Site-Directed Mutagenesis of Lysine 319 in the Lactose Permease of *Escherichia coli*

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ABSTRACT: Lys319, which is on the same face of putative helix X as His322 and Glu325 in the lactose permease of *Escherichia coli*, has been replaced with Leu by oligonucleotide-directed, site-specific mutagenesis. Although previous experiments suggested that the mutation does not alter permease activity, we report here that K319L permease is unable to catalyze active lactose accumulation or lactose efflux down a concentration gradient. The mutant does catalyze facilitated influx down a concentration gradient at a significant rate; however, the reaction occurs without concomitant H^+ translocation. The mutant also catalyzes equilibrium exchange at about 50% of the wild-type rate, but it exhibits poor counterflow activity. Finally, flow dialysis and photoaffinity labeling experiments with *p*-nitrophenyl α-D-galactopyranoside indicate that K319L permease probably has a markedly decreased affinity for substrate. The alterations described are not due to diminished levels of the mutated protein in the membrane, since immunological studies reveal comparable amounts of permease in wild-type and K319L membranes. It is proposed that Lys319, like Arg302, His322, and Glu325, plays an important role in active lactose transport, as well as substrate recognition.

The lactose (lac) permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the concomitant translocation of β-galactosides and H^+ with a stoichiometry of unity [i.e., symport or cotransport; see Kaback (1989, 1992) and Kaback et al. (1990) for reviews]. Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport. On the basis of circular dichroic studies and hydropathy analysis (Foster et al., 1983), a secondary structure was proposed in which the permease has a short hydrophilic N terminus, 12 transmembrane hydrophobic domains in α-helical configuration connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail. Evidence confirming some of the general features of the model and demonstrating that both the N and C termini are on the cytoplasmic face of the membrane has been obtained from laser Raman (Vogel et al., 1985) and Fourier transform infrared (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished work) spectroscopy, from limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986; Page & Rosenbusch, 1988), from immunological studies with monoclonal (Carrasco et al., 1982, 1984a; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibodies (Seckler et al., 1983, 1984, 1986; Carrasco et al., 1984b; Danho et al., 1985), and from chemical labeling (Page & Rosenbusch, 1988). Furthermore, exclusive support for the topological predictions of the 12-helix motif has been obtained from analyses of a large number of *lac* permease–alkaline phosphatase (*lacY–phoA*) chimeras (Calamia & Manoil, 1990).

Site-directed mutagenesis studies on lac permease have been useful for identifying residues that are important for active transport and/or substrate recognition (Kaback, 1989, 1992; Kaback et al., 1990). Out of more than 200 individual site-directed mutations, over 90% retain the ability to catalyze lactose accumulation against a significant concentration gradient. Therefore, it is unlikely that individual amino acid replacements cause global conformational alterations in the permease. More specifically, none of the eight Cys [see Menick et al. (1987) and van Iwaarden et al. (1991)], six Trp (Menezes et al., 1990), or 12 Pro residues (Consler et al., 1991) in the permease is mandatory for transport activity, and out of 14 Tyr residues, only two are essential (Roepe & Kaback, 1989a).[1] On the other hand, site-directed mutagenesis of Arg302 in putative helix IX (Menick et al., 1987), His322 in helix X (Padan et al., 1985; Püttner et al., 1986, 1989; King & Wilson, 1989a,b, 1990; Brooker, 1990, 1991), and Glu325 in helix X (Carrasco et al., 1986, 1989) indicates that these residues play an important role in the translocation mechanism and/or substrate recognition. Moreover, differences in the properties of these mutants with respect to various translocation reactions catalyzed by the permease has led to the suggestion that Arg302, His322, and Glu325 may function as components of a H^+ translocation pathway that acts in part like the charge-relay in the serine proteases, although it is also possible that these residues form part of a coordination site for H_3O^+ [see Kaback et al. (1990) for a discussion].

K319[2] permease was described initially as part of a study focusing on Arg302 (Menick et al., 1987), and observations were presented demonstrating ostensibly that the mutation has no effect on the ability of lac permease to catalyze active

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[1] Although Tyr26 is completely inactive when replaced with Phe, permease with Cys in place of Tyr26 catalyzes active lactose transport essentially as well as wild-type permease (M. Sahin-Toth, B. Persson, J. Schwieger, and H. R. Kaback, unpublished information).

[2] Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating that 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.

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lactose accumulation. However, sequencing techniques for double-stranded plasmid DNA were not used routinely at the time the mutant was constructed. Presumably as a result of incomplete plaque purification, propagation of wild-type *lacY* DNA is sometimes observed after subcloning mutated DNA fragments from the replicative form of M13mp19*lacY* DNA. For this reason, plasmid DNA encoding the early site-directed mutants in lac permease constructed in this laboratory have been subjected to double-stranded DNA sequencing. Although the other mutants described were verified, sequencing of pK319L double-stranded DNA revealed the presence of a wild-type Lys codon (AAA) at position 319. Therefore, pK319L was reconstructed and the entire *lacY* gene was sequenced after cloning into the plasmid vector. In addition, mutants were constructed with Arg, His, or Cys in place of Lys319.

In contradistinction to previous findings (Menick et al., 1987), K319L permease catalyzes neither lactose accumulation against a concentration gradient nor lactose efflux down a concentration gradient, but it does exhibit facilitated influx of lactose without concomitant H^+ translocation. Moreover, K319L permease catalyzes equilibrium exchange at a significant rate, but exhibits poor counterflow activity possibly because of decreased affinity for substrate on the outer surface of the membrane. Although characterized in less detail, permease with Cys in place of Lys319 is also unable to catalyze active lactose transport.

MATERIALS AND METHODS

Materials

[1-^{14}C]Lactose and *p*-nitro[2-^{3}H]phenyl α-D-galactopyranoside (NPG)[3] were synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group, Hoffmann-La Roche, Inc.). All other materials were reagent grade and were obtained from commercial sources.

Methods

Bacterial Strains. The following strains of *E. coli* K-12 were used: JM101 *supE*, *thi*, *D(lac–proAB)*, [F′ *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}ZDM15] (Yanisch-Perron et al., 1985); T206 [*lacI*$^+$*O*$^+$*Z*$^{-Y-}$(*A*), *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR*/F′, *lacI*^{q}*O*$^+$*Z*^{U118}(*Y*$^+$*A*$^+$) harboring plasmid pGM21 [*lacD*(*I*)-*O*$^+$*P*$^+$*D*(*Z*)*Y*$^+$*D*(*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*, *supE-44*, *l*/*F*$^-$ (Boyer & Roulland-Dussoix, 1969); LJ915, HB101 (*ptsEI*$^-$) (generously provided by M. H. Saier, Jr.); BMH71-18*mutL*, [*D*(*lac–pro*), *supE*, *thi*/*proA*$^+$*B*$^+$, *lacI*^{q} *lacZ*DM15, MutL::Tn10] (Kramer et al., 1984).

Site-Directed Mutagenesis. A 23-base oligonucleotide complementary to the sense wild-type *lacY* sequence, but containing a two-base mismatch, was synthesized on an Applied Biosystems synthesizer and purified by polyacrylamide gel electrophoresis (Menick et al., 1987). The codon for Leu (CTA) was substituted for the Lys(AAA) codon at position 319 by annealing a given mutagenic oligonucleotide primer to single-stranded M13mp19 template DNA containing the *lacY* gene, synthesizing heteroduplex DNA overnight at 14 °C, and transforming the repair-deficient strain BMH71-18*mutL* (Kramer et al., 1984) with the resultant heteroduplex DNA. Relevant procedures are described in detail in Sarkar et al. (1986). Replacement of Lys319 with Cys was performed as described in Kunkel (1985).

Colonies were screened by hybridization with ^{32}P-labeled mutagenic primer, and the presence of the mutation was verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978). The replicative form of M13mp-19*lacY* DNA containing the mutation was isolated from positive colonies by alkaline lysis, restricted with *Eco*R1, and cloned into the plasmid pACYC184 to produce pK319L. Orientation of the fragment was determined by *Hin*cII restriction analysis (Sarkar et al., 1986). The entire *lacY* gene was sequenced to ensure that no secondary mutation(s) contributed to the phenotype. *E. coli* HB101 (*Z*$^+$*Y*$^-$) was transformed with given plasmids, and the cells were grown initially on eosin-methylene blue (EMB) plates containing 25 mM lactose as a qualitative estimate of permease activity (Miller, 1972). *E. coli* T184 (*Z*$^-$*Y*$^-$) was also transformed, and transport activity was assayed quantitatively with [1-^{14}C]-lactose.

DNA Sequencing. Double-stranded plasmid DNA was sequenced by using the dideoxynucleotide method (Sanger et al., 1977; Sanger & Coulson, 1978) after alkaline denaturation (Hattori & Sakaki, 1986). A synthetic primer complementary to the region of *lacY* approximately 50 bases downstream from codon 319 were used. In addition, the entire *lacY* gene was sequenced by using six synthetic deoxyoligonucleotide primers complementary to appropriate regions of the gene.

Growth of Bacteria. E. coli T184 were grown aerobically in Luria broth at 37 °C to an OD_{420} of 0.5, and isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM. The cells were grown for an additional 90 min, harvested by centrifugation, washed with 50 mM potassium phosphate (KP_i, pH 7.5)/10 mM $MgSO_4$, and resuspended in the same solution to an OD_{420} of 10.0 (approximately 1 mg of protein/mL).

Preparation of Membrane Vesicles. Right-side-out (RSO) membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975) from *E. coli* T184 harboring given plasmids. Cells were grown under constant aeration at 37 °C to an OD_{420} of approximately 1.0 in Cohn-Rickenberg medium (Miller, 1972) containing 12.5 μg/mL tetracycline. Permease expression was then induced by addition of IPTG to 0.2 mM, and cells were harvested after an additional 90 min and stored overnight at 4 °C as a pellet before vesicle preparation.

Lactose Transport Assays. Active lactose transport was measured in intact cells as described (Trumble et al., 1984). Aliquots (50 μL) of cells prepared as described above were incubated at room temperature with [1-^{14}C]lactose (10 mCi/mM) at a concentration of 0.4 mM, and uptake was quenched at a given time with 50 mM KP_i (pH 5.5)/100 mM LiCl, followed by filtration through Whatman GF/F glass microfiber filters. Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Downhill influx of [1-^{14}C]lactose was measured in *E. coli* LJ915 (*lacZ*$^+$*Y*$^-$, *ptsEI*$^-$) transformed with given plasmids as described (Bibi et al., 1991).

Exchange and efflux measurements were performed on RSO vesicles equilibrated on ice overnight with 50 mM KP_i (pH 7.3)/20 μM valinomycin/0.20 μM nigericin/10 mM [1-^{14}C]-lactose (10 mCi/mM) Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979). Aliquots (2–3 μL) of the equilibrated samples were diluted rapidly into a 100-fold excess of 50 mM KP_i (pH 7.3) alone (efflux) or 50 mM KP_i (pH 7.3) containing

[3] Abbrevations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EMB, eosin-methylene blue; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; NPG, *p*-nitrophenyl α-D-galactopyranoside; OD, optical density; RSO, right-side-out; TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside.

10 mM lactose (exchange), and the reactions were quenched with 50 mM KP_i (pH 5.5)/100 mM LiCl/20 mM $HgCl_2$ and filtered.

Lactose counterflow was carried out with RSO vesicles equilibrated overnight with 50 mM KP_i (pH 7.3)/20 μM valinomycin/0.2 μM nigericin/10 mM lactose (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979). Aliquots (3 μL) were diluted 100-fold into 50 mM KP_i (pH 7.3) containing 0.4 mM [1-^{14}C]lactose (10 mCi/mmol), and the reactions were quenched as described above.

Lactose-Induced H^+ Translocation. H^+ transport on addition of lactose to *E. coli* T184 expressing wild-type or K319L permease was measured essentially as described (West, 1970; West & Mitchell, 1972). Cells were washed twice in 120 mM KCl and suspended in 120 mM KCl containing 30 mM KSCN. An aliquot (1.5 mL containing approximately 5 mg of protein/mL) was placed in a closed vessel fitted with a glass combination pH electrode. The suspension was then gassed continuously with N_2 for 30 min when an aliquot of an anaerobic solution of lactose, maltose, or β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG) was added to a final concentration of 4 mM. External pH was measured with a Radiometer pH meter (PHM84) and recorded continuously on a REC80 Servograph chart recorder modified to expand the scale. To quantitate the pH changes observed, 10 nmol of anaerobic HCl was added for calibration at the end of each experiment.

Flow Dialysis. [^{3}H]NPG bound specifically to nonenergized RSO membrane vesicles was measured by flow dialysis (Rudnick et al., 1976). Aliquots (200 μL) of vesicles resuspended in 50 mM KP_i (pH 7.3)/20 μM valinomycin/0.2 μM nigericin (approximately 2 mg/mL protein) were stirred in the upper chamber of a flow dialysis cell and dialyzed against 50 mM KP_i (pH 7.3) flowing through the lower chamber at a rate of 3.3 mL/min. After the upper chamber was equilibrated with 20 μM [^{3}H]NPG, TDG was added to a final concentration of 10 mM. The concentration of [^{3}H]-NPG in the dialysate was quantitated by liquid scintillation spectrometry.

Photoaffinity Labeling. Photoaffinity labeling of wild-type or K319L permease was carried out with [2-^{3}H]NPG in RSO vesicles (Kaczorowski et al., 1980). A suspension of vesicles [1 mg/mL protein in 100 mM KP_i (pH 8.5)] was incubated at 25 °C under N_2, and after temperature equilibration, D-lactate was added to a final concentration of 20 mM. Where indicated, TDG was also added to a final concentration of 20 mM. The samples were then irradiated at λ > 300 nm in the presence of 0.2 or 20 μM [2-^{3}H]NPG. At given times, aliquots were precipitated with 10% trichloroacetic acid and filtered. Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Immunoblot Analysis. Immunoblot experiments were carried out as described (Carrasco et al., 1984) by using monoclonal antibody 4A10R.

Protein Determinations. Protein was assayed as described by Peterson (1977).

RESULTS

Verification of Mutations by DNA Sequencing. After reconstruction of K319L permease by site-directed mutagenesis in M13mp19, the *lacY*-containing fragment was restricted from the replicative form of M13mp19*lacY* DNA and ligated into plasmid pACYC184. After propagation in *E. coli* T184, recombinant plasmid DNA was isolated and subjected to double-stranded DNA sequencing as described in Methods. Except for the presence of a Leu codon (CTA) in place of the Lys codon at position 319 (AAA), the sequence was identical to that reported for wild-type *lacY* (Büchel et al., 1980).

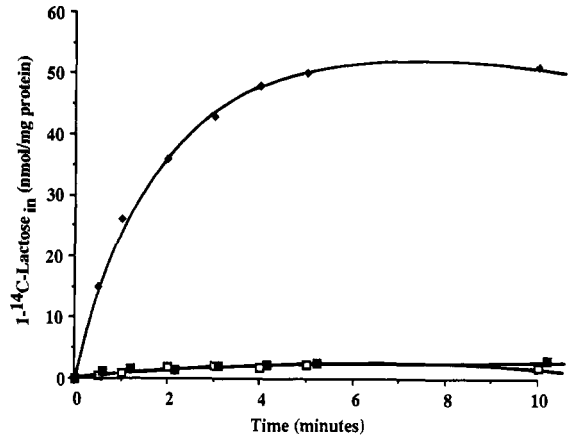

FIGURE 1: Active transport of lactose in *E. coli* T184 expressing no permease (□), wild-type permease (◆), or K319L permease (■). See Materials and Methods for experimental details.

Colony Morphology. E. coli HB101 (Z^+Y^-) harboring pGM21, which encodes wild-type permease, grows as dark red colonies on eosin-methylene blue (EMB) plates containing 25 mM lactose (Miller, 1972), indicating that lactose is transported and metabolized. In contrast, cells harboring pACYC184, the identical plasmid without a *lacY* insert, grow as white colonies. *E. coli* HB101 harboring pK319L appear as red colonies on EMB/25 mM lactose, indicating that permease with Leu in place of Lys319 is minimally able to translocate lactose down a concentration gradient into the cell. Similar results were obtained with a permease mutant containing Cys in place of Lys319 (Sahin-Tóth et al., 1992).

Active Lactose Transport. As a more quantitative measure, [1-^{14}C]lactose transport was assayed in *E. coli* T184 transformed with pACYC184 (no *lacY* insert), pGM21 (pACYC containing wild-type *lacY*), or pK319L (Figure 1). Cells expressing wild-type permease transport lactose rapidly and achieve a steady-state level of accumulation within 5–6 min. In contrast, cells expressing K319L permease do not transport lactose to a significant extent, since the dissacharide is accumulated to the same negligible level in cells transformed with pACYC184 containing no *lacY* insert. Although not shown, similar results are obtained with cells expressing K319C permease (Sahin-Tóth et al., 1992) or when respiration-driven lactose transport is studied in RSO membrane vesicles. Immunoblot analysis of membranes from cells expressing wild-type or K319L permease reveals that comparable amounts of permease are present in both preparations (Figure 2). Thus, the absence of transport activity in cells or vesicles containing K319L permease is not due to defective insertion of the mutant permease into the membrane or to enhanced rates of degradation after insertion.

Facilitated Diffusion and Lactose-Induced H^+ Translocation. E. coli HB101/pK319L grows as red colonies on EMB/25 mM lactose, indicating that the mutated permease may catalyze facilitated influx down a concentration gradient. To examine this possibility more thoroughly, "downhill" transport assays were performed with *E. coli* LJ915 (*lacZ*$^+$*Y*$^-$, *ptsEI*$^-$) with the following considerations taken into account: (i) Since *E. coli* LJ915 expressing wild-type permease catalyzes active transport, the cells were "uncoupled" by addition of 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). (ii) Cells overexpressing hydrophobic membrane proteins such as lac permease are fragile and might release cytosolic β-galactosidase into the medium during the transport assays. Since the enzyme has a high turnover number, release of even small

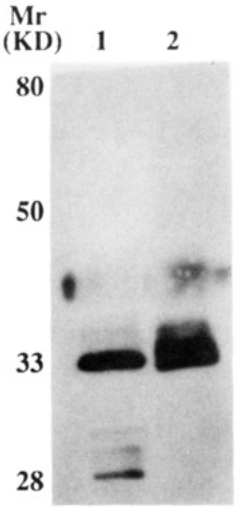

FIGURE 2: Western blot of *E. coli* T184 expressing wild-type permease (lane 1) or K319L permease (lane 2). Positions of prestained molecular mass markers (Bio-Rad) are indicated. Membranes prepared from induced cultures were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and electroblotted, and the blot was incubated with monoclonal antibody 4A10R. After incubation with horseradish peroxidase–protein A and a short incubation with the fluorescent substrate, the blot was exposed to film for 30 min.

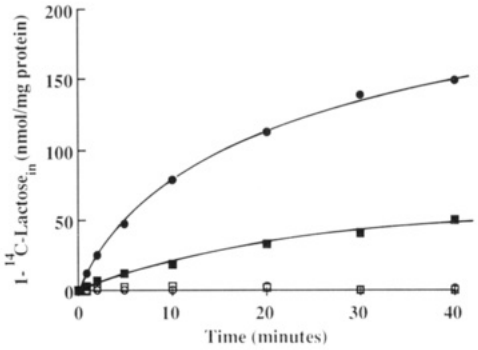

FIGURE 3: Downhill lactose translocation by *E. coli* LJ915 expressing wild-type (●) or K319L (■) permease in the presence of 20 μM CCCP. (○) and (□) denote lactose translocation by cells expressing wild-type and K319L permease, respectively, in the presence of 2 mM *N*-ethylmaleimide.

amounts of enzyme would result in significant hydrolysis of [1-^{14}C]lactose in the external medium, generating [^{14}C]-glucose, which could then be taken up by the phosphoenolpyruvate phosphotransferase system (PTS). Hence the use of *E. coli* LJ915 which is defective in enzyme I of the PTS and unable to transport glucose. Since β-galactosidase is expressed in this strain, any [^{14}C]lactose that enters is rapidly cleaved, thereby providing a "sink" that drives accumulation of radioactivity. Clearly, uptake of radioactivity from [1-^{14}C]-lactose in LJ915/pK319L occurs at a significant, albeit slower, rate than that observed in LJ915/pGM21, and in both instances, uptake is blocked by *N*-ethylmaleimide (Figure 3).

In a parallel experiment, lactose-induced H^+ movements were monitored with a pH electrode (Figure 4). When lactose is added to an anaerobic suspension of *E. coli* expressing wild-type permease, alkalinization of the medium is observed, and the pH tracing reaches maximum displacement in 1–2 min. In marked contrast, cells expressing K319L permease do not alkalinize the medium upon addition of lactose. Therefore, K319L permease catalyzes facilitated influx at a significant rate, but the process does not occur in symport with H^+. It is also noteworthy that no change in external pH is observed upon addition of maltose, although *E. coli* HB101/pK319L grow as red colonies on maltose indicator plates (data not shown). Similarly, no change in pH is observed upon addition

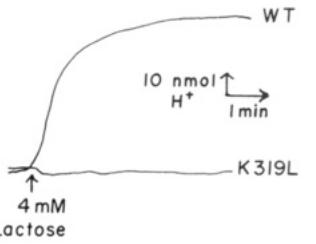

FIGURE 4: Lactose-induced H^+ translocation. Changes in external pH were measured continuously in *E. coli* T184 expressing wild-type or K319L permease on addition of 4 mM lactose, as indicated. Although not shown, similar results were obtained with *E. coli* T184/pK319L on addition of 4 mM maltose or TDG. See Materials and Methods for experimental details.

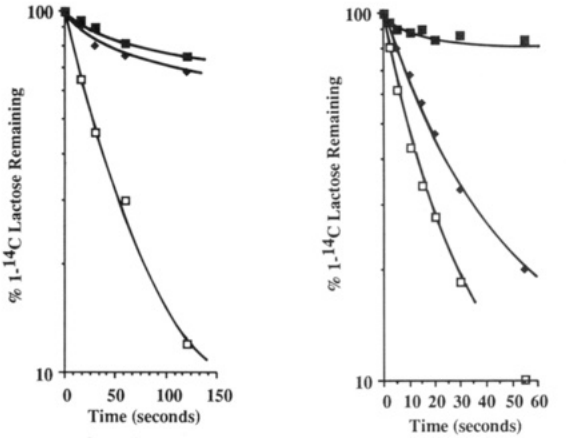

FIGURE 5: (A, left) Permease-mediated downhill lactose efflux in *E. coli* T184 RSO membrane vesicles containing wild-type (□) or K319L permease (◆) at room temperature. Also shown are data for vesicles prepared from *E. coli* T184 containing no permease (■) (i.e., T184 transformed with pACYC devoid of a *lacY* insert). (B, right) Equilibrium lactose exchange in *E. coli* RSO membrane vesicles harboring wild-type (□), K319L permease (◆), or no permease (■) at room temperature. See Materials and Methods for experimental details.

of TDG, although it has been reported (Brooker, 1991) that the double mutant A177V/K319N exhibits TDG/H^+ symport with a reduced stoichiometry.

Efflux, Exchange, and Counterflow. Lactose efflux down a concentration gradient is permease-mediated and occurs in symport with H^+ (Kaczorowski & Kaback, 1979; Viitanen et al., 1983). In contrast, equilibrium exchange and counterflow do not involve net H^+ translocation, although the permease may recycle in the protonated state [see Carrasco et al. (1986, 1989)]. When RSO vesicles containing wild-type permease are equilibrated with [1-^{14}C]lactose and diluted into medium devoid of lactose (Figure 5A) or into medium containing 10 mM lactose (Figure 5B), efflux and exchange occur at rates comparable to those observed previously (i.e., $t_{1/2}$ ca. 25 s and 10 s, respectively; Kaczorowski & Kaback, 1979). In contrast, vesicles containing K319L permease are markedly defective with respect to efflux (Figure 5A, $t_{1/2} \gg 150$ s) but catalyze exchange at about 50% the rate of wild type (Figure 5B, $t_{1/2}$ ca. 20 s). Finally, although K319L vesicles catalyze equilibrium exchange at a significant rate, the preparations catalyze entrance counterflow at only about 10% the rate of wild type (Figure 6).

NPG Binding and Photoaffinity Labeling. NPG is a potent competitive inhibitor of lactose transport that binds to lac permease with a K_D of about 20 μM, which corresponds of its K_i with respect to lactose transport (Rudnick et al., 1976). Flow dialysis experiments carried out with [^{3}H]NPG and vesicles containing K319L permease fail to reveal significant binding activity at pH 5.5, 7.5, or 9.0 (data not shown). Thus,

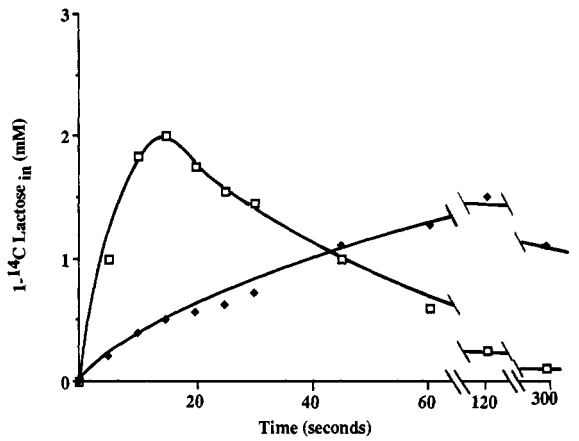

FIGURE 6: Lactose entrance counterflow in *E. coli* T184 RSO membrane vesicles containing wild-type (□) or K319L (◆) permease. Note the compressed *x*-axis after 60 s. See Materials and Methods for experimental details.

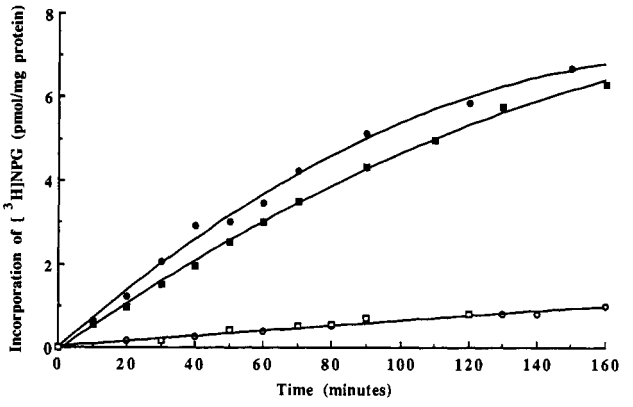

FIGURE 7: Photoaffinity labeling of *E. coli* T184 RSO membrane vesicles containing wild-type (●) or K319L (■) permease with [^{3}H]-NPG at a final concentration of 0.2 μM. Also shown are data obtained from similar experiments where photoaffinity labeling was carried out in the presence of 20 mM TDG (open symbols). See Materials and Methods for experimental details.

when [2-^{3}H]NPG is added to the upper chamber of the flow dialysis apparatus to a final concentration of 15 μM, no ligand is competitively dissociated upon addition of 10 mM unlabeled NPG. On the other hand, K319L permease is not totally devoid of binding activity, as evidenced by photoaffinity-labeling experiments with [2-^{3}H]NPG (Figure 7). Under anaerobic reducing conditions, photolysis of the nitrophenyl ether leads to specific labeling of the permease presumably by nucleophilic aromatic photosubstitution (Kaczorowski et al., 1980). When RSO vesicles containing K319L permease 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. Interestingly, however, the rate of photolabeling observed with K319L vesicles is slower than that observed with wild-type vesicles [3.0 pmol NPG h^{-1} (mg of protein)$^{-1}$ versus 3.6, respectively, at 0.2 μM NPG (Figure 7) and 51 pmol h^{-1} (mg of protein)$^{-1}$ versus 95, respectively at 20 μM NPG (not shown)]. Taken as a whole, the results support the contention that K319L permease has a reduced affinity for substrate.

DISCUSSION

In contrast to previous findings (Menick et al., 1987), the results presented here indicate clearly that mutagenization of Lys319 in putative helix X of lac permease leads to marked inactivation of active lactose transport in a manner suggesting that the permease is uncoupled. Thus, like other site-directed uncoupled mutants (Padan et al., 1985: Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987), lac permease with Leu in place of Lys319 does not catalyze active lactose transport or efflux down a concentration gradient, but it confers a wild-type phenotype on cells harboring the mutations with respect to colony appearance on lactose indicator plates. Moreover, similar properties are observed when Lys319 is replaced with Cys (Sahin-Tóth et al., 1992). Consistently, K319L permease catalyzes facilitated influx of lactose, but concomitant H^+ translocation is not observed. In addition, K319L permease catalyzes equilibrium exchange at a significant rate, but it catalyzes counterflow poorly. Finally, although the mutant permease does not bind NPG demonstrably by flow dialysis, it is specifically photoaffinity labeled by the nitrophenyl ether, thereby indicating reduced affinity for substrate. Thus, it seems reasonable to conclude that Lys319 is involved in both the translocation mechanism and substrate recognition.

It is noteworthy that although K319L exhibits an uncoupled phenotype, its behavior is unique relative to the uncoupled mutants described previously (Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987). In addition to being grossly defective in all of the translocation reactions involving net translocation of H^+, mutants in Arg302 (putative helix IX) or His322 (helix X) do not catalyze equilibrium exchange or counterflow, nor do they exhibit high-affinity binding of NPG, while mutants in Glu325 catalyze equilibrium exchange and counterflow at least as well as wild-type permease and bind NPG with only a slightly elevated K_d. In contrast, K319L permease catalyzes equilibrium exchange at about 50% the rate of wild-type permease, but it is significantly more defective with respect to counterflow. In addition, K319L permease does not bind NPG under equilibrium conditions (i.e., flow dialysis), but is clearly photoaffinity labeled by the same ligand, indicating that the affinity of K319L permease for substrate is severely reduced [see Püttner et al. (1989)]. Since the only operational difference between equilibrium exchange and counterflow is the difference in external substrate concentrations, it is attractive to postulate that Lys319 is involved in ligand binding on the external face of the membrane. In this regard, evidence has been presented indicating that lac permease contains more than one binding site (Lolkema & Walz, 1990; Lolkema et al., 1991; Persson & Kaback, 1991).

Collins et al. (1989) have reported the isolation of permease mutants with Asn in place of Lys319 as one of a number of mutants that leads to enhanced recognition of maltose and diminished recognition of cellobiose. Although data have not been presented, *E. coli* HB101 harboring K319L permease grows as red colonies on maltose indicator plates. However, the cells do not accumulate radiolabeled maltose to any extent whatsoever, and inward H^+ translocation is not observed upon addition of maltose to an anaerobic suspension of *E. coli* T184 transformed with pK319L. Clearly, therefore, although the mutant permease may exhibit enhanced recognition of maltose, it does not catalyze maltose/H^+ symport.

Recently, King et al. (1991) demonstrated that mutants in lac permease containing either Asn in place of Asp237 or Thr in place of Lys358 are inactive with respect to melibiose transport. Interestingly, second-site revertants of D237Q yield neutral amino acid replacements in Lys358, and reciprocally, second-site revertants of K358T yield neutral amino acid replacements in Asp237. On the basis of these elegant observations, it was concluded that Asp237 is salt-bridged to Lys358. As part of an extensive mutagenesis study using a lac permease mutant devoid of Cys residues (van Iwarden et

al., 1991), it has been shown (Sahin-Tóth et al., 1992) that replacement of Asp240 with Cys, like replacement of Lys319 with Cys, leads to marked inactivation of active lactose accumulation. Remarkably, when the mutations are combined in the same molecule (i.e., D240C/K319C), however, the double mutant catalyzes lactose accumulation against a gradient. Moreover, Lee et al. (1992) have also described a functional interaction between Lys319 and Asp240. Possibly, therefore, Lys319 may be involved in a salt bridge with Asp240, and the phenotype of the K319L mutant may be due to disruption of this interaction.

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Registry No. Lys, 56-87-1; Leu, 61-90-5; Cys, 52-90-4; H^+, 12408-02-5; lactose, 63-42-3.