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Site-Directed Mutagenesis of Tyrosine Residues in the *lac* Permease of *Escherichia coli*

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ABSTRACT: By using oligonucleotide-directed, site-specific mutagenesis, each of the 14 Tyr residues in the *lac* permease of *Escherichia coli* was replaced with Phe, and the activity of each mutant was studied with respect to active transport, equilibrium exchange, and efflux. Ten of the mutations have no significant effect on permease activity. Of the four mutations that alter activity, replacement of Tyr26 or Tyr336 with Phe severely decreases all modes of translocation, and the binding affinity of the mutant permeases for *p*-nitrophenyl α-D-galactopyranoside is markedly decreased (i.e., K_D is increased). In addition, the Phe336 mutant permease is inserted into the membrane to a lesser extent than wild-type permease, as judged by immunoblot experiments. Permease containing Phe in place of Tyr236 catalyzes lactose exchange approximately 40% as well as wild-type permease but does not catalyze active transport or efflux. Finally, permease with Phe in place of Tyr382 catalyzes equilibrium exchange normally, but exhibits low rates of active transport and efflux without being uncoupled, thereby suggesting that replacement of Tyr382 with Phe alters a kinetic step involving translocation of the unloaded permease across the membrane.

The *lac* permease of *Escherichia coli* is a 46.5-kDa hydrophobic transmembrane protein that catalyzes the coupled translocation of a single β-galactoside molecule with a single H^+ (i.e., symport or cotransport) [cf. Kaback (1983, 1986) for reviews]. The polypeptide is encoded by the *lacY* gene, and it has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and demonstrated to be fully functional as a monomer. On the basis of circular dichroism studies and hydropathy analysis (Foster et al., 1983), a secondary structure model for the permease has been proposed in which the polypeptide is organized into 12 hydrophobic α-helical domains that traverse the membrane in zigzag fashion, connected by more hydrophilic, charged segments (Figure 1). Evidence supporting some of the general features of the model 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from laser Raman (Vogel et al., 1985) and Fourier transform infrared (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished results) spectroscopy, from limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), from binding studies with

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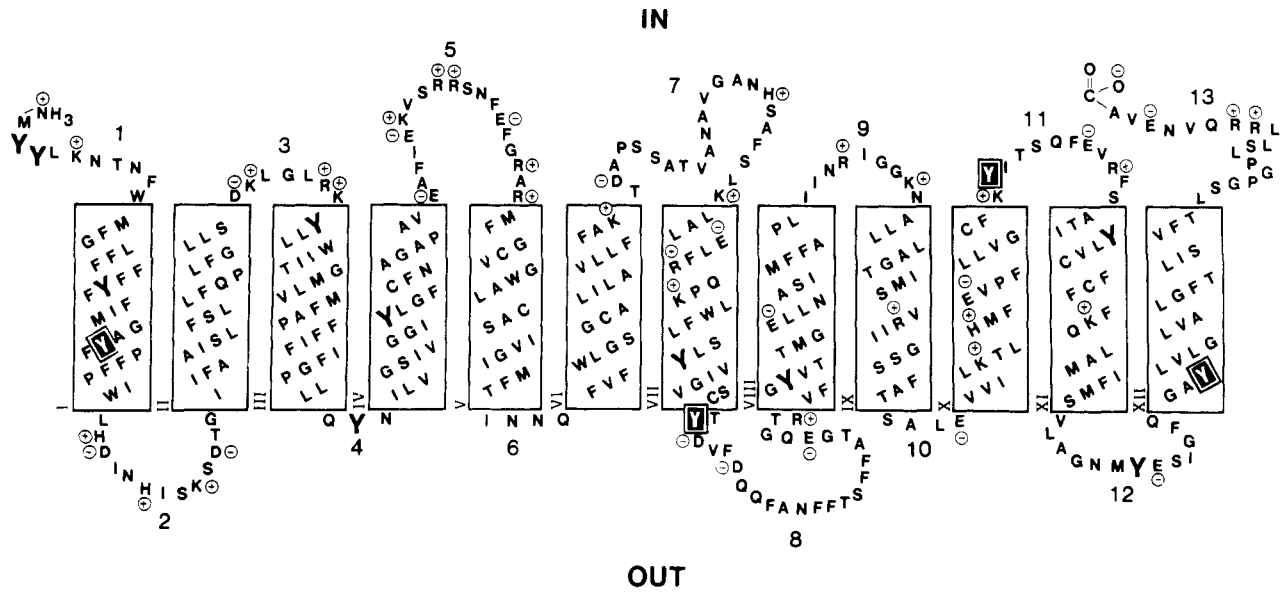

FIGURE 1: Secondary structure model of *lac* permease based on the hydropathy plot of the deduced amino acid sequence (Foster et al., 1983). Tyr residues not essential for activity are emboldened; essential Tyr residues are boxed.

monoclonal (Carrasco et al., 1982, 1984; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibodies (Seckler et al., 1983, 1986; Seckler & Wright, 1984; Carrasco et al., 1985; Danho et al., 1985), from chemical labeling (Page & Rosenbusch, 1988), and, most recently, from *lacY–phoA* fusion analyses (J. Calamia and C. Manoil, unpublished information).

Application of oligonucleotide-directed, site-specific mutagenesis to the study of *lac* permease has begun to delineate residues that are important for activity [cf. Kaback (1987, 1988) for reviews]. Thus, Arg302, His322, and Glu325 have been shown to be essential for lactose/H^+ symport, and it has been hypothesized that these residues may function in a charge-relay type of mechanism. On the other hand, of the eight Cys residues in the permease, only Cys154 is important for activity, and it does not appear to be involved in either substrate binding or H^+ translocation. Moreover, although Cys148 is required for substrate protection against *N*-ethylmaleimide inactivation, replacement of this residue with Ser or Gly has little or no effect on activity. At present, out of approximately 70 independent site-directed permease mutants, more than 60% exhibit no significant change in activity. Thus, it seems unlikely that individual amino acid replacements indiscriminately cause deleterious conformational changes in the permease.

Since Tyr residues are potential H^+ donors or acceptors in membrane proteins that transport H^+ (Rothschild et al., 1986; Dollinger et al., 1986; Roepe et al., 1987, 1988) and since they are often found in substrate recognition sites [e.g., see Wright (1984)], we have now replaced each of the 14 Tyr residues in *lac* permease with Phe in order assess the importance of the tyrosyl hydroxyl moiety for lactose transport and/or substrate recognition. Of the 14 mutations, 10 have no effect whatsoever on the ability of the permease to catalyze active transport. Each of the four mutants that diminish or abolish active lactose transport is further characterized with respect to permease-mediated lactose efflux down a concentration gradient, equilibrium exchange, and binding of the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside (NPG).[1]

Materials and Methods

Materials

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

Methods

Bacterial Strains. The following strains of *E. coli* K-12 were used: JM101, *supE, thi,* Δ(*lacproAB*), [F′, *traD36, proAB, lacI*^{q}*ZΔM15*] (Yanish-Perron et al., 1985); T206 [*lac I*$^+$*O*$^+$,*Z*$^-$,*Y*$^-$(*A*),*rpsL,met*$^-$,*thr*$^-$,*recA,hsdM,hsdR/F′,lac-I*^{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, *hsd S20* (r^-_B, m^-_B), *rec A13, ara-14, proA2, lacY1, galK2, rpsL20*(*Sm*^{r}),*xyl-5, mtl-1, supE-44,* λ$^-$/*F*$^-$ (Boyer & Roulland-Dussoix, 1969); BMH71-18 *MutL*[Δ(*lacpro*),*supE,-thi/proA*$^+$*B*$^+$,*lacI*^{q}*lacZΔM15/MutL::Tn10*] (Kramer et al., 1984).

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis using M13mp19 as the cloning vector was carried out essentially as described (Sarkar et al., 1986) with given modifications. Deoxyoligonucleotide primers complementary to the coding strand of *lacY* DNA with the exception of a single mismatch were synthesized on an Applied Biosystems synthesizer and purified by polyacrylamide gel electrophoresis (Table I). In each case, Phe codons (TTT or TTC), were substituted for given Tyr codons (TAT or TAC, respectively). Oligonucleotides were annealed to single-stranded M13mp19 template containing the coding strand of *lacY*, and heteroduplex DNA was synthesized overnight at 14 °C. In order to maximize the yield of mutant recovery, the repair-deficient strain *E. coli* BMH71-18 *mutL* (Kramer et al., 1984) was transformed with the resultant heteroduplex DNA.

Colonies were screened by hybridization with appropriate ^{32}P-labeled mutagenic primers, and the mutations were verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978). The replicative form of M13 DNA containing specified mutations was isolated by alkaline lysis and restricted with *Eco*RI, and the fragment containing *lacY* was

[1] Abbreviations: [^{3}H]NPG, *p*-nitro[2-^{3}H]phenyl α-D-galactopyranoside; OD, optical density; EMB, eosin–methylene blue; KP_i, potassium phosphate; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *m*-fluorophenylhydrazone.

Table I: DNA Sequence Analyses of Tyr to Phe Mutants in the *lac* Permease of *E. coli*

codon	mutagenic primer[a]	DNA codon change
2	3′-TTAGGTAATACAA*GATAAATTTTTT-5′	TAC to TTC
3	3′-GGTAATACATGAA*AAATTTTTTGTG-5′	TAT to TTT
19	3′-AGAAAAAGAAAAA*GAAAAAATAGTA-5′	TAC to TTC
26	3′-AGTACCCTCGGAA*GAAGGGCAAAAA-5′	TAC to TTC
75	3′-CCGACGCGTTTAA*GGACGACACCTA-5′	TAC to TTC
101	3′-GTGACAATGTTAA*GTTGTAAAATCA-5′	TAC to TTC
113	3′-AACCACCATAAAA*AGATCCGAAAAC-5′	TAT to TTT
228	3′-AAAACAGTGACAA*ACAATAACCGCA-5′	TAT to TTT
236	3′-AAAGGACGTGGAA*GCTACAAAAACT-5′	TAC to TTC
263	3′-CCCATAAACCGAA*GCATTGCTGTTA-5′	TAC to TTC
336	3′-CGACGAAATTTAA*ATAATGGTCGGT-5′	TAT to TTT
350	3′-GTCGCTGCTAAAA*AGACCAGACAAA-5′	TAT to TTT
373	3′-GCCCGTTATACAA*ACTTTCGTAGCC-5′	TAT to TTT
382	3′-AGGTCCCGCGAAA*AGACCACGACCC-5′	TAT to TTT

[a] Sequences of mutagenic primers used to replace Tyr codons with Phe codons at specified positions in the *lacY* gene. In each case, an asterisk follows the base changed.

cloned into the plasmid pACYC184. Orientation of *lacY* in the recombinant plasmid was determined by *Hin*cII restriction enzyme analysis (Sarkar et al., 1986). In all cases where the activity of the mutated permease was compromised, the entire *lacY* gene was sequenced to ensure that no secondary mutations contributed to the phenotype. With the exception of the Y236F[2] mutant, each of the other mutants exhibited a *lacY* DNA sequence identical with that reported by Büchel et al. (1980) except for the given base change (cf. Table I). When sequencing the Y236F mutant, an additional mutation was found (Y19F). Thus, plasmid pY236F/Y19F was restricted with *Ava*I, and the fragment containing the Y263F mutation was isolated and ligated to the appropriate fragment from *Ava*I-digested pGM21 (pACYC184 containing wild-type *lacY*) (Püttner et al., 1989).

E. coli HB101 (Z^+Y^-) was transformed with plasmids encoding mutant *lac* permeases, 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). In addition, *E. coli* T184 (Z^-Y^-) was transformed, and transport was measured quantitatively with [1-^{14}C]lactose.

Transport Measurements. Active transport of lactose was measured by using *E. coli* T184 harboring given plasmids as described (Trumble et al., 1984). Cells were grown at 37 °C to an optical density at 420 nm (OD_{420}) of 0.5, induced with 0.2 mM isopropyl 1-thio-β-D-galactoside (IPTG), and grown for another 90 min. Cells were harvested by centrifugation, washed extensively with 50 mM potassium phosphate (KP_i, pH 7.5)/10 mM magnesium sulfate, and resuspended in the same salt solution to an OD_{420} of 10.0 (approximately 1 mg of protein/mL). Aliquots (50 μL) of the cell suspensions were incubated at room temperature, and [1-^{14}C]lactose (10 mCi/mmol) was added to a final concentration of 0.4 mM. At given times, the reactions were terminated by rapid dilution with 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM lithium chloride/20 mM mercuric chloride and immediately filtered through Whatman GF/F glass fiber filters. Radioactivity retained on the filters was assayed by liquid scintillation spectrometry.

Efflux and equilibrium exchange measurements were performed on intact cells grown as described above and treated with 2 mM ethylenediaminetetraacetic acid (EDTA) for 1 min at 37 °C (Leive et al., 1965). After EDTA treatment, the cells were immediately placed at 4 °C, washed with 50 mM KP_i (pH 7.5)/10 mM magnesium sulfate, and equilibrated overnight with 10 mM [1-^{14}C]lactose (10 mCi/mmol) in the presence of 0.2 μM carbonyl cyanide *m*-fluorophenylhydrazone (FCCP). Aliquots (2 μL) of the cell suspensions were then diluted rapidly into a 100-fold excess of 50 mM KP_i (pH 7.5)/10 mM magnesium sulfate without (efflux) or with 10 mM unlabeled lactose (equilibrium exchange). At specified times, the reactions were terminated and assayed as described above.

Immunoblot Analyses. Immunoblots were carried out as described (Herzlinger et al., 1985) with monoclonal antibody 4A10R and ^{125}I-labeled protein A.

Binding of [^{3}H]NPG. Binding of NPG was determined in right-side-out membrane vesicles by using the method of Hsu and Fox (1970). Right-side-out membrane vesicles were prepared as described (Kaback, 1971).

Results

Verification of Mutations by DNA Sequencing. The *lacY* gene was cloned initially from pGM21 into the replicative form of M13mp19 DNA, and single-stranded phage DNA was isolated and used as a template for site-directed mutagenesis. Subsequently, single-stranded phage DNA containing mutated *lacY* was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) using appropriate primers complementary to regions of *lacY* 50–100 bases downstream from the mutations. As shown by the sequence analyses summarized in Table I, the mutated *lacY* genes described contain given base changes at the sites predicted. In addition, the entire *lacY* gene was sequenced in pY382F, pY336F, pY236F, and pY26F by using six synthetic primers complementary to appropriate regions of *lacY*. In each case, with the exception of the base change described, the sequences were identical with that reported by Büchel et al. (1980).

Qualitative Effects of Tyr to Phe Mutations on Lactose Transport. When *E. coli* HB101 (Z^+Y^-) is transformed with pGM21, which encodes wild-type permease, and grown on EMB/lactose, the cells form red colonies. In contrast, when the same cells are transformed with pACYC184, the identical plasmid with no *lacY* insert, the cells grow as white colonies. Clearly, therefore, when the cells express functional permease, lactose is allowed access to the intracellular milieu where the disaccharide is rapidly hydrolyzed by β-galactosidase and metabolized, thereby acidifying the external medium which produces the color change observed. *E. coli* HB101 transformed with pY2F, pY3F, pY19F, pY75F, pY101F, pY113F, pY228F, pY263F, pY350F, or pY373F grows as red colonies

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

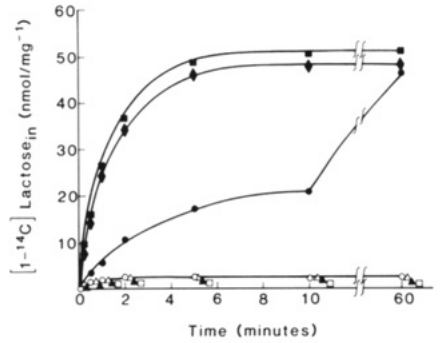

FIGURE 2: Active transport of lactose in *E. coli* T184 expressing no *lac* permease. (○) T184-pACYC; (■) wild-type permease (T206) or *lac* permease with Phe replacements for given Tyr residues (Y2F, Y3F, Y19F, Y101F, Y113F, Y263F, Y373F); (♦) Y228F, Y350F, Y75F; (●) Y382F; (△) Y26F; (▲) Y236F; (□) Y336F. See Materials and Methods for experimental details.

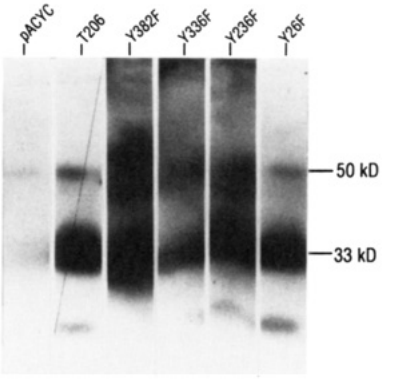

FIGURE 3: Estimation of *lac* permease in membranes from *E. coli* T184, T206, and given Tyr mutants. Membrane fractions (15 μg of protein) from T184-pACYC (lane 1), T206 (lane 2), Y382F (lane 3), Y336F (lane 4), etc. were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the protein bands were electroblotted onto nitrocellulose. The material was then incubated sequentially with monoclonal antibody 4A10R and ^{125}I-labeled protein A and radioautographed for 3 h as described (Herzlinger et al., 1985).

that are indistinguishable from *E. coli* HB101/pGM21. In contrast, cells transformed with pY236F or pY382F grow as pink colonies, and cells transformed with pY26F or pY336F form white colonies that are distinguishable from HB101/pACY184. Qualitatively, therefore, of the 14 Tyr mutants described, only 4 appear to have a significant effect on permease activity.

Active Lactose Transport. As shown in Figure 2, *E. coli* Y2F, Y3F, Y19F, Y75F, Y101F, Y113F, Y228F, Y263F, Y350F, or Y373F accumulate [1-^{14}C]lactose in a manner comparable to that of *E. coli* T206. Thus, cells expressing wild-type permease or each of the mutations accumulate lactose rapidly and approach a similar steady-state level of accumulation within 4–5 min. On the other hand, *E. coli* Y382F transports lactose at about 30–40% of the rate of *E. coli* T206; within 1 h, however, the cells achieve a steady-state level of accumulation comparable to T206. Finally, permease with Y26F, Y236F, or Y336F is devoid of activity, as judged by the observation that cells containing these mutated permeases accumulate lactose in a manner indistinguishable from that of cells devoid of a functional *lacY* gene (i.e., *E. coli* T184 transformed with pACYC184 which has no *lacY* insert).

The decreased transport activity of the mutants cannot be attributed to decreased expression of *lac* permease. Immunoblots carried out on membrane fractions from cells harboring plasmids with specified *lacY* genes exhibit intensities at 33 kDa similar to that observed with membranes from *E. coli* T206, with the sole exception of Y336F which is less intense (Figure 3). It should be emphasized, however, that transport activity is virtually nil in Y336F, although a significant amount of permease is present.

Efflux and Equilibrium Exchange. Lactose efflux down a concentration gradient and equilibrium exchange are useful for studying permease turnover because specific steps in the overall catalytic cycle can be delineated [cf. Kaback (1987, 1988)]. When EDTA-treated cells are equilibrated with lactose in the presence of FCCP and diluted 100-fold, it is apparent that Y382F permease catalyzes efflux at 25–30% the rate of the wild-type [Figure 4; $t_{1/2} \sim 2$ min for Y382F versus ~30 s for wild-type (T206)]. In contrast, Y26F, Y236F, or Y336F permease exhibits little or no ability to catalyze efflux, since cells containing the mutated permeases approximate the rate of efflux observed in cells devoid of permease (i.e., T184/pACYC184). Since permease-mediated efflux, like active transport, involves net H^+ translocation, it is not surprising that the mutant permeases are correspondingly defective in both modes of translocation. Alternatively, equilibrium exchange does not involve net H^+ translocation,

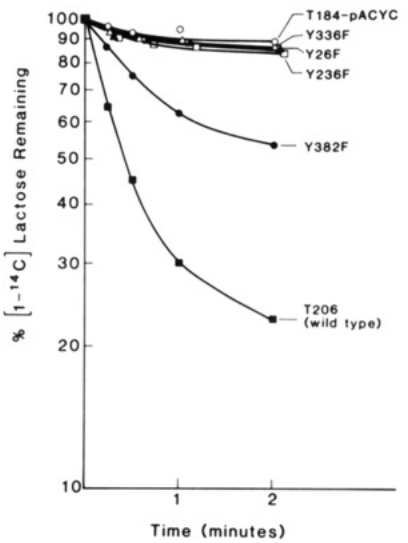

FIGURE 4: Permease-mediated downhill lactose efflux in EDTA-treated *E. coli* T184 harboring plasmids encoding given permeases. See Materials and Methods for experimental details.

and the ability of the permease to catalyze this reaction reflects the translocation of the ternary complex between the permease, lactose and H^+ (Kaback, 1987, 1988). Thus, it is interesting that Y382F permease catalyzes equilibrium exchange at almost the same rate as wild-type permease (Figure 5; $t_{1/2} \sim 10$ and 15 s, respectively, for wild-type permease and the Y382F mutant). Moreover, Y236F permease, which is completely defective with respect to active transport and efflux, exhibits low but significant equilibrium exchange activity (Figure 5; $t_{1/2} \sim 2$ min), while the Y26F and Y336F mutants exhibit essentially no exchange activity.

[^{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, monoclonal antibody 4B1, and 4B1 Fab fragments indicate that 1 mol of NPG is bound/mol of permease (Herzlinger, 1984). Scatchard analyses of NPG binding data performed with right-side-out membrane vesicles from T206 yield a K_D value of 22 μM (Table II). Each of the Tyr mutants that exhibit altered transport activity manifests significantly elevated K_D's (i.e., decreased affinity) for NPG. Thus, the Y382F mutant, which

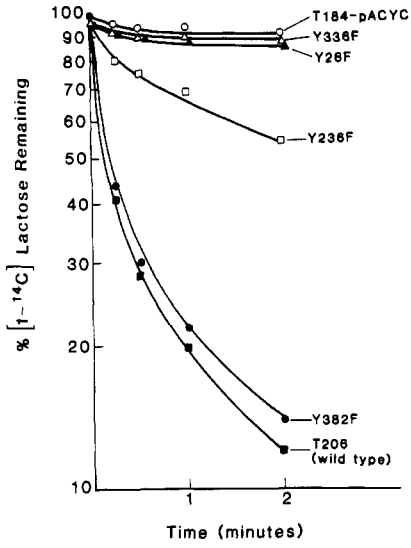

FIGURE 5: Equilibrium exchange in EDTA-treated *E. coli* T184 harboring plasmids encoding given permeases. See Materials and Methods for experimental details.

Table II: NPG Binding Constants[a]

mutant	K_D
wild type	22 μM
Y26F	>750 μM
Y236F	170 μM
Y336F	>1 mM
Y382F	100 μM

[a] K_D values for binding of NPG were obtained from Scatchard plots of binding data obtained as described under Materials and Methods. In each case, right-side-out membrane vesicles were used.

exhibits significant transport activity, has a K_D of 100 μM; Y236F, which catalyzes equilibrium exchange at a slow rate, has a K_D of 170 μM; and Y26F and Y336F, which exhibit no transport activity whatsoever, have K_D's that are only marginally significant (i.e., greater than 750 μM and 1 mM, respectively).

Discussion

A secondary structure of *lac* permease with each of the 14 Tyr residues highlighted is shown in Figure 1. Tyr residues whose replacement with Phe causes significant alterations in permease activity are boxed; those that have no effect on activity are emboldened. Interestingly, Tyr336 is in the immediate proximity of putative helix X which contains His322 and Glu325, two residues obligatory for lactose/H^+ symport that may be directly involved in H^+ translocation (Padan et al., 1985; Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Lee et al., 1989).

Permease with Y26F exhibits no transport activity whatsoever and manifests a dramatic decrease in affinity for NPG. Tyr26 is present in a region of the permease in which other mutations have been shown to cause profound effects on binding and transport (Overath et al., 1987). Specifically, mutation of Gly24 and/or Pro28 to Arg and Ser, respectively, leads to inactivation of transport with an increase in NPG binding affinity. It seems plausible to suggest, therefore, that the region of putative helix I disposed toward the external surface of the membrane may form part of a substrate recognition site. Brooker and Wilson (1985) isolated *lacY* mutants that transport maltose and found only Y236F, -N, -S, or -H and A177V or -T, suggesting that these residues are at or near the sugar binding site. As shown here, Y236F permease is unable to catalyze active transport of lactose or efflux and is markedly defective in equilibrium exchange. In addition, the mutant exhibits a significant increase in K_D for NPG. All of the effects are consistent with the contention that Tyr236 may be a binding site residue.

As shown by immunoblot analysis (Figure 3), the amount of Y336F permease in the membrane is less than that observed with wild-type permease or the other mutants described which suggests that Y336F permease either is inserted into the membrane less efficiently or is proteolyzed more rapidly after it is inserted. In either case, the result suggests that Tyr336 may be a conformational determinant. It should be emphasized, however, that Y336F permease is present to a significant extent. Thus, the virtually complete loss of transport and binding activity cannot be attributed to total absence of permease. Tyr336 is in a five-residue segment in hydrophilic domain 11 (Figure 1) with the sequence Lys, Tyr, Ile, Thr, Ser. This segment exhibits general similarity to a sequence found in the sugar binding site of the arabinose binding protein of *E. coli* whose structure has been refined to 1.7-Å resolution (Quiocho & Vyas, 1984). Furthermore, Lys335 and Ser339 have recently been subjected to site-directed mutagenesis, and the mutated permeases, like Y336F permease, exhibit decreased affinity for NPG (P. D. Roepe and H. R. Kaback, unpublished experiments). Thus, we conclude tentatively that Tyr336 may also be a component of a substrate binding site.

Y382F permease catalyzes active lactose transport at about 30–40% the rate of wild-type permease but eventually accumulates the disaccharide against a concentration gradient to essentially the same extent as wild-type permease. Since the stoichiometry between lactose and H^+ translocation is 1:1 and the steady-state level of lactose accumulation is in equilibrium with the H^+ electrochemical gradient (Zilberstein et al., 1979; Felle et al., 1980), the observation that Y382F permease catalyzes lactose accumulation to a normal steady-state argues strongly that the mutated permease couples lactose and H^+ translocation normally. Therefore, it seems more likely that Y382F permease catalyzes lactose/H^+ symport with a stoichiometry of 1:1, but with a reduced turnover number. Interestingly, Y382F permease catalyzes equilibrium exchange essentially as well as wild-type permease, indicating that translocation of fully loaded permease is not impaired. Since the mutant appears to be coupled and catalyzes equilibrium exchange, it seems reasonable to conclude that a defect in the translocation of the unloaded permease is responsible for the reduced rate of transport observed.

Finally, it is noteworthy that of the 10 nonessential Tyr residues identified in the present work, only 4 are conserved in the sequence of the *lac* permease of *Klebsiella pneumoniae* (McMorrow et al., 1988). Moreover, of the four Tyr residues found to be essential for activity, each is conserved in the *Klebsiella* permease.

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Registry No. L-Tyr, 60-18-4; H^+, 12408-02-5; *lac* permease, 9033-40-3; lactose, 63-42-3; *p*-nitrophenyl α-D-galactopyranoside, 7493-95-0.

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