Substitution of Glutamine-60 with Glutamic Acid Causes the *lac* Permease of *Escherichia coli* To Become Temperature Sensitive

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ABSTRACT: The *lac Y* gene of *Escherichia coli* was modified by oligonucleotide-directed, site-specific mutagenesis so that Gln-60 is replaced with Glu. Although the replacement introduces a negative charge into a putative hydrophobic, transmembrane α-helical segment of the *lac* permease, lactose/H^+ symport is unimpaired. However, the modified permease is more susceptible to heat inactivation. That is, upon incubation at 45 °C, Glu-60 permease loses activity with a $t_{1/2}$ of 20 min relative to a $t_{1/2}$ of 50 min with wild-type permease.

The H^+/β-galactoside symport (cotransport) in *Escherichia coli* is catalyzed by the *lac* permease, a transmembrane protein encoded by the *lac Y* gene (Kaback, 1983, 1985, 1986; Overath & Wright, 1983). The *lac Y* gene has been cloned (Teather et al., 1978) and sequenced (Büchel et al., 1980), and the permease has been purified to a single polypeptide species, reconstituted into proteoliposomes, and shown to be completely functional (Viitanen et al., 1984). It is apparent, therefore, that the product of the *lac Y* gene is solely responsible for β-galactoside transport.

On the basis of circular dichroic measurements with purified *lac* permease and a hydropathic analysis of amino acid sequence, as deduced from the DNA sequence of *lac Y*, a secondary structure model has been proposed in which the polypeptide is organized into 12 hydrophobic, α-helical segments that traverse the membrane in a zig-zag manner connected by hydrophilic charged segments (Foster et al., 1983; Kaback, 1985). Although largely unproven, preliminary support for certain aspects of the model has been provided by proteolysis experiments and antibody binding studies with right-side-out (RSO)[1] and inside-out membrane vesicles.

If a specific transmembrane α-helix in the permease is important for activity, introduction of a charged residue into that stretch of hydrophobic amino acids should alter the activity of the protein due to a secondary structural alteration. By utilizing oligonucleotide-directed, site-specific mutagenesis of *lac Y* (Sarkar et al., 1986), Gln-60 has been replaced with Glu, thereby introducing a negatively charged amino acid residue into the second putative transmembrane α-helix of the proposed model. Cells harboring the mutated gene in a recombinant plasmid catalyze active lactose transport as well as cells harboring wild-type *lac Y* in the plasmid. However, permease with Glu in place of Gln-60 is significantly more unstable than the wild-type molecule at 45 °C.

Materials and Methods

The following reagents were purchased from commercial sources: T4 DNA ligase, bacterial alkaline phosphatase, *Eco*RI, *Hin*dIII, *Hin*cII, *Taq*I, and dideoxynucleotide sequencing materials were from BRL; [γ-^{32}P]ATP, [α-^{32}P]-dATP, [1-^{14}C]lactose, ^{125}I-protein A, and T4 polynucleotide kinase were from Amersham; DNA polymerase I (Klenow fragment) was from BRL, Boehringer/Mannheim, and New England Biolabs; nitrocellulose (BA85) and Elutip-d columns were from S & S; Sea Plaque and Sea Kem agarose were from FMC. All other chemicals were reagent grade obtained from commercial sources as described (Sarkar et al., 1986).

Bacterial Strains. The following strains of *E. coli* K12 were used: JM101 (Δ*lacpro*, *supE*, *thi*/*F'*, *traD36*, *proAB*, *lacI*^{q}*ZΔM15*) (Messing, 1983); T206 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^+$(*A*$^+$), *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR*/*F'*, *lacI*^{q}*O*$^+$*Z*^{U118} (*Y*$^+$*A*$^+$)] harboring plasmid pGM21 [lacΔ(*I*)*O*$^+$*P*$^+\Delta$(*Z*)*Y*$^+\Delta$(*A*), *tet*^{r}] (Teather et al., 1980); T184 (T206 cured of plasmid pGM21) (Teather et al., 1980); HB101 [*hsdS20* (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(*Sm*^{r}), *xyl-5*,*mtl-1*, *supE44*, λ^-/*F*$^-$] (Boyer & Roulland-Dussoix, 1969).

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis was carried out essentially as described (Zoller & Smith, 1983) with the following modifications (Sarkar et al., 1986). An 18-base oligodeoxynucleotide (5'-GCGGTTC*GAATAATAGCG-3') was synthesized and used as the mutagenic primer. A 16-base oligodeoxynucleotide primer (hybridization probe primer, New England BioLabs) was used in addition to the mutagenic primer to increase the yield of closed-circular (cc) heteroduplex DNA. The single-stranded (ss) template used for mutagenesis was M13mp19 DNA containing the antisense strand of the 2.3 kbp *Eco*RI restriction fragment from the plasmid pGM21 which contains the *lac Y* gene. Enrichment for cc heteroduplex was carried out either by alkaline sucrose density gradient centrifugation (Zoller & Smith, 1983) or by acid–phenol extraction (Zasloff et al., 1978).

Phage harboring the mutation were identified initially by dot-blot hybridization using the ^{32}P-labeled mutagenic primer, plaque purified, and the replicative form (RF) was isolated from *E. coli* JM101 by alkaline lysis (Maniatis et al., 1982). Since the base change described introduces a new *Taq*I site into *lac Y*, the mutation was verified further by *Taq*I restriction enzyme analysis of the RF DNA using the manufacturer's protocol. Finally, the mutation was confirmed by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) using a synthetic sequencing primer.

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient across the membrane; kbp, kilobase pair(s); ss, single stranded; cc, closed circular; RF, replicating form; RSO, right side out; PMS, phenazine methosulfate; Mab, monoclonal antibody; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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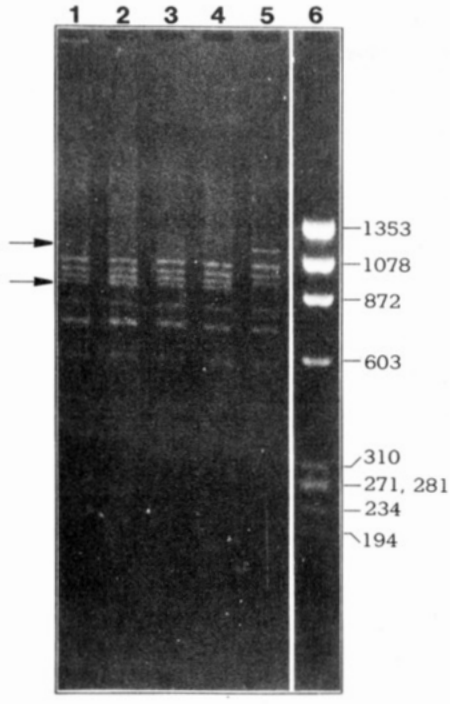

FIGURE 1: *Taq*I restriction enzyme analysis of the recombinant wild-type and mutant mp19-*lacY* RF DNAs. Recombinant RF DNA was prepared by an alkaline lysis procedure (Maniatis et al., 1982). Five microliters of each RF DNA was incubated with 3.5 units of *Taq*I restriction enzyme at 65 °C for 2.5 h in a total volume of 50 μL of 1× core buffer [BRL 10× core buffer: 500 mM Tris·HCl (pH 8.0)/100 mM $MgCl_2$/500 mM NaCl] and electrophoresed on a 2% agarose gel containing 0.8 μg/mL ethidium bromide. Lanes 1–4 are *Taq*I-digested RF DNA from four randomly selected mutants which were positive on dot-blot hybridization; lane 5 is *Taq*I-digested mp19-*lacY* wild-type recombinant RF DNA, and lane 6 contains φX174 RF DNA/*Hae*III fragments (molecular weight markers, BRL); fragment sizes of the markers are indicated in base pairs. The top arrow in the left-hand side of the figure indicates the *Taq*I-restricted fragment of the wild-type (lane 5) DNA that is absent in all of the *Taq*I-restricted mutant RF DNAs (lanes 1–4). The appearance of a new restriction fragment in the mutants is shown by the lower arrow (lanes 1–4).

The mutated 2.3 kbp *lac Y* fragment was cloned into the original vector pACYC184, and *E. coli* T184 was transformed with the recombinant plasmid as described (Sarkar et al., 1986; Trumble et al., 1984). Recombinant plasmids containing *lac Y* were then isolated from several transformed cells, and the orientation of the mutant *lac Y* was determined by *Hinc*II restriction enzyme analysis (Sarkar et al., 1986). Plasmids in which the orientation of the mutated 2.3 kbp fragment is identical with that in pGM21 were selected (Teather et al., 1980).

Growth of Cells and Membrane Preparation. *E. coli* T206, T184, and E60 (T184 harboring the recombinant plasmid pE60) were grown and induced with isopropyl 1-thio-β-D-galactopyranoside as described (Teather et al., 1980). RSO vesicles were prepared by osmotic lysis (Kaback, 1971; Short et al., 1975).

Lactose Transport. Respiration-driven accumulation of [1-^{14}C]lactose (0.3 mM final concentration; 59 mCi/mmol) was measured in RSO membrane vesicles with reduced phenazine methosulfate (PMS) as the electron donor (Kaback, 1974).

Immunochemical Assays. To quantitate the amount of permease in the membrane of transformed cells, binding studies were performed on RSO membrane vesicles with ^{125}I-labeled monoclonal antibody (Mab) 4B1 (Carrasco et al., 1986; Herzlinger et al., 1985). Alternatively, the amount of

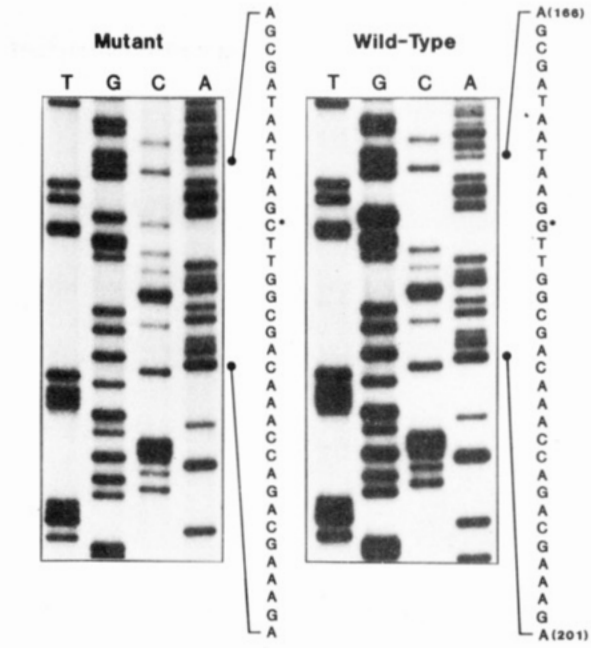

FIGURE 2: Dideoxynucleotide sequencing of recombinant M13mp19 ssDNA containing wild-type or mutant *lac Y* (Gln-60 → Glu). Sequencing was performed as described (Sanger et al., 1977; Sanger & Coulson, 1978) using a synthetic primer (′5-TAAAAC-CAAAGCCTAGAT-3′) that is complementary to base pairs 338–355 of the *lac Y* antisense strand. The autoradiograph shows the sequence in the region of the mutation with asterisks denoting the base change.

permease was determined by immunoblotting with anti-carboxyl terminus antibody and ^{125}I-labeled protein A (Carrasco et al., 1986; Herzlinger et al., 1985).

Protein Determinations. Protein was assayed according to Lowry et al. (1951) using crystalline bovine serum albumin as standard.

RESULTS

*Taq*I restriction enzyme analysis of RF DNA from four randomly selected mutants that were positive on dot-blot hybridization demonstrates that a new *Taq*I site has been created (Figure 1). Furthermore, dideoxynucleotide sequencing of wild-type and mutant ssDNA in the region of the mutation provides clear evidence that G at position 178 of the wild-type *lac Y* has been changed to C, thereby changing the codon for Gln at residue 60 of the *lac* permease (CAA) to a Glu codon (GAA) (Figure 2).

Respiration-driven lactose transport in RSO membrane vesicles from *E. coli* T206 and E60 is shown in Figure 3. In the absence of electron donors, lactose transport proceeds slowly, and negligible amounts of the disaccaride are accumulated over the time course of the experiment. However, when $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) is generated by means of reduced PMS oxidation (Ramos et al., 1976; Ramos & Kaback, 1977a,b), both the initial rate and steady-state level of lactose accumulation increase dramatically, and, importantly, E60 vesicles catalyze transport as well as T206 vesicles within experimental error. Although data will not be shown, it is also notable that the amount of *lac* permease in the membrane of E60 is comparable to that in the membrane of T206, as judged by direct binding assays with Mab 4B1 and by immunoblot analyses with anti-carboxyl terminus antibody and ^{125}I-labeled protein A.

Since insertion of a negatively charged amino acid residue into a hydrophobic, transmembrane α-helix would be expected to represent a destabilizing influence, permease activity (i.e., the initial rate of transport) was measured in T206 and E60

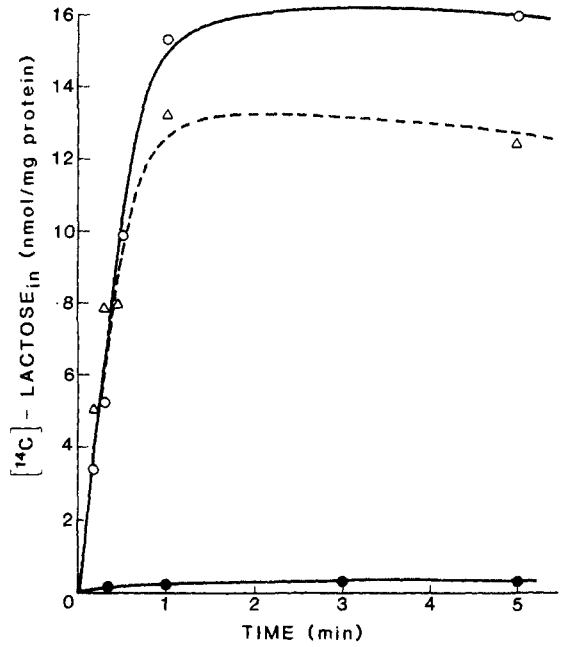

FIGURE 3: Time course of [^{14}C]lactose uptake in T206 and E60 RSO membrane vesicles. Aliquots (50 μL) of T206 (○) or E60 (△) vesicles containing 300 μg of protein in 50 mM potassium phosphate (pH 6.6)/10 mM $MgSO_4$ were assayed for [^{14}C]lactose transport at 25 °C in the presence of 20 mM ascorbate and 1 mM PMS under oxygen. Reactions were initiated by the addition of [^{14}C]lactose (59 mCi/mmol) to a final concentration of 0.3 mM and terminated by the addition of 3 mL of 100 mM potassium phosphate (pH 7.5)/100 μM lithium chloride/2 mM mercuric chloride. Samples were then filtered immediately on nitrocellulose filters (0.45 μm, Whatman) and washed once with the same solution. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. (●) Transport in the absence of ascorbate/PMS.

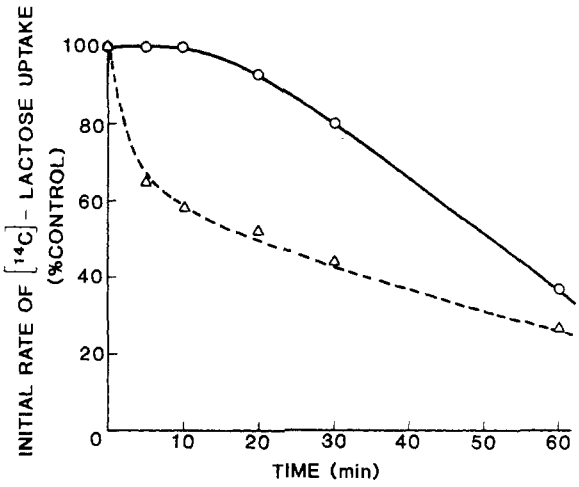

FIGURE 4: Heat inactivation of the initial rate of lactose uptake in T206 (○) and E60 (△) membrane vesicles. Vesicles were incubated at 45 °C, and aliquots were withdrawn at a given time and kept on ice. Samples were then assayed for lactose transport as described under Figure 3. Initial rates of lactose uptake were calculated from the early linear portion of the uptake curves of each assay.

vesicles after given periods of incubation at 45 °C (Figure 4). As shown, the initial rate of lactose transport in T206 vesicles decreases slowly, and after about 50 min, half of the activity remains (i.e., $t_{1/2} \approx 50$ min). With E60 vesicles, on the other hand, activity decreases relatively rapidly, exhibiting a $t_{1/2}$ of about 20 min.

DISCUSSION

The second transmembrane α-helical segment in the putative secondary structure model of the *lac* permease is (Foster et al., 1983; Kaback, 1985) H_2N...Ile^{47}-Ile-Phe-Ala-Ala-Ile-Ser-Leu-Phe-Ser-Leu-Leu-Phe-Gln^{60}-Pro-Leu-Phe-Gly-Leu-Leu-Ser...COOH. The segment is 21 amino acid residues in length, and 16 of the 21 residues are unequivocally hydrophobic in nature. Since the folding of proteins into defined three-dimensional structures is thought to be dependent on the primary amino acid sequence (Anfinsen, 1973) and is generally considered to be a prerequisite for function, it seems highly unlikely that Gln-60 is at or near the active site(s) of the permease because replacement of this residue with Glu has no effect on catalytic activity. Furthermore, it should be stressed that this conclusion holds regardless of the ultimate veracity of the secondary structure model.

Neither Gln nor Glu is found commonly in the interior of proteins, presumably because of the energetic cost of burying such residues in a nonaqueous environment (Engelman & Steitz, 1981). The enhanced hydrogen-bonding capability of the amino function together with the carbonyl group in Gln is consistent with the expectation of a high energy requirement for transfer from water to a nonaqueous environment. Similarly, with Glu, energy is required to protonate the carboxyl group and to remove the protonated group from contact with the aqueous environment. Thus, replacement of Gln-60 with Glu may not alter the folding of the permease in so gross a manner as to affect catalytic activity because the energy required to bury either Gln or Glu may be comparable. On the other hand, exchange of a charged residue such as Glu for an uncharged residue such as Gln might well affect thermal stability if substitution takes place in a hydrophobic region of the protein (Hecht et al., 1984; Fermi & Perutz, 1981). In other words, the finding that replacement of Gln-60 with Glu leads to decreased heat stability of the permease is consistent with the notion that the residue is buried in a hydrophobic environment.

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Optically Detected Magnetic Resonance Studies of Porcine Pancreatic Phospholipase A_2 Binding to a Negatively Charged Substrate Analogue†

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ABSTRACT: The direct binding of porcine pancreatic phospholipase A_2 and its zymogen to 1,2-bis(heptanylcarbamoyl)-*rac*-glycerol 3-sulfate was studied by optical detection of triplet-state magnetic resonance spectroscopy in zero applied magnetic field. The zero-field splittings of the single Trp^3 residue undergo significant changes upon binding of phospholipase A_2 to lipid. Shifts in zero-field splittings, characterized mainly by a reduction of the *E* parameter from 1.215 to 1.144 GHz, point to large changes in the Trp^3 local environment which accompany the complexing of phospholipase A_2 with lipid. This may be attributed to Stark effects caused by the binding of a charged group near Trp^3 in the enzyme–lipid complex. The cofactor, Ca^{2+}, which is strongly bound to the enzyme active site, has an influence on the bonding, as reflected by smaller zero-field splitting shifts. A relatively small change in the Trp environment was observed for the interaction of the zymogen with lipid.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides. The pancreatic enzymes have an absolute requirement for Ca^{2+} ions, which bind to the active site (Dijkstra et al., 1981a). In mammals PA_2[1] is secreted by the pancreas as its zymogen, $proPA_2$, which is converted subsequently to the active enzyme by trypsin, resulting in the loss of a heptapeptide from the N-terminal part (de Haas et al., 1968b). Although both PA_2 and $proPA_2$ are able to catalyze the hydrolysis of molecularly dispersed substrates at a fairly slow rate, only the active enzyme becomes superactivated in the presence of certain organized lipid–water interfaces (Volwerk & de Haas, 1982). Regardless of the various hypotheses, no generally accepted explanation has been given to date for the extremely effective heterogeneous catalysis. A particular surface region of PA_2 has been postulated as an interface recognition site, which is distinct from the active site but is important nonetheless for binding of the protein to the organized lipid substrate.

In order to identify the amino acid side chains of PA_2 involved in the recognition of, and interaction with, lipid–water interfaces, numerous direct binding studies have been carried out with micellar solutions of nondegradable substrate analogues (Volwerk & deHaas, 1982). Mostly neutral phosphocholine-containing lipids have been employed in order to

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; C_{16}-PN, *n*-hexadecylphosphocholine; cmc, critical micellar concentration; diC_7-dicarbamoyl-GS, 1,2-bis(heptanylcarbamoyl)-*rac*-glycerol 3-sulfate sodium salt; D-diC_7GS, 2,3-diheptanoyl-*sn*-glycerol 1-sulfate sodium salt; D-diC_8GS, 2,3-dioctanoyl-*sn*-glycerol 1-sulfate sodium salt; EDTA, ethylenediaminetetraacetic acid; ODMR, optical detection of triplet-state magnetic resonance; PA_2, porcine pancreatic phospholipase A_2; $proPA_2$, porcine pancreatic prophospholipase A_2; SDS, sodium *n*-dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; zfs, zero-field splitting(s).

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