39 functions normally, while permease with Arg in place of His322 appears to be able to catalyze downhill lactose translocation at high substrate concentrations without H^+ translocation and permease with Arg in place of His205 is completely defective. Subsequently, His205 or His322 in *lac* permease was replaced with Asn or Gln, and it was demonstrated with intact cells that permease with H205N[2] or H205Q catalyzes active transport like wild-type permease, while permease with H322N or H322Q, like permease with H322R, is unable to do so (Püttner et al., 1986). On the basis of these findings, it was suggested that His322 is involved specifically in the coupling between lactose and H^+ translocation. Most recently, evidence has been obtained suggesting that Arg302 (Menick et al., 1987b), His322 (Püttner et al., 1986), and Glu325 (Carrasco et al., 1986), neighboring residues in putative helices IX and X of the permease, play an important role in lactose/H^+ symport, possibly as components in a charge-relay.

This paper further documents the transport properties of permease molecules with Arg substituted for His35 and His39, His205, or His322. In addition, the properties of permeases with Asn, Gln, or Lys in place of His322 are described. The results demonstrate the following: (i) Purified, reconstituted H35-39R permease behaves in a fashion indistinguishable from that of wild-type permease. (ii) While H205R permease was thought to be totally defective in transport, sequencing of the entire *lac Y* gene in pH205R reveals two additional mutations in the 5′ end of the gene. When this portion of the gene is replaced with a restriction fragment from wild-type *lac Y*, it is apparent that permease with H205R, like permease with H205N or H205Q (Püttner et al., 1986), catalyzes lactose/H^+ symport normally. (iii) Permease with Arg, Asn, Gln, or Lys in place of His322 is "uncoupled" and catalyzes downhill lactose transport without concomitant H^+ translocation, thereby providing further evidence that a His residue at position 322 of the permease is obligatory for lactose/H^+ symport.

Experimental Procedures

Materials

[1-^{14}C]Lactose was purchased from Amersham/Searle; *p*-nitro[6-^{3}H]phenyl α-D-galactopyranoside was synthesized by Yu-Ying Liu of the Isotope Synthesis Group of Hoffmann-La Roche, Inc., under the direction of Arnold Liebman. All other materials were of reagent grade and obtained from commercial sources as described (Sarkar et al., 1986).

Methods

Bacterial Strains. The following strains of *Escherichia coli* K-12 were used: JM101, *supE, thi,* Δ(*lac-proAB*), [F′ *traD36, proA*$^+$*B*$^+$, *lacI*^{q}ZΔ*M15*] (Yanish-Perron et al., 1985); JM109, *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* λ$^-$, Δ(*lac-proAB*), [F′ *traD36, proA*$^+$*B*$^+$, *lacI*^{q}ZΔ*M15*] (Yanish-Perron et al., 1985); BMH71-18 *mutL,* Δ(*lac-pro*), *supE, thi/proA*$^+$*B*$^+$, *lacI*^{q}ZΔ*M15/MutL::Tn10* (Kramer et al., 1984); T206, *lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*), *rpsL,met*$^-$,*thr*$^-$,*recA,hsdM,hsdR* [*F′, lacI*^{q}*O*$^+$*Z*^{U118}(*Y*$^+$*A*$^+$)] harboring plasmid pGM21 [*lac*Δ(*I*)*O*$^+$*P*$^+$Δ(*Z*)*Y*$^+$Δ(*A*),*tet*^{r}] (Teather et al., 1980); T184 [T206 cured of plasmid pGM21] (Teather et al., 1980); HB101, *hsdS20* (*r*^-_B, *m*^-_B), *recA13, ara-14, proA2, lacY1, galK2, rpsL20*(Sm^r), *xyl-5, mtl-1, supE44,* λ$^-$/F$^-$ (Boyer & Roulland-Dussoix, 1969).

Oligonucleotide-Directed, Site-Specific Mutagenesis. Site-directed mutagenesis using M13mp19 as a cloning vector was performed essentially as described (Sarkar et al., 1986) or with specified modifications. Sequences of the mutagenic primers with His codons changed to Arg, Asn, or Gln codons were published elsewhere (Padan et al., 1985; Püttner et al., 1986). For replacement of His322 with Lys, a mutagenic primer (5′-TTCAAACATT*TT*CAGCGTTTTCAG-3′) was used which contained two mismatches (*). Mismatch repair was minimized by transfecting the heteroduplex DNA into the mutator strain *E. coli* BMH71-18 *mutL* (Kramer et al., 1984). Mutations were verified by dideoxynucleotide sequencing as described (Padan et al., 1985; Püttner et al., 1986). In addition, the entire *lac* Y gene in plasmids pH205R and pH322R was sequenced by use of six synthetic oligonucleotide primers complementary to appropriate regions of *lac Y*. Unless stated otherwise, each of the *lac Y* genes described has a nucleotide sequence identical with that described by Büchel et al. (1980) with the exception of given base change(s).

Replacement of the 5′ End of lac Y in pH205R. Although it was presumed that the *lac Y* gene in pH205R contained a

[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 wild-type *lac* permease. The sequence is followed by a second letter denoting the amino acid replacement at this position (e.g., H205N designates that His205 is replaced with Asn).

single base change in codon 205 (Padan et al., 1985), sequencing of the entire gene revealed two additional cryptic mutations in the 5′ end (L. Steinke and H. R. Kaback, unpublished information). For this reason, the 5′ end of *lac Y* was restricted from the gene and replaced with the analogous fragment from wild-type *lac Y* in the following manner: Plasmids pGM21 (encoding wild-type permease) and pH205R (containing an A → G alteration in codon 205 of *lac Y* and two additional base changes in codons 26 and 29) were cleaved with restriction endonuclease *Ava*I. Two fragments (~2.7 and ~3.8 kbp) resulting from a restriction site in *lac Y* (codon 70) and another located approximately 2.92 kbp from the *Eco*RI site on the vector pACYC184 were obtained. After separation by agarose gel electrophoresis and isolation of the fragments, the 3.8-kbp fragment from pGM21 was ligated to the 2.7-kbp fragment from pH205R. The cryptic strain *E. coli* HB101 (Y^-Z^+) was transformed with the ligation mixture. Cells plated on lactose/eosin–methylene blue (EMB) indicator plates (Miller, 1972) grew as dark red colonies. Sequencing of *lac Y* in the construct with six sequencing primers revealed a single base change (A → G) in codon 205.

Growth of Cells and Preparation of Membranes. *E. coli* T206, H35-39R, H205R, H322R, H322N, H322Q, or H322K (i.e., *E. coli* T184 harboring given plasmids) was grown and induced with isopropyl 1-thio-β-D-galactopyranoside as described (Teather et al., 1980). For preparation of crude membranes, cells were disrupted by passage through a French pressure cell at 20000 psi (1 psi = 6.895 kPa), and the membrane fraction was recovered by differential centrifugation (Newman et al., 1981). Right-side-out (RSO) membrane vesicles were prepared by osmotic lysis as described (Kaback, 1971; Short et al., 1975).

Purification and Reconstitution of lac Permease. *lac* permease containing given mutations was purified from appropriate *E. coli* membranes and reconstituted into proteoliposomes containing *E. coli* phospholipids as described [cf. Viitanen et al. (1986)]. The final preparations contained 50 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol (DTT), 37.5 mg of phospholipid/mL, and 50–90 μg of protein/mL.

Proteoliposomes were thawed at room temperature and sonicated in a bath-type sonicator for 8–15 s (Viitanen et al., 1986). Where indicated, proteoliposomes were concentrated by centrifugation for 1 h at 45000 rpm in a Beckman Type 50 Ti rotor ($175000g_{max}$). The pellet was resuspended in 50 mM potassium phosphate (pH 7.5) containing 1 mM DTT to a given protein concentration.

Permease Activity. Permease activity was assayed qualitatively by transforming the cryptic strain HB 101 (Z^+Y^-) with a given plasmid, followed by growth on lactose/EMB indicator plates.

Transport Assays. Transport of [1-^{14}C]lactose in intact cells was assayed as described (Trumble et al., 1984).

Active transport of [1-^{14}C]lactose or [U-^{14}C]proline in RSO membrane vesicles was measured under oxygen in the presence of reduced phenazine methosulfate (PMS) by rapid filtration (Kaback, 1971, 1974). Efflux, exchange, and counterflow assays were carried out as described (Kaczorowski & Kaback, 1979). Lactose-facilitated diffusion and lactose-induced H^+ influx were measured as reported previously (Patel et al., 1982).

All assays with proteoliposomes containing purified *lac* permease were conducted at pH 7.5 and 25 °C. Efflux, exchange, counterflow, and ΔΨ-driven active transport were carried out as described [cf. Viitanen et al. (1984, 1986)]. For measurements of facilitated diffusion, sonicated proteoliposomes were concentrated 3–5-fold to a protein concentration of about 360 μg/mL, and valinomycin and nigericin were added to final concentrations of 20 and 2 μM, respectively. Lactose-induced H^+ influx was measured in the presence of 20 μM valinomycin (Newman et al., 1981; Foster et al., 1982).

Binding of [^{3}H]NPG. Binding of [^{3}H]NPG to RSO membrane vesicles was assayed under nonenergized conditions by flow dialysis (Rudnick et al., 1976). The upper chamber contained 0.2 mL of RSO membrane vesicles (5–6 mg of protein) in 50 mM potassium phosphate (pH 7.5). The same buffer was pumped through the lower chamber at 3.5 mL/min, and 1.5-mL fractions were collected. Assays were initiated by addition of given concentrations of [^{3}H]NPG to the upper chamber. After equilibration was achieved, TDG was added to the upper chamber as indicated. Specific binding of [^{3}H]NPG was quantitated from the increase in the dialyzable concentrations of [^{3}H]NPG after addition of excess TDG. K_D and B_{max} values for NPG binding were calculated according to Scatchard (1949) from experiments using [^{3}H]NPG concentrations ranging from 5.19 to 49.6 μM.

Photoaffinity Labeling of RSO Membrane Vesicles with NPG. Photolabeling of RSO membrane vesicles with [^{3}H]-NPG was carried out under anaerobic reducing conditions (Kaczorowski et al., 1980).

Binding of Monoclonal Antibodies (Mab). In order to estimate the amount of permease in the membrane, immunoblot analyses were performed with Mab 4A10R and ^{125}I-labeled protein A (Herzlinger et al., 1985). Alternatively, the permease was assayed by direct binding with Mab 4B1 (Carrasco et al., 1982).

Protein Determinations. Protein in proteoliposomes was measured by a modification (Newman et al., 1981) of the method of Schaffner and Weissman (1973); for membrane vesicles, the method described by Lowry et al. (1951) was used with bovine serum albumin as standard.

Results

Arg Replacements

Active Transport. Proteoliposomes reconstituted with purified *lac* permease catalyze lactose accumulation when a membrane potential (ΔΨ, interior negative) is imposed by means of a potassium diffusion gradient in the presence of valinomycin (Newman et al., 1981; Foster et al., 1982; Viitanen et al., 1983). As shown in Figure 1, proteoliposomes containing purified wild-type or H35-39R permease accumulate lactose in the presence of ΔΨ at almost identical rates and to similar steady-state levels of accumulation. In contrast, proteoliposomes containing *lac* permease with H322R do not catalyze ΔΨ-driven lactose transport. Thus, the steady-state level of lactose accumulation in proteoliposomes reconstituted with H322R permease is virtually identical with that of proteoliposomes containing wild-type permease in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or *p*-(chloromercuri)benzenesulfonate (*p*CMBS). The observations are qualitatively similar to those reported with intact cells (Padan et al., 1985) and RSO membrane vesicles (data not shown) and verify the conclusion that replacement of His35 and His39 with Arg has no discernible effect on permease activity, while substitution of Arg for His322 causes inactivation of $\Delta\bar{\mu}_{H^+}$ driven lactose accumulation.

Previous experiments (Padan et al., 1985) described the replacement of His205 with Arg and demonstrated that *E. coli* T184 or HB101 transformed with pH205R was unable to transport lactose. Upon sequencing of the entire *lac Y* gene

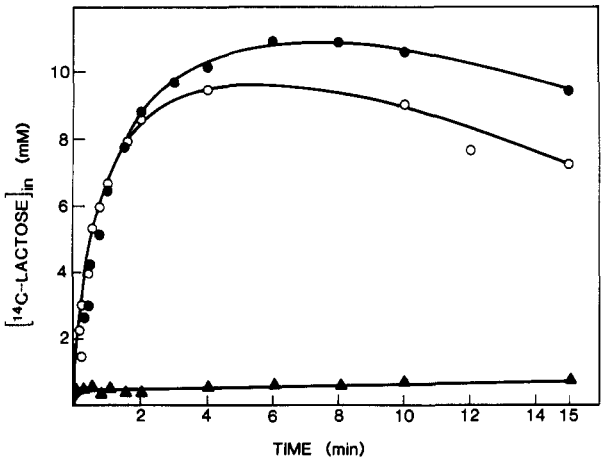

FIGURE 1: Membrane potential ($\Delta\Psi$) driven lactose transport in proteoliposomes reconstituted with purified *lac* permease. *lac* permease was purified from membranes of a given strain of *E. coli* and reconstituted into proteoliposomes at a protein concentration of 50 μg/mL (wild-type T206, H35-39R permease) or 90 μg/mL (H322R permease). Lactose transport was measured as described (Viitanen et al., 1986) by diluting 1 μL of proteoliposomes containing 20 μM valinomycin into 200 μL of 50 mM sodium phosphate (pH 7.5) containing 0.3 mM [1-^{14}C]lactose (19 mCi/mmol). (●) T206; (○) H35-39R; (▲) H322R or T206 in the presence of 20 μM CCCP or 1 mM *p*CMBS.

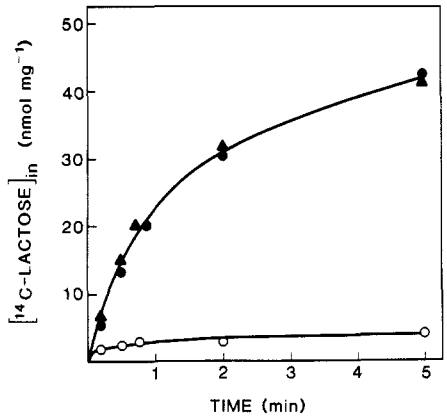

FIGURE 2: Lactose transport in *E. coli* T184 transformed with pACYC184 (○), pGM21 (●), or pH205R (▲). Reactions were initiated by the addition of [1-^{14}C]lactose (10 mCi/mmol) to a final concentration of 0.4 mM, terminated by addition of 3 mL of 0.1 M potassium phosphate (pH 5.5) containing 0.1 M lithium chloride, and filtered immediately (Trumble et al., 1984).

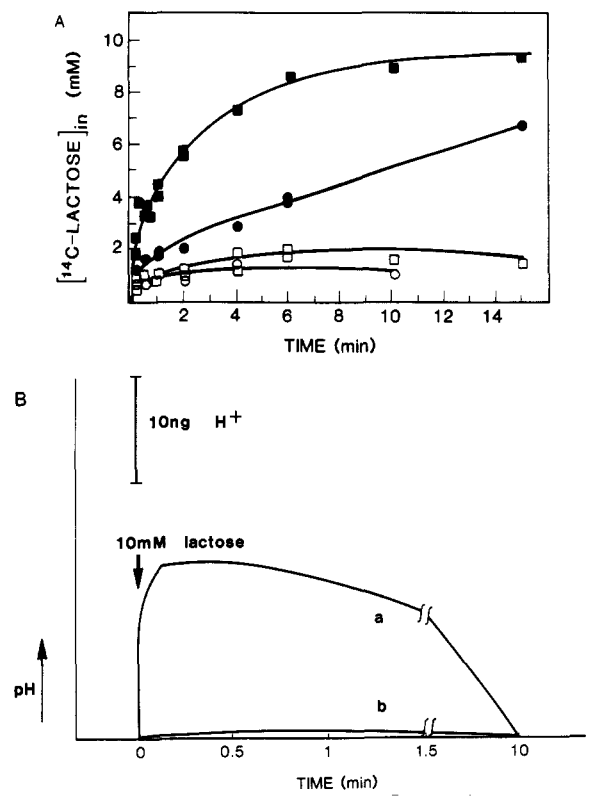

FIGURE 3: Facilitated diffusion of lactose (A) and lactose-induced H^+ influx (B) in proteoliposomes reconstituted with purified permeases. (A) Proteoliposomes containing purified wild-type (■) or H322R permease (●) were resuspended in 50 mM potassium phosphate (pH 7.5)/1 mM DTT at a protein concentration of 360 μg/mL, and valinomycin and nigericin were added to final concentrations of 20 μM and 2 μM, respectively. Aliquots (1 μL) were diluted rapidly into 100 μL of 50 mM potassium phosphate (pH 7.5) containing [1-^{14}C]lactose (3.8 mCi/mmol) at a final concentration of 7.5 mM. At the times indicated, reactions were terminated and filtered immediately. Control experiments were performed in the presence of *p*CMBS at a final concentration of 2.5 mM [(□) wild-type permease; (○) H322R permease]. (B) A 2.0-mL suspension of proteoliposomes containing 4.5 μg of (a) wild-type or (b) H322R permease in 150 mM KCl/10 mM $MgSO_4$ and 20 μM valinomycin was placed in a closed electrode vessel that was continuously flushed with a stream of water-saturated nitrogen. The reaction was started by addition of lactose to a final concentration of 10 mM, and the pH of the solution was monitored continuously as described (Patel et al., 1982). The pH change was calibrated at the end of each experiment by addition of 10 μL of 1.0 mM HCl. When nigericin (2 μM final concentration) was added to proteoliposomes containing wild-type permease, data identical with those of curve b were obtained (not shown).

in this site-directed mutant, however, it was revealed that, besides the A → G mutation in codon 205, the gene contains two additional mutations, T → C in codon 26 (Tyr → His) and T → G in codon 29 (Phe → Val) (L. Steinke and H. R. Kaback, unpublished information). For this reason, the 5′ end of *lac Y* was restricted from pH205R DNA and replaced with an analogous fragment from pGM21 which contains wild-type *lac Y*, and the reconstructed *lac Y* gene was sequenced again to ascertain the presence of a single mutation in codon 205. In contrast to earlier observations, when *E. coli* T184 is transformed with this construct containing a single mutation encoding Arg in place of His205, the cells transport lactose as well as cells transformed with pGM21 (Figure 2).

Facilitated Diffusion and Lactose-Induced H^+ Translocation. Although permease encoded by pH322R does not catalyze active transport, transformation of the cryptic strain *E. coli* HB101 (Y^-Z^+) with the plasmid causes the cells to grow as red colonies in the presence of 20 mM lactose on EMB plates (Padan et al., 1985). Thus, it was suggested that permease with H322R facilitates lactose movements down a concentration gradient at high substrate concentration without concomitant H^+ translocation. This conclusion receives strong support from measurements of facilitated diffusion and lactose-induced H^+ movements in proteoliposomes reconstituted with purified permeases.

When 10 mM lactose is added to a suspension of proteoliposomes reconstituted with purified wild-type permease in the presence of valinomycin and nigericin, the internal lactose concentration equilibrates with the external medium within 6–8 min (Figure 3A). On the other hand, in proteoliposomes with H322R permease, equilibration proceeds at about 40% of the rate observed with wild-type permease. Since the rate of equilibration is markedly diminished by *p*CMBS in both preparations (equilibration does occur, however, in about 3 h), it is apparent that downhill lactose translocation over the time course of the experiments is almost entirely permease mediated and that the rate of passive influx is relatively insignificant. Clearly, therefore, *lac* permease with H322R is

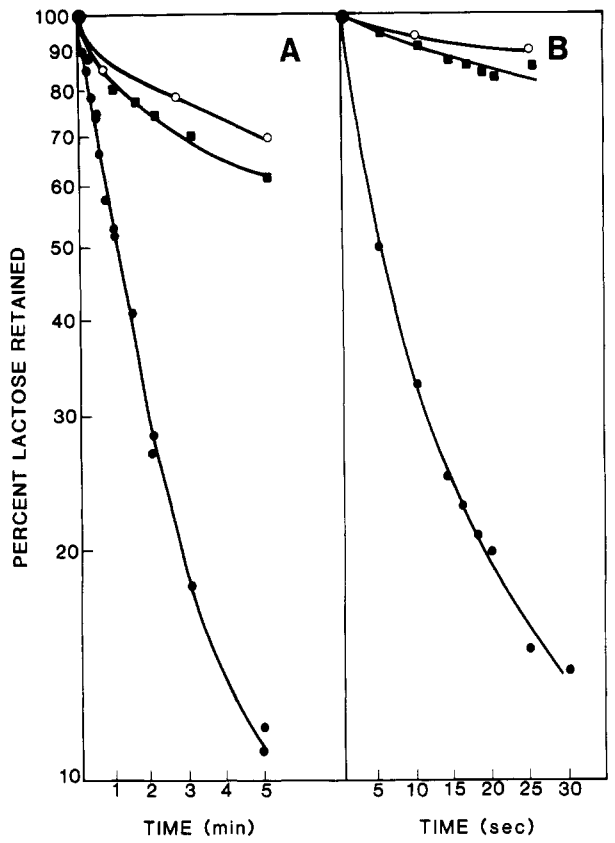

FIGURE 4: Lactose efflux (A) and exchange (B). Valinomycin (final concentration 20 μM) and [1-^{14}C]lactose (11.8 mCi/mmol; 10 mM final concentration) were added to suspensions of proteoliposomes reconstituted with purified wild-type or H322R permease. After equilibration at room temperature for 1 h, 1-μL aliquots were rapidly diluted into 200 μL of 50 mM potassium phosphate (pH 7.5) (A; efflux) or into the same buffer containing 10 mM unlabeled lactose (B; exchange). At the times indicated, reactions were terminated as described (Garcia et al., 1983). (●) Proteoliposomes containing wild-type permease; (○) proteoliposomes containing wild-type permease in the presence of 2.5 mM *p*CMBS; (■) proteoliposomes containing H322R permease.

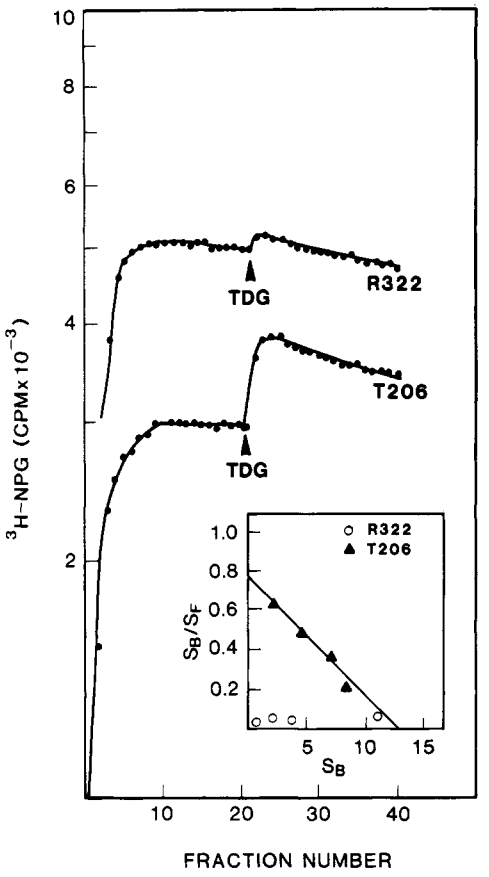

FIGURE 5: Binding of [^{3}H]NPG to RSO membrane vesicles. Flow dialysis was carried out with 0.2 mL of T206 or H322R membrane vesicles (5–6 mg of protein) suspended in 0.1 M potassium phosphate (pH 7.5) as described under Experimental Procedures. [^{3}H]NPG (9.46 Ci/mmol) was added at fraction 1 to a final concentration of 25 μM. As indicated by arrows, TDG was added to the upper chamber to a final concentration of 20 mM. The insert shows a Scatchard plot (Scatchard, 1949) of similar experiments carried out at [^{3}H]NPG concentrations ranging from 5.19 to 49.6 μM with T206 and H322R membrane vesicles, as indicated.

able to catalyze facilitated diffusion at a significant rate.

In a parallel experiment, lactose-induced H^+ movements were monitored with a pH electrode (Figure 3B). As shown previously (Foster et al., 1982), when lactose is added to proteoliposomes containing wild-type *lac* permease in the presence of valinomycin, transient alkalinization of the medium is observed, and the pH tracing reaches maximum displacement in 15–30 s and slowly returns to base line. Moreover, alkalinization is abolished when nigericin is added to the reaction mixture. In marked contrast, proteoliposomes containing H322R permease do not exhibit transient alkalinization upon addition of lactose, and the pH trace may be superimposed on that of proteoliposomes with wild-type permease assayed in the presence of nigericin or CCCP. Therefore, H322R permease catalyzes facilitated diffusion at a significant rate, but the process does not occur in symport with H^+. In other words, H322R permease is uncoupled.

Efflux, Exchange, and Counterflow. Lactose efflux from RSO membrane vesicles (Kaczorowski & Kaback, 1979) and proteoliposomes (Viitanen et al., 1983) is permease-mediated and occurs in symport with H^+. In contrast, equilibrium exchange and counterflow do not involve net H^+ translocation, although the permease may recycle in the protonated state [cf. Carrasco et al. (1986)]. When proteoliposomes reconstituted with purified wild-type permease are equilibrated with 10 mM [1-^{14}C]lactose, treated with valinomycin, and diluted into medium devoid of lactose (Figure 4A) or into medium containing 10 mM lactose (Figure 4B), efflux and exchange occur at rates similar to those described previously [i.e., $t_{1/2} \sim 1$ min and 5 s, respectively; cf. Garcia et al. (1983) and Viitanen et al. (1983)]. Although data are not shown, similar results were obtained with proteoliposomes reconstituted with H35-39R permease. In contrast, H322R permease is markedly defective in efflux (Figure 4A, $t_{1/2} > 10$ min), and exchange is also impaired (Figure 4B). Finally, proteoliposomes containing H322R permease are completely devoid of counterflow activity, while proteoliposomes with H35-39R permease catalyze counterflow in essentially the same manner as proteoliposomes with wild-type permease (data not shown). Since RSO membrane vesicles from *E. coli* H322R (Püttner et al., 1986) exhibit very similar behavior, it is evident that the effects documented here with purified, reconstituted permeases cannot be attributed to artifacts produced during isolation and purification but are a consequence of the mutations described.

[^{3}H]NPG Binding. NPG is a potent competitive inhibitor of lactose transport that binds to *lac* permease with a K_D of about 20 μM, which corresponds to its K_i with respect to lactose transport (Rudnick et al., 1976). Furthermore, comparative binding studies with NPG, Mab 4B1, and Mab 4B1 Fab fragments indicate that 1 mol of NPG is bound per mole of permease (Herzlinger et al., 1985). Scatchard analyses of NPG binding data performed with RSO membrane vesicles from T206 (Figure 5, inset) and H35-39R (not shown) yield

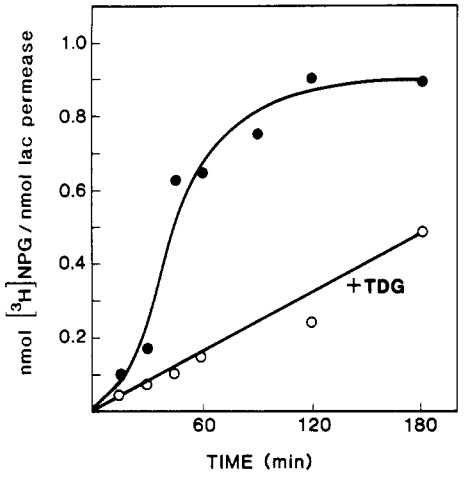

FIGURE 6: [^{3}H]NPG photoaffinity labeling of H322R membrane vesicles. RSO vesicles were resuspended to 1.5 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6) and preequilibrated with an argon atmosphere in the presence of 20 mM lithium D-lactate (Kaczorowski et al., 1980). [^{3}H]NPG (10 Ci/mmol) was added to a final concentration of 20 μM, and the sample was illuminated. At various times, 50-μL aliquots were removed and mixed with 3 mL of ice-cold 10% trichloroacetic acid. Precipitated protein was collected by filtration and assayed for radioactivity by liquid scintillation spectrometry. A parallel experiment was carried out in the presence of 20 mM TDG (○). Although data are not shown, after 60-min illumination in the absence of TDG essentially all of the radioactivity is associated with the 33-kDa band identified as *lac* permease [cf. Kaczorowski et al. (1980)].

K_D values of 16 μM and 17.5 μM, respectively. In contrast, H322R membrane vesicles manifest minimal binding activity which prohibits precise determination of K_D. However, the small signal obtained upon addition of TDG (Figure 5) is reproducible at various NPG concentrations, and Scatchard analysis suggests that the K_D for NPG may be markedly increased.

The contention that H322R permease is not totally devoid of binding activity is strengthened by photolabeling experiments with NPG (Figure 6). Under anaerobic reducing conditions, photolysis of the nitrophenyl ether leads to highly specific labeling of the permease presumably by nucleophilic aromatic photosubstitution (Kaczorowski et al., 1980). When RSO membrane vesicles containing H322R are irradiated in the presence of [^{3}H]NPG, the vesicles exhibit time-dependent incorporation of radioactivity that is blocked to a large extent by addition of excess TDG. Furthermore, sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography demonstrates that essentially all of the radioactivity incorporated into the vesicles comigrates with the 33-kDa protein that has been identified as the product of *lac Y* [not shown; cf. Kaczorowski et al. (1980)].

Asn, Gln, and Lys Replacements

In order to more fully elucidate the role of His322 in lactose/H$^+$ symport, lactose transport was studied in RSO membrane vesicles containing permeases in which His322 was replaced with Asn, Gln, or Lys.

Influx of Lactose. Although data will not be shown, transformation of *E. coli* HB101 with pH322N, pH322Q, or pH322K yields red colonies on EMB at 25 mM lactose and also at 12 mM lactose but white colonies on EMB containing 6 mM lactose. However, T184 cells transformed with pH322N or pH322Q do not catalyze lactose accumulation (Püttner et al., 1986). The latter observation is confirmed and extended by measurements of lactose transport in RSO membrane vesicles (Figure 7). Both the initial rate and

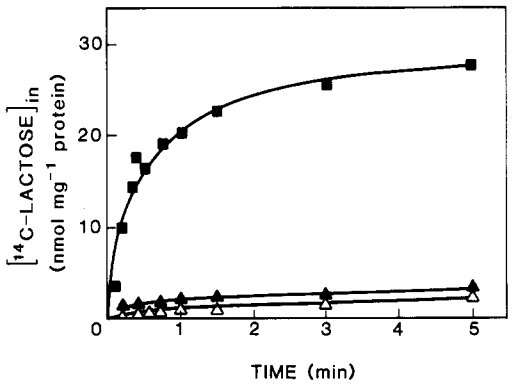

FIGURE 7: Respiration-driven lactose transport in RSO membrane vesicles from *E. coli* T206 (■) or H322N, H322Q, or H322K (▲). Aliquots (50 μL) of RSO vesicles containing 100 μg of membrane protein were assayed for lactose uptake at indicated times with 0.4 mM [1-^{14}C]lactose (19.2 mCi/mmol) in the presence (■, ▲) or absence (△) of reduced PMS as described (Kaback, 1971, 1974). The differences between H322N, H322Q, and H322K vesicles were insignificant (▲).

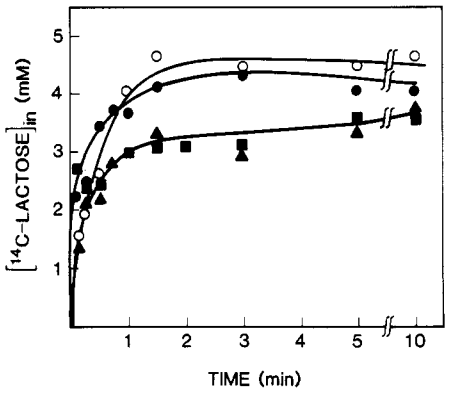

FIGURE 8: Lactose-facilitated diffusion in RSO membrane vesicles from *E. coli* T206 (●), H322N (▲), H322Q (■), or H322K (○). Aliquots (100 μL) of RSO membrane vesicles (9 mg of protein/mL) were incubated at 25 °C, and at zero time, [1-^{14}C]lactose (3 mCi/mmol) was added to a final concentration of 8 mM. At indicated times, samples were quenched and assayed as described (Patel et al., 1982). The data were corrected for control samples in which *lac* permease was inactivated by addition of 2 mM $HgCl_2$.

steady-state level of respiration-driven lactose transport are negligible in H322N, H322Q, and H322K vesicles. In contrast, each vesicle preparation transports proline normally, and the specific permease content of each preparation is comparable to that of vesicles containing wild-type permease, as evidenced by immunoblot analyses [data not shown; cf. Herzlinger et al. (1985)]. Therefore, permease with Asn, Gln, or Lys in place of His322, like H322R permease, appears to be uncoupled, a conclusion substantiated by direct measurements of downhill lactose translocation and lactose-induced H$^+$ movements. Permease with H322N, H322Q, or H322K catalyzes downhill lactose translocation in a manner similar to that observed with wild-type permease (Figure 8). However, none of the mutated permeases exhibits transient alkalinization of the external medium on addition of lactose (data not shown; cf. Figure 3B).

Efflux, Exchange, and Counterflow. lac permease with H322R is grossly defective in each mode of translocation, although it catalyzes downhill lactose influx without concomitant H$^+$ translocation (Püttner et al., 1986; cf. Figure 3). Interestingly, permease with H322N, H322Q, or H322K catalyzes efflux at rates that are only marginally slower than that of wild-type permease (Figure 9A; $t_{1/2}$ for efflux is ~18 s for T206, ~25 s for H322N, ~30 s for H322Q, and ~25

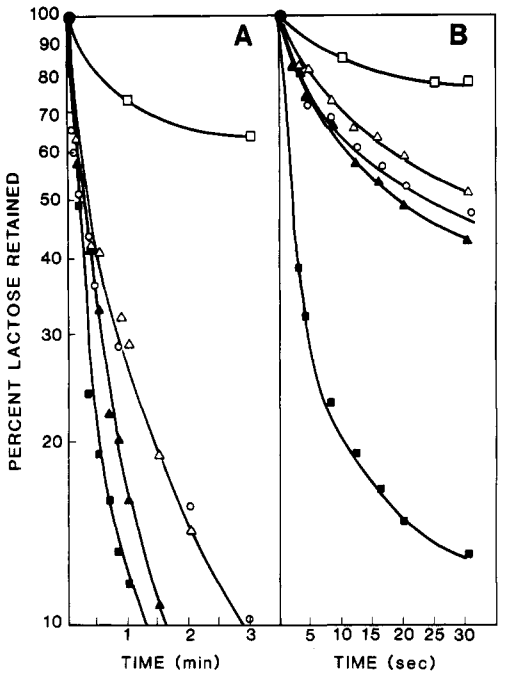

FIGURE 9: Lactose efflux (A) and exchange (B) in RSO membrane vesicles from *E. coli* T206 (■), H322N (▲), H322Q (△), or H322K (○). Membrane vesicles (~30 mg of protein/mL) containing approximately the same amount of permease (0.4 nmol/mg of protein) were equilibrated at 4 °C overnight with 10 mM [1-^{14}C]lactose (5.9 mCi/mmol). Aliquots (2 μL) were then rapidly diluted into media devoid of lactose (A; efflux) or media containing equimolar concentrations of unlabeled lactose (B; exchange). At the times indicated, reactions were terminated by adding 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). Similar experiments were performed in the presence of 1 mM *p*CMBS (□). Data are expressed as a percentage of lactose retained by using zero time points for normalization.

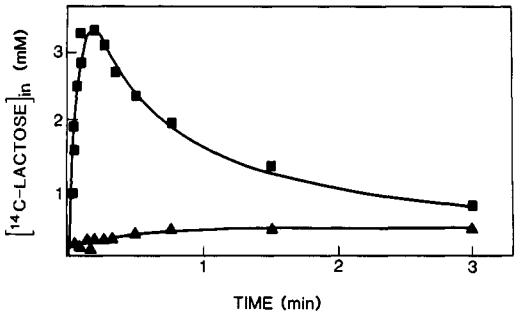

FIGURE 10: Lactose counterflow at saturating external lactose concentrations. Membrane vesicles from *E. coli* T206 (■) or H322N, H322Q, or H322K (▲) were equilibrated with 10 mM lactose as described under Experimental Procedures. Aliquots (2 μL) were then diluted into 400 μL of 0.1 M potassium phosphate (pH 6.6) containing 0.43 mM [1-^{14}C]lactose (9 mCi/mmol), and the samples were assayed at indicated times as described (Kaczorowski & Kaback, 1979). Differences between H322N, H322Q, and H322K vesicles were insignificant (▲).

s for H322K). Like permease with H322R, however, permease with H322N, H322Q, or H322K is markedly defective with respect to equilibrium exchange (Figure 9B; $t_{1/2}$ for exchange is ~3 s for T206, ~20 s for H322N, ~32 s for H322Q, and ~24 s for H322K) and does not catalyze counterflow (Figure 10).

Although permease with H322N, H322Q, or H322K clearly mediates efflux of lactose down a concentration gradient, the process does not occur in symport with H^+. Thus, the rate of efflux from vesicles containing either H322N or H322Q permease is not significantly affected by pH, by imposition of ΔΨ (interior negative), or by replacement of protium with deuterium (Table I). In contrast, the rate of efflux from vesicles containing wild-type permease is increased when the external pH is increased from pH 5.5 to pH 7.5 and decreased when ΔΨ (interior negative) is imposed or when protium is replaced with deuterium [in addition, cf. Kaczorowski and Kaback (1979), Kaczorowski et al. (1979), and Viitanen et al. (1983)].

Table I: Effect of pH, ΔΨ, or D_2O on Lactose Efflux in RSO Membrane Vesicles from T206, H322N, or H322Q

	$t_{1/2}$ of efflux (s)			
	control (pH 7.5)	pH 5.5[a]	ΔΨ (interior negative)[b]	pD 7.5[c]
T206	9	21	25	19
H322N	13	11	21	13
H322Q	19	20	15	17

[a] Aliquots of membrane vesicles in 0.1 M potassium phosphate (pH 5.5) were equilibrated with 10 mM [1-^{14}C]lactose (6 mCi/mmol) in the presence of 20 μM valinomycin and rapidly diluted into 0.1 M potassium phosphate (pH 5.5), as described (Kaczorowski & Kaback, 1979). At given times, samples were diluted with 3 mL of 0.1 M potassium phosphate (pH 5.5)/0.1 M lithium chloride/20 mM $HgCl_2$ and immediately filtered. [b] To test the effect of ΔΨ (interior negative) on lactose efflux, similar experiments were carried out, except that aliquots of membrane vesicles in 0.1 M potassium phosphate (pH 7.5)/20 μM valinomycin were diluted into 0.1 M sodium phosphate (pH 7.5), as described (Kaczorowski et al., 1979). [c] Efflux in the presence of D_2O was performed as described (Kaczorowski et al., 1979; Viitanen et al., 1983).

[^{3}H]Binding and Photoaffinity Labeling. RSO membrane vesicles from *E. coli* H322N or H322Q behave like H322R membrane vesicles with respect to NPG binding and photoaffinity labeling. Vesicles containing the mutated permeases exhibit low equilibrium binding of [^{3}H]NPG; however, both preparations exhibit normal photoaffinity labeling (data not shown). Thus, permease with Asn or Gln in place of His322, like permease with H322R, appears to bind NPG with markedly decreased affinity.

Discussion

The results presented here confirm and extend the previous conclusions of Padan et al. (1985) and Püttner et al. (1986) that, of the four His residues in *lac* permease, only His322 is important for lactose/H^+ symport. Thus, it was shown earlier with intact cells and RSO membrane vesicles that replacement of His35 and His39 with Arg has no discernible effect on permease activity, while replacement of His205 or His322 with Arg leads to complete loss of lactose/H^+ symport. Interestingly, however, evidence was presented suggesting that permease with Arg in place of His322 may facilitate downhill lactose movements at high substrate concentrations without concomitant H^+ translocation. As demonstrated conclusively by data presented here, purified H35-39R permease reconstituted into proteoliposomes catalyzes all modes of translocation as well as wild-type permease. Furthermore, purified permease containing H322R does not catalyze active transport, efflux, equilibrium exchange, or counterflow but facilitates downhill lactose influx along a concentration gradient at high substrate concentrations, albeit at a slower rate than wild-type permease and without concomitant H^+ translocation. It is also noteworthy in this regard that the permease has been "engineered" recently in such a manner that His35 and His39 were replaced with Arg and His205 was replaced with Gln (Püttner & Kaback, 1988). The molecule which has a single His residue at position 322 catalyzes lactose/H^+ symport in a fashion indistinguishable from that of wild-type permease. In brief, therefore, His322 appears to be required for each

translocation reaction catalyzed by the permease that involves protonation or deprotonation [cf. Carrasco et al. (1986) and Kaback (1987, 1988)].

The importance of His322 in lactose/H^+ symport is further highlighted by studies carried out with mutant permeases containing Asn, Gln, or Lys at position 322. Although the studies were performed with RSO membrane vesicles, rather than proteoliposomes reconstituted with purified permeases, the results provide clear support for the contention that an imidazole group is required at position 322 for coupling between lactose and H^+ translocation.

The amino acid changes at position 322 introduced so far create mutant permeases which are grossly defective with respect to active transport, exchange, and counterflow. In addition, permease with H322R exhibits impaired efflux which contrasts with the ability of permease with H322N, H322Q, or H322K to catalyze this reaction at rates comparable to that of wild-type permease. As opposed to wild-type permease which catalyzes lactose efflux in symport with H^+ (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Garcia et al., 1983; Viitanen et al., 1983), permease with H322N or H322Q appears to catalyze efflux without concomitant H^+ translocation, as evidenced by the observations that the rate of efflux is not influenced by pH, by imposition of a $\Delta\Psi$ (interior negative), or by replacement of protium with deuterium. Thus, permease with H322N, H322Q, or H322K is phenomenologically similar to permease with H322R (Püttner et al., 1986) or R302L (Menick et al., 1987b) with the single exception that the former catalyze downhill lactose efflux.

Although previous experiments (Padan et al., 1985) indicated that the permease will not tolerate replacement of His205 with Arg, permeases with Gln or Asn in place of His205, which were created independently, catalyze lactose/H^+ symport normally (Püttner et al., 1986). As discussed, the original *lac Y* gene encoding H205R permease has been shown to contain two additional mutations in the 5′ end of the gene, and when this portion of the gene is replaced with the analogous portion from wild-type *lac Y* gene, it is apparent that H205R is not defective in lactose/H^+ symport. In addition to emphasizing the importance of sequencing the entire gene after site-directed mutagenesis, the results strengthen the argument that His322 is the only His residue in the permease that is critical for activity. It is also noteworthy that this conclusion is consistent with the results of Garcia et al. (1982) demonstrating that chemical modification of a single His residue is sufficient to inactivate the permease.

Binding studies demonstrate that the K_D for NPG is essentially the same in T206 and H35-39R permeases, and transport studies show that H205R transports lactose normally. It is unlikely, therefore, that His35, His39, or His205 is directly involved in binding. In contrast, H322R permease probably manifests an increased K_D for NPG, since the altered permease exhibits a small but significant amount of NPG binding by flow dialysis and it can be photolabeled with NPG. Photoaffinity labeling with NPG is thought to involve a short-lived triplet-state intermediate that reacts rapidly with residues at the binding site, thereby forming a dead-end complex (Kaczorowski et al., 1980). Thus, it is not surprising that H322R permease can be photolabeled but exhibits minimal binding under equilibrium conditions. It is particularly interesting that permease molecules with Leu in place of Arg302 also appear to exhibit an increase in K_D for NPG (D. R. Menick, L. Patel, and H. R. Kaback, unpublished information), while permease with Ala in place of Glu325 exhibits a relatively normal K_D for the ligand (Püttner & Kaback, 1988; Carrasco et al., 1989). On the basis of the transport properties of site-directed mutants and modeling studies (Püttner et al., 1986; Carrasco et al., 1986; Menick et al., 1987b), it has been suggested that Arg302, His322, and Glu325 are sufficiently close to hydrogen bond and that they may be involved in H^+ translocation as part of a charge-relay mechanism [cf. Kaback (1987, 1988) for reviews]. Since site-directed mutagenesis of Arg302 and His322 also appears to cause a decrease in binding affinity, it is tempting to speculate that the pathways for H^+ and lactose may overlap (i.e., that Arg302 and His322 may also be components of the substrate-binding site and that protonation of His322 may be required for high-affinity binding). In the absence of a high-resolution structure, however, it may be impossible to distinguish between this possibility and one involving alterations in binding affinity secondary to long-range conformational changes resulting from protonation or deprotonation of critical residues.

ACKNOWLEDGMENTS

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Registry No. His, 71-00-1; Arg, 74-79-3; Asn, 70-47-3; Gln, 56-85-9; Lys, 56-87-1; NPG, 7493-95-0; H^+, 12408-02-5; lactose, 63-42-3; lactose permease, 9068-45-5.

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Characterization of Site-Directed Mutants in the *lac* Permease of *Escherichia coli*. 2. Glutamate-325 Replacements

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ABSTRACT: *lac* permease with Ala in place of Glu325 was solubilized from the membrane, purified, and reconstituted into proteoliposomes. The reconstituted molecule is completely unable to catalyze lactose/H^+ symport but catalyzes exchange and counterflow at least as well as wild-type permease. In addition, Ala325 permease catalyzes downhill lactose influx without concomitant H^+ translocation and binds *p*-nitrophenyl α-D-galactopyranoside with a K_D only slightly higher than that of wild-type permease. Studies with right-side-out membrane vesicles demonstrate that replacement of Glu325 with Gln, His, Val, Cys, or Trp results in behavior similar to that observed with Ala in place of Glu325. On the other hand, permease with Asp in place of Glu325 catalyzes lactose/H^+ symport about 20% as well as wild-type permease. The results indicate that an acidic residue at position 325 is essential for lactose/H^+ symport and that hydrogen bonding at this position is insufficient. Taken together with previous results and those presented in the following paper [Lee, J. A., Püttner, I. B., & Kaback, H. R. (1989) *Biochemistry* (third paper of three in this issue)], the findings are consistent with the idea that Arg302, His322, and Glu325 may be components of a H^+ relay system that plays an important role in the coupled translocation of lactose and H^+.

The preceding paper (Püttner et al., 1989) confirms and extends earlier observations (Padan et al., 1979, 1985; Patel et al., 1982; Garcia et al., 1982; Püttner et al., 1986) focusing on the importance of His322 in lactose/H^+ symport by the *lac* permease. Thus, evidence was presented indicating that His322 may be directly involved in lactose-coupled H^+ translocation.

Although a high-resolution structure for *lac* permease is not available, recent studies (Costello et al., 1984, 1987; Li & Tooth, 1987) suggest the presence of a notch or groove within the molecule. Therefore, the number of amino acid residues directly involved in substrate and H^+ translocation may be fewer than the number of residues required to span the full thickness of the membrane.

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