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Registry No. ATPase, 9000-83-3; ATP, 56-65-5.

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lac Permease of *Escherichia coli*: Arginine-302 as a Component of the Postulated Proton Relay

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ABSTRACT: The *lac* permease of *Escherichia coli* was modified by site-directed mutagenesis such that Arg-302 in putative helix IX was replaced with Leu. In addition, Ser-300 (helix IX) was replaced with Ala, and Lys-319 in putative helix X was replaced with Leu. Permease with Leu at position 302 manifests properties that are similar to those of permease with Arg in place of His-322 [Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483]. Thus, permease with Leu-302 is markedly defective in active lactose transport, efflux, exchange, and counterflow but catalyzes downhill influx of lactose at high substrate concentrations without H^+ translocation. In contrast, permease molecules with Ala at position 300 or Leu at position 319 catalyze lactose/H^+ symport in a manner indistinguishable from that of wild-type permease. By molecular modeling, Arg-302 may be positioned in helix IX so that it faces the postulated His-322/Glu-325 ion pair in helix X. In this manner, the guanidino group in Arg-302 may interact with the imidazole of His-322 and thereby play a role in the H^+ relay suggested to be involved in lactose/H^+ symport [Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486].

lac permease of *Escherichia coli* is a hydrophobic transmembrane protein encoded by the *lac Y* gene that catalyzes symport of β-galactosides with H^+ [cf. Kaback (1986a,b) and Wright et al. (1986) for reviews]. Therefore, when a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] is generated across the cytoplasmic membrane (interior negative and alkaline), the permease utilizes free energy released from downhill trans-

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; Dns^6-Gal, 6-(*N*-dansylamino)hexyl 1-thio-β-D-galactopyranoside; ss, single stranded; cc, closed circular; RF, replicative form; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RSO, right side out; PMS, phenazine methosulfate; Mab, monoclonal antibody.

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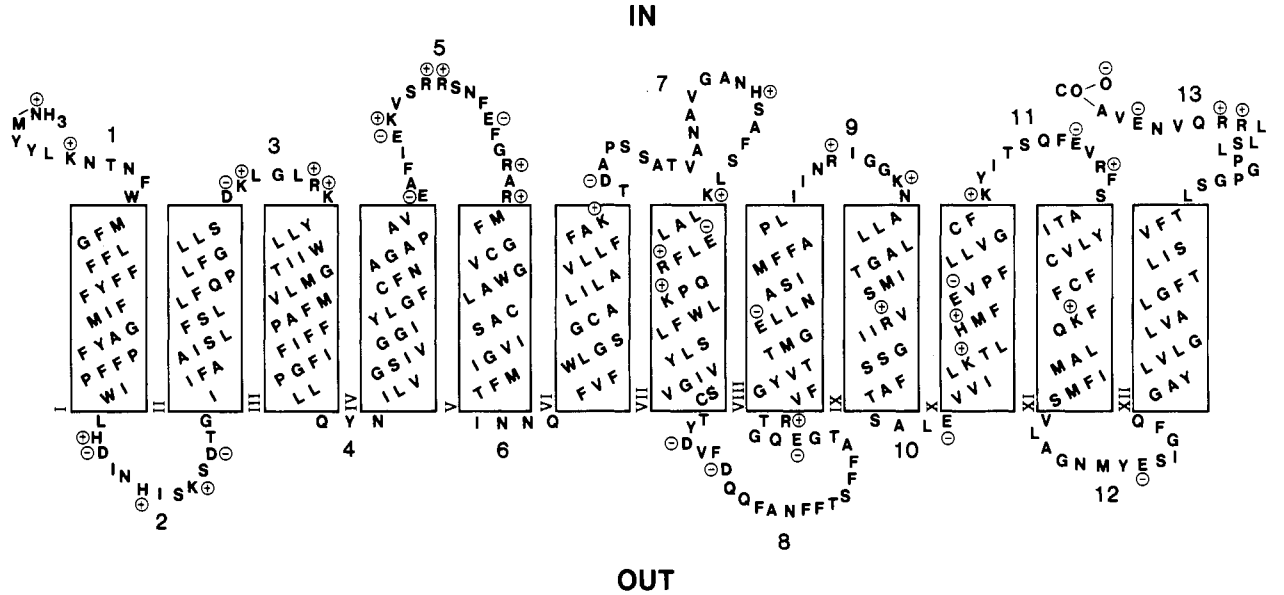

FIGURE 1: Secondary structure of *lac* permease as predicted from the hydropathy profile of Foster et al. (1983). Hydrophobic segments are shown in boxes as transmembrane α-helical domains connected by hydrophilic segments containing most of the charged residues as well as the residues commonly found in β-turns.

location of H^+ in response to $\Delta\bar{\mu}_{H^+}$ to drive uphill accumulation of β-galactosides against a concentration gradient. Conversely, when a concentration gradient of substrate is created in the absence of $\Delta\bar{\mu}_{H^+}$, the permease utilizes free energy released from downhill translocation of substrate to drive H^+ uphill with 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, and the permease has been purified to a single polypeptide species, reconstituted into proteoliposomes, and shown to be completely functional, thereby demonstrating that the *lac Y* gene product is solely responsible for β-galactoside transport. Secondary structure models for the permease suggest that the polypeptide is organized into 12–14 hydrophobic segments in α-helical conformation that traverse the membrane in zigzag fashion, connected by more hydrophilic, charged segments (Figure 1). Evidence supporting certain general aspects of these models has been obtained from circular dichroic and laser Raman spectroscopy, from proteolysis studies, and from binding studies with monoclonal and site-directed polyclonal antibodies [cf. Kaback (1986a,b) and Wright et al. (1986)].

During the past few years, oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) has been used to introduce specific amino acid changes into *lac* permease (Sarkar et al., 1986). Recent applications of the technique suggest that His-322 and Glu-325, neighboring residues in putative helix X (Figure 1), may be ion paired and may function as components of a H^+ relay system similar to that postulated for the serine proteases [Püttner et al., 1986; Carrasco et al., 1986; cf. Kaback (1987) for a review]. Furthermore, Cys residues, long thought to play a critical role in permease function, are probably not directly involved in either substrate binding or H^+ translocation (Menick et al., 1987; Kaback, 1987).

In this paper, we demonstrate that replacement of Arg-302 with Leu yields permease with the same properties as permease with Arg substituted for His-322. On the basis of molecular modeling studies, it is suggested that Arg-302, which is in putative helix IX, may be in close proximity to the postulated His-322/Glu-325 ion pair in helix X. Other experiments demonstrate that replacement of Ser-300 (helix IX) or Lys-319 (helix X) with Ala or Leu, respectively, has no effect on lactose/H^+ symport, thereby highlighting the importance of specific amino acid residues in this region of the permease.

Table I: Bacterial Strains and Plasmids

strain	relevant genotype (chromosome/F′/plasmid)	ref
JM101	supE, thi, Δ(lac-proAB)/traD36, proAB lacI^{q}ZΔM13/–	*a*
JM109	recA1, endA1, gyrA96, thi, hsdRΔ7, supE44, relA1, λ$^-$, Δ(lac-proAB)/traD36, proAB, lacI^{q}ZΔM15/–	*a*
T184	LacI$^+$O$^+$Z$^-$Y$^-$/lacI^{q}Z^{U118}(Y$^+$)/–	*b*
BW313	dut, ung, thi-1, relA, spoT1/lysA/–	*c*
BMH71-18 *mutL*	Δ(lac-pro), supE, thi/proA$^+$B$^+$, lacI^{q}lacZΔM15/MutL::Tn10	*d*
HB101	hsdS20, (r_B^-, m_B^-) recA13, ara-14, proA2, lacY1, galK2, rps(sm^r), xyl-5, mtl-1, supE44, λ$^-$/F$^-$/–	*e*

[a] Yanisch-Perron et al. (1985). [b] Hobson et al. (1977). [c] Kunkel (1985). [d] Kramer et al. (1984). [e] Boyer Roulland-Dussoix (1969).

Materials and Methods

Materials

6-(*N*-Dansylamino)hexyl 1-thio-β-D-galactopyranoside (Dns^6-Gal) was synthesized as described Schuldiner et al., 1975). All other materials were of reagent grade and were obtained from commercial sources (Sarkar et al., 1986).

Methods

Bacterial Strains. Bacterial strains are listed in Table I with the relevant genotypes.

Oligonucleotide Synthesis. The oligonucleotides described were synthesized on an Applied Biosystems Model 380B DNA synthesizer and purified by polyacrylamide gel electrophoresis as described in the Applied Biosystems manual.

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1986) with one of the following modifications to improve the frequency of mutant recovery: (i) For the Ser-300 or Lys-319 replacements with Ala or Leu, respectively, template single-stranded (ss) DNA was 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 syn-

Table II: DNA Sequence Analysis of Mutants in *lac Y*

plasmid	mutagenic primer	DNA sequence change	protein change
pGM21		*lac* Y^+	
pA300	5′-TACAGC*CATAATAGTGCC-3′	codon 300, TCT → GTC	Ser-300 → Ala
pL302	5′-GCCAATAATAA*GTACAGACAT-3′	codon 302, CGT → CTT	Arg-302 → Leu
pL319	5′-ATGCAGCGTTA*A*CAGAATAAC-3′	codon 319, AAA → TTA	Lys-319 → Leu

thesized in vitro as described (Sarkar et al., 1985) and transfected into *E. coli* JM109 (ung^+). Mutant phage were screened as described below. (ii) For replacement of Arg-302 with Leu, mismatch repair was minimized by transfecting the heteroduplex into the mutator strain *E. coli* BMH71-18 *mutL* (Kramer et al., 1984). Phage harboring the mutation were identified initially by colony–blot hybridization with the appropriate ^{32}P-labeled mutagenic primer (Carter et al., 1984). Phage from positive colonies were plaque purified, and the mutation was verified by dideoxyoligonucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) utilizing a synthetic primer complementary to a region of *lac Y* 50–150 bases downstream from the site of the mutation.

Double-stranded *lac Y* DNA from each mutant was restricted from M13mp19 replicative form (RF) DNA and ligated into the *Eco*RI site of pACYC184 DNA. The resulting plasmids (Table II) were used to transform *E. coli* T184 (Z^-Y^-) or HB101 (Z^+Y^-), as indicated.

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* T184 harboring the appropriate plasmid were grown and induced with isopropyl 1-thio-β-D-galactopyranoside (IPTG) as described (Teather et al., 1980). Cells were harvested at midlog phase, washed, and resuspended in 0.1 M potassium phosphate (pH 7.5) to an A_{420} of 10. Right-side-out (RSO) membrane vesicles prepared as described (Kaback, 1971, 1974) were resuspended to a final concentration of 7.2 mg of protein/mL in 0.1 M potassium phosphate (pH 6.6). Aliquots were frozen and stored in liquid nitrogen for subsequent use.

Transport Assays. Transport of [1-^{14}C]lactose in intact cells was assayed by rapid filtration (Trumble et al., 1984). Transport of [1-^{14}C]lactose or [U-^{14}C]proline in RSO membrane vesicles was measured in the presence of reduced phenazine methosulfate (PMS) under oxygen (Kaback, 1971, 1974). Efflux, exchange, and facilitated diffusion in RSO vesicles were assayed as described (Kaczorwski & Kaback, 1979; Patel et al., 1982). The concentrations and specific activities of the radioactive substrates used are given in the figure legends. Entrance counterflow was measured by lactose efflux induced enhancement of Dns^6-Gal fluorescence (Schuldiner et al., 1975; Kaczorowski et al., 1979). RSO membrane vesicles were concentrated and equilibrated with 20 mM lactose as described. An aliquot (10 μL) of the suspension was then rapidly diluted into 2 mL of 0.1 M potassium phosphate (pH 7.5)/0.01 M magnesium sulfate containing 10 μM Dns^6-Gal in a 1 × 1 cm quartz cuvette that had been placed in the sample compartment of a Perkin-Elmer MPF66 spectrophotofluorometer. Fluorescence was recorded continuously at 500 nm (excitation 340 nm) with the emission and excitation slits set at 6 nm.

Quantitation of Permease. In order to estimate the amount of permease in the membrane, immunoblot analyses were performed with monoclonal antibody (Mab) 4A10R and ^{125}I-labeled protein A (Herzlinger et al., 1985).

Protein Determinations. Protein was measured as described (Lowry et al., 1951) with bovine serum albumin as standard.

RESULTS

Verification of Mutations by DNA Sequencing. The *lac Y* gene in each plasmid used was cloned initially from pGM21 into the replicative form of M13mp19 DNA, and ss phage DNA was isolated and used as a template for site-directed mutagenesis. Subsequently, ss phage DNA containing mutated *lac Y* was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with appropriate primers complementary to regions of *lac Y* 50–150 bases downstream from the mutations. The sequence analyses summarized in Table II demonstrate that the mutated *lac Y* genes contain changes in given codons such that Ser-300, Arg-302, or Lys-319 in the permease is replaced with Ala, Leu, or Leu, respectively.

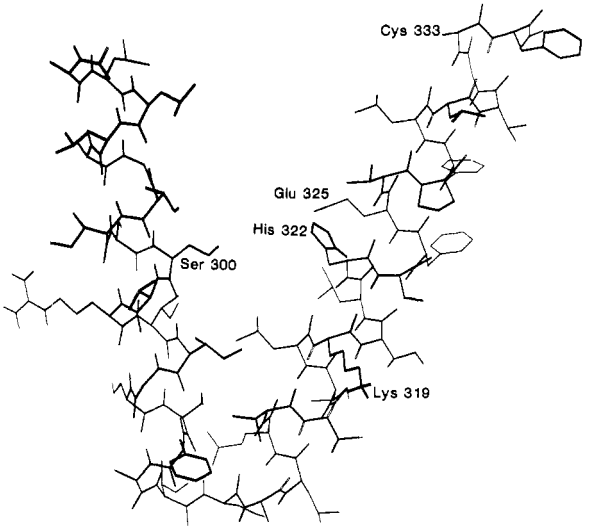

FIGURE 2: Molecular modeling of putative helices IX and X in the *lac* permease. The study was performed by Dr. Vincent Madison on a Evans-Sutherland computer on the basis of the hydropathy profile of Foster et al. (1983).

Replacement of Ser-300 with Ala or Lys-319 with Leu. In the charge–relay postulated for chymotrypsin (Blow et al., 1969), Asp and His function to increase the nucleophilicity of the Ser residue acylated during peptide bond cleavage. There are six Ser and Thr residues in putative helix IX, which is in close proximity to the region of the permease containing His-322 and Glu-325 (i.e., helix X; Figure 1). Furthermore, when the amino acid sequence of putative helices IX and X is subjected to molecular modeling (Figure 2), it is apparent that Ser-300 is the most likely hydroxyl-containing amino acid residue to be implicated if a triad similar to that suggested for chymotrypsin is involved in H^+ translocation. However, when *E. coli* T184 is transformed with a plasmid encoding *lac* permease with Ala in place of Ser-300, both the initial rate of transport and the steady-state level of accumulation are comparable to those observed when the same cells are transformed with plasmid encoding wild-type *lac* permease [Figure 3, compare (□) with (●)].

Molecular modeling of putative helix X suggests in addition that Lys-319 is on the same face of the helix as His-322/Glu-325, separated by one helical turn from His-322 (Figures 1 and 2). Thus, Lys-319 might also be thought to play an important role in lactose/H^+ symport. However, replacement of this residue with Leu has no effect on active lactose transport [Figure 3, compare (○) with (●)].

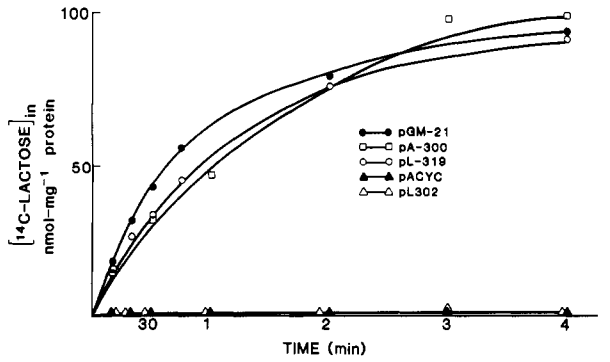

FIGURE 3: Lactose transport in *E. coli* T184 transformed with pACYC/T184 (▲), pGM21 (●), pA300 (□), pL302 (△), or pL319 (○). Reactions were initiated by the addition of [1-^{14}C]lactose (17.5 mCi/mmol) to a final concentration of 0.4 mM and terminated by the addition of 3 mL of 0.1 M potassium phosphate (pH 5.5) containing 0.1 M lithium chloride. Samples were filtered immediately on Whatman GF/F filters and washed with the same solution. Radioactivity retained on the filters was determined by liquid scintillation spectrometry.

Effect of Arg-302 to Leu Mutation on Active Lactose Transport. Like the His-322 to Arg mutation (Padan et al., 1985), *E. coli* HB101 (Z^+Y^-) transformed with pL302, the plasmid encoding permease with Leu in place of Arg-302, grow as red colonies on eosin methylene blue (EMB) indicator plates containing 25 mM lactose [data not shown; cf. Padan et al. (1985)]. That is, the cells are indistinguishable from HB101 transformed with pGM21 (i.e., HB101 transformed with either plasmid is no longer cryptic). Nonetheless, it is apparent that *E. coli* T184 transformed with pL302 is totally defective with respect to active lactose transport [Figure 3; compare (△) with (●)]. The rate and steady-state level of accumulation in T184/pL302 are the same as those observed in T184 transformed with pACYC184 (▲), the identical plasmid without the *lac Y* gene. Importantly, however, *E. coli* L302 membranes contain a normal complement of permease, as evidenced by immunoblot analyses (data not shown). Therefore, permease containing Leu in place of Arg-302 apparently retains the ability to facilitate lactose entry at high substrate concentrations although it no longer catalyzes lactose/H^+ symport, conclusions that will be documented further below.

Studies with RSO membrane vesicles prepared from *E. coli* T206 and L302 confirm the argument that permease with Leu in place of Arg-302 is specifically defective in catalyzing lactose accumulation against a gradient (Figure 4). As shown in panel A, when $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) is generated in the presence of reduced PMS, T206 vesicles accumulate lactose at a linear rate for about 20 s and achieve a steady-state at about 1.5 min. In contrast, L302 vesicles with or without reduced PMS transport lactose at the same low rate and to the same miniscule level as observed in T206 vesicles in the absence of electron donor. Both vesicle preparations, however, accumulate proline when $\Delta\bar{\mu}_{H^+}$ is generated (Figure 4B).

Effect of Arg-302 to Leu Mutation on Efflux, Exchange, and Counterflow. Efflux, exchange, and counterflow are useful for studying permease turnover because specific steps in the overall catalytic cycle can be delineated (Kaback, 1986a,b, 1987; Püttner et al., 1986; Carrasco et al., 1986). As described for the Arg-322 mutation (Padan et al., 1985; Püttner et al., 1986), the rate of efflux is almost negligible in RSO vesicles from L302 (Figure 5A; $t_{1/2} \approx 10$ s for T206 vesicles and >90 s for L302 vesicles). Similarly, the rate of exchange is also markedly diminished in L302 vesicles (Figure 5B; $t_{1/2} \approx 2$ s for T206 vesicles and ≫16 s in L302 vesicles). Finally, permease with Leu in place of Arg-302 is unable to catalyze counterflow (Figure 6). In the experiments shown, T206 and

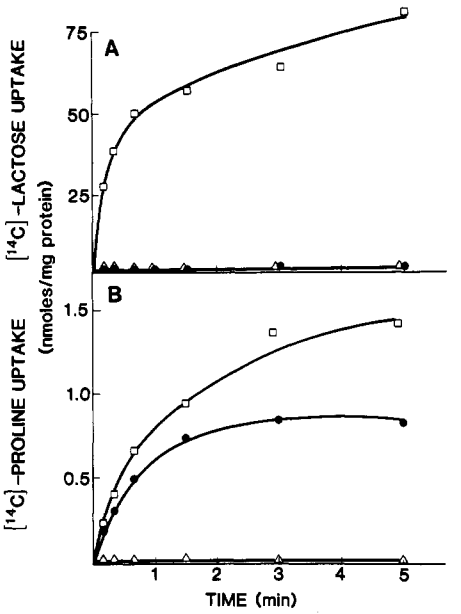

FIGURE 4: Time course of lactose (A) and proline (B) transport in T206 and L302 membrane vesicles. (A) Aliquots (50 μL) of T206 (□) or L302 (●) vesicles containing 100 μg of protein in 0.05 M potassium phosphate (pH 6.6)/0.01 M magnesium sulfate were assayed for [1-^{14}C]lactose transport at 25 °C in the presence of 20 mM ascorbate and 1 mM PMS under oxygen with 0.38 mM [1-^{14}C]lactose (19.5 mCi/mmol). The reactions were terminated by rapid filtration as described (Kaback, 1971, 1974). (△) Transport in the absence of ascorbate/PMS. (B) T206 (□) and L302 (●) membrane vesicles were incubated with ascorbate/PMS as described above, and [U-^{14}C]proline (286 mCi/mmol) was added to 6.94 μM final concentration to initiate the reactions. (△) Transport in the absence of ascorbate/PMS.

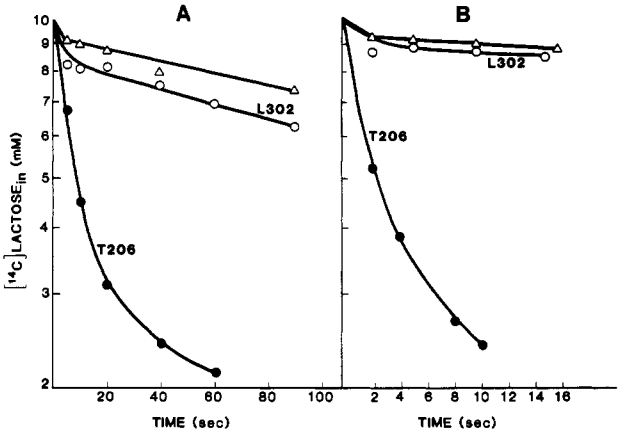

FIGURE 5: Lactose efflux (A) and exchange (B) in RSO membrane vesicles of *E. coli* T206 (●) and L302 (○). (A) Membrane vesicles were concentrated to 35 mg of protein/mL in 0.1 M potassium phosphate at pH 7.5. A small aliquot of [1-^{14}C]lactose (19 mCi/mmol) was added to each suspension to a final concentration of 10 mM, and valinomycin was added at a final concentration of 20 μM. After equilibration for 3 h at room temperature, 2-μL aliquots were rapidly diluted into 400 μL of 0.1 M potassium phosphate (pH 7.5) at 25 °C. At the times shown, the samples were diluted rapidly with 1.5 mL of 0.1 M potassium phosphate, pH 5.5, containing 0.1 M lithium chloride and 20 mM mercuric chloride and immediately filtered as described. (B) Experiments were carried out as described in (A), but 10 mM unlabeled lactose was included in the medium into which the concentrated, equilibrated vesicles were diluted and the temperature was maintained at 18 °C. Controls (△) were performed with T206 and L302 vesicles in which *lac* permease was inactivated by treatment with pCMBS (2 mM) for 20 min at 25 °C prior to loading with lactose. Data for both efflux and exchange were normalized to zero-time points determined as described (Kaczorowski & Kaback, 1979).

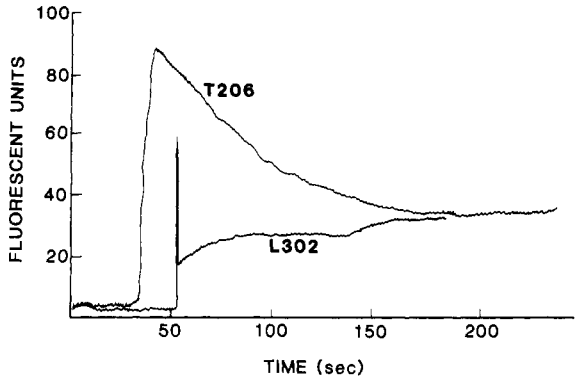

FIGURE 6: Lactose/Dns^6-Gal counterflow. RSO membrane vesicles from *E. coli* T206 and L302 were concentrated to 40 mg/mL in 0.1 M potassium phosphate (pH 7.5)/0.01 M magnesium sulfate. Valinomycin, nigericin, and lactose were added to final concentrations of 20 μM, 2 μM, and 20 mM, respectively, and the samples incubated at 4 °C overnight. Counterflow was initiated by diluting 10 μL of equilibrated vesicles into 2 mL of 0.1 M potassium phosphate (pH 7.5)/0.01 M magnesium sulfate containing 10 μM Dns^6-Gal, and fluorescence emission was recorded continuously at 500 nm (excitation 340 nm) as described under Methods. A control in which 20 mM TDG was added to L302 vesicles prior to Dns^6-Gal yielded a trace identical with that shown for L302.

L302 vesicles loaded with lactose were tested for efflux-induced Dns^6-Gal fluorescence enhancement, and it is apparent that a typical overshoot is observed with T206 membranes [cf. Schuldiner et al. (1975) and Kaczorowski et al. (1979)], while L302 vesicles exhibit no counterflow activity.

Permease with Leu-302 Catalyzes Downhill Lactose Influx without H$^+$. Although cells and vesicles containing L302 permease are grossly defective in active lactose accumulation, HB101 transformed with pL302 grows as red colonies on EMB/lactose plates, suggesting that permease with this mutation is able to catalyze downhill lactose influx at high substrate concentrations without H$^+$ translocation. In order to study this possibility more quantitatively, facilitated diffusion was measured in T206 and L302 vesicles (Figure 7A). In the presence of 10 mM lactose, both vesicle preparations take up the disaccharide relatively rapidly for about 1 min. The rates then decrease, and the intravesicular pools achieve complete equilibration in about 10 min. In contrast, when the permeases are inactivated with *p*-(chloromercuri)benzenesulfonate (pCMBS), the rate of facilitated diffusion is markedly inhibited, but the intravesicular pools equilibrate with lactose eventually by passive diffusion (i.e., in about 3 h; data not shown).

In the corollary experiment, lactose-induced H$^+$ influx was measured in lightly buffered suspensions of RSO vesicles (Patel et al., 1982). When lactose is added to T206 vesicles, transient alkalinization of the medium is observed; the pH tracing reaches maximum displacement in 0.5–1.0 min and returns to the base line after approximately 4 min (Figure 7B). On addition of lactose to L302 vesicles, however, transient alkalinization is not observed.

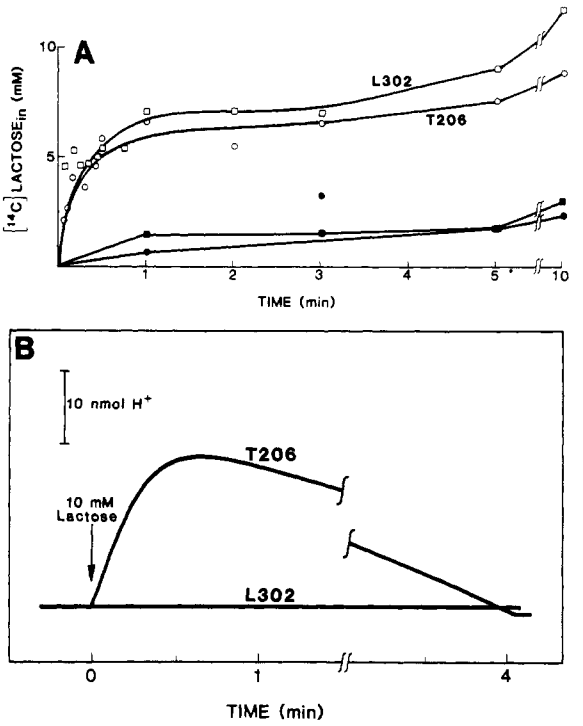

FIGURE 7: (A) Lactose-facilitated diffusion in RSO membrane vesicles from T206 (○) and L302 (□). Aliquots (100 μL) of RSO vesicles (7.2 mg of protein/mL) were incubated at 25 °C, and at zero time [1-^{14}C]lactose (5.9 mCi/mmol) was added to a final concentration of 10 mM. At the indicated times, samples were quenched and assayed as described (Kaback, 1971). Controls were performed with T206 (●) and L302 (■) vesicles in which *lac* permease was inactivated by treatment with pCMBS (2 mM) for 20 min at 25 °C. (B) Lactose-induced H$^+$ influx in *E. coli* T206 and L302 RSO membrane vesicles. A 2.5-mL suspension of T206 or L302 RSO membrane vesicles containing 3 mg of protein in 0.15 M KCl was placed in a closed electrode vessel. The reaction was started by addition of lactose to a final concentration of 10 mM, and the pH of the solution was recorded continuously as described (Patel et al., 1982). The pH change was calibrated at the end of each experiment by addition of 10 μL of 10 mM HCl.

Discussion

The recent findings of Püttner et al. (1986) and Carrasco et al. (1986) focus on His-322 and Glu-325, neighboring residues in putative helix X of the *lac* permease, as central components of a H$^+$ relay that may play a critical role in lactose/H$^+$ symport. In brief, site-directed mutagenesis was utilized to replace His-322 with Arg, Asn, or Gln and Glu-325 with Ala, and the properties of the mutated permeases were compared to those of the wild-type molecule with respect to various modes of translocation. Permease will not tolerate replacement of His-322 with Arg, Asn, or Gln, and permease with Arg-322 is defective in all steps in the overall kinetic mechanism that involve protonation or deprotonation, leading to the conclusion that an imidazole at position 322 is critical for lactose-coupled H$^+$ translocation. In contrast, replacement of Glu-325 with Ala results in a permease that is defective in all steps involving net H$^+$ translocation but catalyzes exchange and counterflow normally. Thus, permease with Ala at position 325 may not be able to accept H$^+$ from His-322, a conclusion that is consistent with the recent demonstration that permease with Gln in place of Glu-325 also does not catalyze lactose/H$^+$ symport [cf. Kaback (1987)].

In any case, the striking differences between the transport properties of the Arg-322 and Glu-325 permeases provide the basis for a strategy to define other residues involved in lactose/H$^+$ symport both before and after His-322 and Glu-325. Mutations in the pathway prior to His-322 should not catalyze exchange or counterflow, while mutations after Glu-325 should catalyze both reactions. Clearly, this strategy can be used to subclassify uncoupled mutants selected by classical mutagenesis prior to DNA sequencing or to characterize mutants constructed by site-directed mutagenesis.

The notion that lactose-coupled H$^+$ translocation may involve a H$^+$ relay mechanism like that hypothesized for the serine proteases, chymotrypsin in particular (Blow et al., 1969), is based on the positions of His-322 and Glu-325 in a region of the permease thought to be in α-helical conformation and

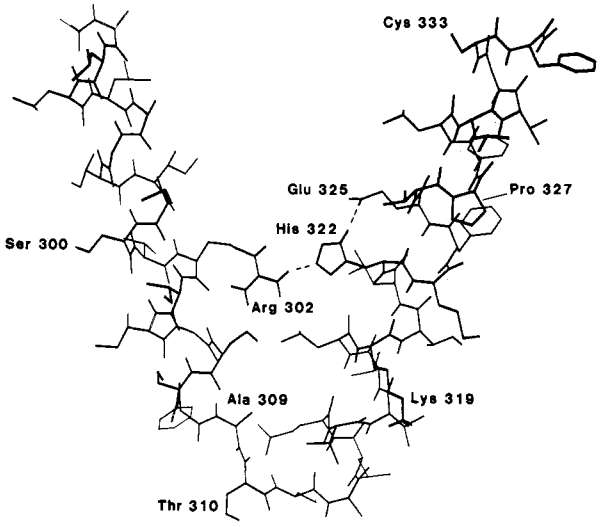

FIGURE 8: Modified molecular model of putative helices IX and X in *lac* permease. The study was performed as described in Figure 2, except that Ala-309 and Thr-310 were transferred from helix IX to hydrophilic segment 10, which connects helices IX and X (cf. Figure 1). By this means, the pitch of helix IX is altered so that Arg-302 is now on the right side of helix IX where the guanidino group hydrogen bonds to the imidazole ring of His-322, which, in turn, hydrogen bonds to Glu-325.

on the transport properties of the Arg-322 and Ala-325 mutants. Other studies [cf. Kaback (1987)] support the hypothesis in that the polarity of His-322/Glu-325, as well as the distance/configuration between the imidazole and the carboxylate, is critical for lactose/H^+ symport.

Although it is impossible to prove or disprove the H^+ relay hypothesis on the basis of currently available evidence, if such a mechanism is involved in lactose/H^+ symport, it would seem reasonable to search for hydroxyl-containing amino acid residues in the vicinity of the postulated His/Glu ion pair in helix X. As indicated in Figure 1, putative helix IX, which must be close to helix X given the length of the connecting hydrophilic segment, contains six Ser and Thr residues. In addition, when the amino acid sequence of helices IX and X is subjected to molecular modeling, it is apparent that Ser-300 might be on the side of helix IX facing His-322/Glu-325 (Figure 2). Importantly, replacement of Ser-300 with Ala does not compromise permease activity. Similarly, replacement of Lys-319 with Leu also has no effect on permease activity. Although these mutations, and in addition replacement of Cys-333 with Ser (Menick et al., 1987; cf. Figure 2), have no effect on lactose/H^+ symport, they emphasize the specificity of His-322/Glu-325 and support the contention that single amino acid changes do not cause drastic conformational alterations within a relatively localized portion of the permease.

Replacement of Arg-302 with Leu leads to a marked defect in the ability of the permease to catalyze lactose/H^+ symport. Furthermore, by use of the strategy outlined above, it is apparent that permease with Leu-302 exhibits properties similar to those of permease with Arg substituted for His-322. Therefore, it appears that Arg-302 is a component of the putative H^+ relay that functions prior to His-322. Interestingly, Arg-302 may be moved from the left to the right side of helix IX simply by transferring two amino acid residues (Ala-309 and Thr-310; Figure 1) from the helix to the hydrophilic segment connecting the helices (Figure 8). Under these circumstances, the guanidino group in Arg-302 is sufficiently close to His-322 to hydrogen bond with the imidazole ring. Minimally, therefore, the putative H^+ relay in the permease would involve interaction between Arg-302, His-322, and Glu-325 (Figure 8). However, it should be noted that in each of the mutants described the altered permeases are defective in lactose/H^+ symport in both directions across the membrane. Given the order of the three residues involved and their respective pK_as, H^+ would move from Glu-325 to His-322 to Arg-302, but not in the reverse direction. Two possibilities are noteworthy in this regard: (i) the pK_as of the respective residues may be conformationally dependent, or (ii) lactose-coupled H^+ translocation may not involve physical movement of H^+ from one residue to the next. Instead, His-322 may be the only residue immediately involved in H^+ translocation, and its pK_a may be poised by the proximities of Glu-325 and Arg-302 (i.e., decreased by Glu and increased by Arg). Insight into these possibilities should be forthcoming from studies with permeases engineered to contain a single His residue at position 322 with either Glu or Ala at position 325 [cf. Kaback (1987)].

Finally, it should be emphasized that L302 permease and each of the other "uncoupled" permeases studied thus far, whether isolated by classical mutagenesis (Wong et al., 1970; Wilson et al., 1970; Wilson & Kutsch, 1972; Herzlinger et al., 1985) or constructed by site-directed mutagenesis (Padan et al., 1985; Püttner et al., 1986; Carrasco et al., 1986), catalyze downhill lactose influx without H^+ at rates similar to that observed for facilitated diffusion with wild-type permease. Remarkably, however, each of the uncoupled permeases exhibits a marked defect with respect to efflux of lactose down a concentration gradient. Clearly, therefore, under certain conditions, the permease functions in a highly asymmetrical fashion, allowing influx of substrate but not efflux.

ADDED IN PROOF

As indicated in Figure 8, the hydroxyl group in Ser-306 may be sufficiently close to Arg-302 to hydrogen bond to one of the guanidino nitrogens. For this reason, Ser-306 was replaced with Ala. Permease with this mutation catalyzes lactose/H^+ symport in a manner indistinguishable from that of wild-type permease. It is also noteworthy that Arg-302 has been replaced with Gln or His, and permease with each of these replacements behaves like permease with Leu in place of Arg-302 (D. R. Menick, L. Patel, and H. R. Kaback, unpublished data).

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Effect of Plasmid RP1 on Phase Changes in Inner and Outer Membranes and Lipopolysaccharide from *Acinetobacter calcoaceticus*: A Fourier Transform Infrared Study†

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ABSTRACT: The successful transfer of the resistance plasmid RP1 into the Gram-negative bacterium *Acinetobacter calcoaceticus* resulted in increased resistance of this microorganism to the antibiotics kanamycin and tetracycline. Microorganisms harboring the RP1 plasmid showed altered fatty acid composition in the lipopolysaccharide fraction and increased outer membrane permeability compared to organisms without the plasmid. Thermotropic gel to liquid crystal lipid phase changes were detected in both inner and outer membranes and purified lipopolysaccharide by Fourier transform infrared spectroscopy. The phase transition temperatures observed in the outer membranes and isolated lipopolysaccharide of the plasmid-containing cells were significantly higher than those of the plasmid-free organisms, while little difference was observed for the inner membranes. The plasmid-induced decrease in outer membrane fluidity may play a mediating role in the mechanisms of antibiotic resistance and susceptibility to host immune cells in Gram-negative microorganisms.

Phase changes in bacterial membranes have been detected by a variety of physical and spectroscopic techniques, such as calorimetry (Steim et al., 1969) and NMR (Smith, 1979), ESR[1] (Davis et al., 1985), and fluorescence (Tecoma et al., 1977) spectroscopy. The structure of the *Acholeplasma laidlawii* plasma membrane has been studied intensively since this organism possesses no cell wall and the fatty acid composition of its membrane lipids can be easily modified by including appropriate fatty acid supplements in the growth

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[1] Abbreviations: ESR, electron spin resonance; FT-IR, Fourier transform infrared; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; PMN, polymorphonuclear leukocyte; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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