Role of Proline Residues in the Structure and Function of a Membrane Transport Protein

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ABSTRACT: By use of site-directed mutagenesis, each prolyl residue in the *lac* permease of *Escherichia coli* at positions 28 (putative helix I), 31 (helix I), 61 (helix II), 89 (helix III), 97 (helix III), 123 (helix IV), 192 (putative hydrophilic region 7), 220 (helix VII), 280 (helix VIII), and 327 [helix X; Lolkema, J. S., et al. (1988) *Biochemistry 27*, 8307] was systematically replaced with Gly, Ala, or Leu or deleted by truncation of the C-terminus [i.e., Pro403 and Pro405; Roepe, P. D., et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3992]. Replacements were chosen on the basis of side-chain helical propensity: Gly, like Pro, is thought to be a "helix breaker", while Ala and Leu are "helix makers". With the exception of Pro28, each prolyl residue can be replaced with Gly or Ala, and Pro403 and -405 can be deleted with the C-terminal tail, and significant *lac* permease activity is retained. In contrast, when Pro28 is replaced with Gly, Ala, or Ser, lactose transport is abolished, but permease with Ser28 binds *p*-nitrophenyl α-D-galactopyranoside and catalyzes active transport of β-galactopyranosyl-1-thio-β-D-galactopyranoside. Replacement of Pro28, -31, -123, -280, or -327 with Leu abolishes lactose transport, while replacement of Pro61, -89, -97, or -220 with Leu has relatively minor effects. None of the alterations in permease activity is due to inability of the mutant proteins to insert into the membrane or to diminished lifetimes after insertion, since the concentration of each mutant permease in the membrane is comparable to that of wild-type permease as judged by immunological analyses. The results indicate that (i) Pro28 is important for substrate binding and recognition, (ii) it is primarily the hydrophobicity and/or size of the side chain at the other positions that is important for *lac* permease activity, and (iii) neither cis/trans isomerization of prolyl residues nor the presence of kinks at these positions is important for membrane insertion, stability, or substrate translocation.

The *lac* permease of *Escherichia coli* is a prototypic membrane transport protein that catalyzes cotransport of a single β-galactoside molecule with a single H^+ (i.e., lactose/H^+ symport) [cf. Kaback (1983, 1989, 1990) and Wright et al. (1986) for reviews]. Encoded by the *lacY* gene which has been cloned and sequenced, this hydrophobic polytopic cytoplasmic membrane protein has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and demonstrated to be solely responsible for β-galactoside transport as a monomer. On the basis of circular dichroic measurements and hydropathy analysis of the primary sequence of the permease (Foster et al., 1983), a secondary structure model has been proposed in which the protein consists of 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion, connected by hydrophilic segments with the N- and C-termini on the cytoplasmic surface (cf. Figure 1). The general features of the model are consistent with other spectroscopic measurements (Vogel et al., 1985),[1] chemical modification (Page & Rosenbusch, 1988), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1984, 1986; Herzlinger et al., 1984). Furthermore, recent studies (Calamia & Manoil, 1990) on a large number of *lac* permease–alkaline phosphatase (*lacY–phoA*) fusion proteins have provided strong exclusive support for the topological predictions of the 12-helix model.

Since prolyl residues are thought to play an important role in the structure and function of many proteins and because certain amino acid residues in putative helices IX and X of *lac* permease are important for lactose/H^+ symport and/or substrate recognition (Padan et al., 1985; Menick et al., 1987; Carrasco et al., 1986, 1989; Püttner et al., 1986, 1989; Lee et al., 1989; Collins et al., 1989), Pro327 in putative helix X was subjected to site-directed mutagenesis (Lolkema et al., 1988). Permease with P327A[2] catalyzes active transport in a manner indistinguishable from wild-type permease, permease with P327G exhibits about one-tenth the initial rate of wild-type permease but catalyzes lactose accumulation to the same steady-state level as wild-type permease, and permease with P327L is inactive.[3] Thus, there is no relationship between permease activity and the helix-breaking (Pro and Gly) or

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[1] In addition to circular dichroic and laser Raman spectroscopy, Fourier-transform infrared studies also show that purified *lac* permease is largely helical (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished work).

[2] Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in wild-type *lac* permease. The sequence is followed by a second letter denoting the amino acid replacement at this position.

[3] Additional site-directed replacements for Pro327 demonstrate that P327S permease has about 47% of the specific activity of wild-type permease, P327T about 8%, and P327C about 4%, and permeases with Val or Ile in place of Pro327 are essentially devoid of activity (J. S. Lolkema, and H. R. Kaback, unpublished work).

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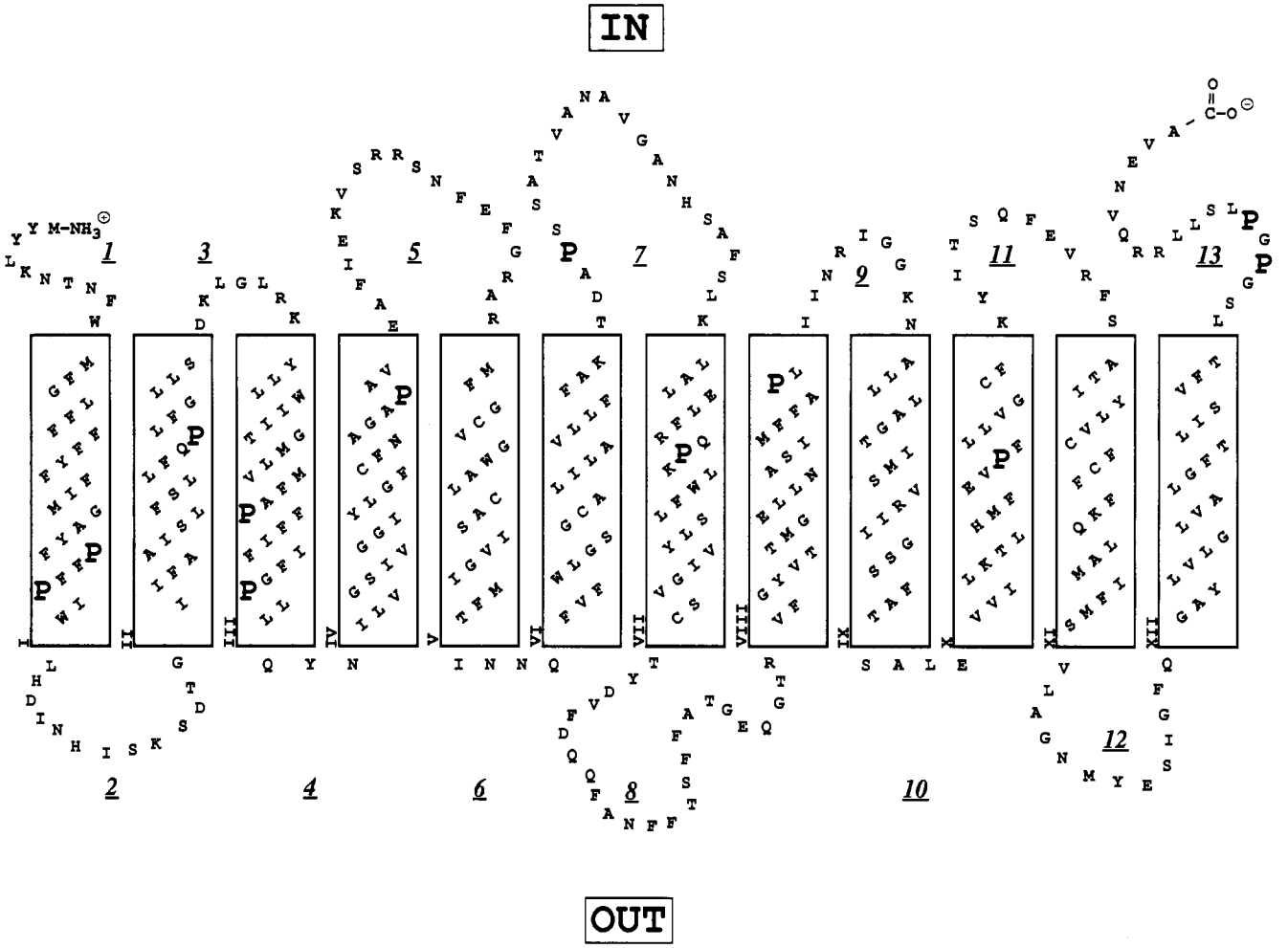

FIGURE 1: Secondary structure model of *lac* permease showing the positions of Pro residues. The model is based on the hydropathy of the deduced amino acid sequence (Foster et al., 1983) with prolyl residues (P) highlighted. The single-letter amino acid code is used.

helix-making (Ala and Leu) properties of the residue at position 327, and the suggestion was made that it is a specific chemical property of the side chain at position 327, rather than cis/trans isomerization of Pro327 or the presence of a kink, that is important. Subsequently, it was demonstrated (Roepe et al., 1989a) that the C-terminal tail of the permease, which contains Pro at positions 403 and 405, can be deleted with no significant effect on either permease activity or the lifetime of the protein.

In this paper, we describe the systematic replacement of each of the remaining nine Pro residues in *lac* permease with Gly, Ala, or Leu. With the exception of Pro192, which is located in putative hydrophilic region 7, each of the other prolyl residues is located in a putative transmembrane helix (Figure 1). Although Pro28 appears to be important for substrate binding and recognition, none of the prolyl residues is essential for membrane insertion, stability, or substrate translocation.

Experimental Procedures

Materials

[1-^{14}C]Lactose and β-D-[^{3}H]galactopyranosyl-1-thio-β-D-galactopyranoside (TDG)[4] were synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group, Hoffmann-La Roche, Inc.). ^{125}I-Labeled protein A was purchased from ICN, [α-^{35}S]dATP from Amersham, and [γ-^{32}P]ATP from New England Nuclear. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) and calf intestine alkaline phosphatase were from Boehringer-Mannheim Biochemicals. Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by polyacrylamide gel electrophoresis or with oligonucleotide purification cartridges (trityl affinity columns). *Ava*I, *Eco*RI, and *Hin*cII restriction endonucleases, Klenow DNA polymerase, polynucleotide kinase, T4 DNA ligase, and T4 DNA polymerase were from New England Biolabs. Sequenase (modified T7 DNA polymerase) and Sequenase reaction kits were from United States Biochemical Corp. Gene-Clean kits were purchased from Bio101. Nitrocellulose membranes (0.2 μm, BA85/20) were from Schleicher & Schuell, and glass microfiber filters (GF/F) were from Whatman. 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,*(*lac-proAB*), [F′*traD36,proA*$^{+}$*B*$^{+}$,-*lacI*^{q}*ZΔ15*] (Yanish-Perron et al., 1985); BMH71-18 *mutL*, Δ(*lac-pro*), *supE,thi*/*proA*$^{+}$*B*$^{+}$,*lacI*^{q}*ZΔM15*/*MutL*::*Tn10* (Kramer et al., 1984); T206 [*lacI*$^{+}$*O*$^{+}$*Z*$^{-}$*Y*$^{-}$(*A*),*rpsL*,*met*$^{-}$, *thr*$^{-}$,*recA*,*hsdM*,*hsdR*/F′, *lacI*^{q}*O*$^{+}$*Z*^{U118}(*Y*$^{+}$*A*$^{+}$)] harboring plasmid pGM21 [*lacΔ*(*I*)*O*$^{+}$*P*$^{+}$Δ(*Z*)*Y*$^{+}$Δ(*A*),*tet*^{r}] (Teather et al., 1980); T184 (T206 cured of plasmid pGM21) (Teather et al., 1980); HB101, *hsdS20* (*r*$^{-}_{B}$, *m*$^{-}_{B}$), *recA13*,*ara*$^{-}$*14*,

[4] Abbreviations: TDG, β-D-galactopyranosyl-1-thio-β-D-galactopyranoside; IPTG, isopropyl 1-thio-β-D-galactopyranoside; EMB, eosin methylene blue; KP_i, potassium phosphate; TBST, 10 mM Tris-HCl/0.9% sodium chloride/0.2% Triton X-100; NPG, nitrophenyl α-D-galactopyranoside.

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

mutant	mutagenic oligonucleotide[a]	codon change
P28A	3′-CGGATGAAG**CGC**AAAAAGGGC-5′	CCG → GCG
P28G	3′-CGGATGAAG**CCC**AAAAAGGGC-5′	CCG → GGG
P28L	3′-CGGATGAAG**GAC**AAAAAGGGC-5′	CCG → CUG
P28S	3′-CGGATGAAG**AGC**AAAAAGGGC-5′	CCG → UCG
P31A	3′-GGCAAAAAG**CGC**TAAACCGAT-5′	CCG → GCG
P31G	3′-GGCAAAAAG**CCC**TAAACCGAT-5′	CCG → GGG
P31L	3′-GGCAAAAAG**GAC**TAAACCGAT-5′	CCG → CUG
P61A	3′-AATAAGGTT**CGC**GACAAACCA-5′	CCG → GCG
P61G	3′-AATAAGGTT**CCC**GACAAACCA-5′	CCG → GGG
P61L	3′-AATAAGGTT**GAC**CGACAAACCA-5′	CCG → CUG
P89A	3′-TACAAACGC**CGC**AAGAAATAA-5′	CCG → GCG
P89G	3′-TACAAACGC**CCC**AAGAAATAA-5′	CCG → GGG
P89L	3′-TACAAACGC**GAC**AAGAAATAA-5′	CCG → CUG
P97A	3′-ATAGAAGCCC**CGT**GACAATGTT-5′	CCA → GCA
P97G	3′-ATAGAAGCCC**CCT**GACAATGTT-5′	CCA → GGA
P97L	3′-ATAGAAGCCC**GAT**GACAATGTT-5′	CCA → CUA
P123A	3′-CGGCCACGC**CGT**CGTCATCTC-5′	CCA → GCA
P123G	3′-CGGCCACGC**CCT**CGTCATCTC-5′	CCA → GGA
P123L	3′-CGGCCACGC**GAT**CGTCATCTC-5′	CCA → CUA
P192A	3′-TGCCTACGC**CGG**AGAAGACGG-5′	CCC → GCC
P192G	3′-TGCCTACGC**CCG**AGAAGACGG-5′	CCC → GGC
P192L	3′-TGCCTACGC**GAG**AGAAGACGG-5′	CCC → CUC
P220A	3′-AAGTCTGTC**CGT**TTTGACACC-5′	CCA → GCA
P220G	3′-AAGTCTGTC**CCT**TTTGACACC-5′	CCA → GGA
P220L	3′-AAGTCTGTC**GAT**TTTGACACC-5′	CCA → CUA
P280A	3′-AAGAAACGC**CGT**GACTAGTAA-5′	CCA → GCA
P280G	3′-AAGAAACGC**CCT**GACTAGTAA-5′	CCA → GGA
P280L	3′-AAGAAACGC**GAT**GACTAGTAA-5′	CCA → CUA

[a] Sequences of mutagenic primers used to replace Pro codons at given positions in the *lacY* gene. In each case, the mutagenized codon is underlined, and the bases changed are in boldface type.

proA2, *lacY1*, *galK2*, *rpsL20*(*Sm*^{r}), *xyl-5*, *mtl-1*, *supE44*, λ^-F^- (Boyer & Roulland-Dussoix, 1969); CJ236, *dut-1*, *ung-1*, *thi-1*, *relA-1* harboring plasmid pCJ105 (*Cm*^{r}); and MV1190, Δ(*lac-proAB*),*thi*, *supE*, Δ(*srl-recA*)*306*::*Tn10* (*tet*^{r}) [F′: *traD36*, *proAB*, *lacI*^{q}*Z*Δ*M15*] (Bio-Rad Laboratories, Muta-Gene Mutagenesis Kit).

Oligonucleotide-Directed Site-Specific Mutagenesis. All site-specific mutations were directed by synthetic oligodeoxynucleotide primers complementary to the antisense strand of the *lacY* gene, except for given base changes. In general, the length of the primers was 21 nucleotides, and the region of the mismatch was in the center of the sequence. Sequences of the primers used are summarized in Table I.

Mutations at position 31 were made essentially by the methods described by Sarkar et al. (1986); all other mutations were made by using the method of Kunkel (1985). Briefly, phosphorylated mutagenic primer was annealed to single-stranded M13mp19 DNA into which a 2.3-kb fragment containing *lacY* had been cloned. Single-stranded template DNA was isolated from the supernatant of M13mp19(*lacY*)-infected *E. coli* JM101 (for mutations at position 31) or from *E. coli* CJ236 (*dut*$^-$,*ung*$^-$). The latter template which had uracil incorporated in place of thymine allows selective propagation of the mutant strand after extension, ligation, and transfection of *E. coli* MV1190 (*dut*$^+$,*ung*$^+$). The primer was extended in vitro using the Klenow fragment of DNA polymerase (for mutations at position 31) or with T4 DNA polymerase and ligated with T4 DNA ligase. *E. coli* JM101 (for mutations at position 31) or MV1190 was transfected with the resultant double-stranded DNA. Transfectants were picked from plaques and screened either by colony-blot hybridization using 3′-^{32}P-labeled mutagenic primer (for mutations at position 31) and/or by dideoxynucleotide sequencing of single-stranded DNA isolated from the supernatant of phage-infected MV1190.

The replicative form of M13mp19(*lacY*) was isolated from transfectants that screened positive for a given mutation, and the DNA fragment containing *lacY* was restricted with *Eco*RI, purified by agarose gel electrophoresis, treated with "glass milk" (Gene Clean), and ligated into pACYC184 that had been linearized with *Eco*RI and treated with calf intestine alkaline phosphatase. The resulting plasmids were then used to transform *E. coli* HB101 (Z^+Y^-), and as a qualitative assay of permease activity, the transformants were plated on indicator medium containing eosin methylene blue (EMB) and 25 mM lactose (Padan et al., 1985). Selected colonies were grown overnight on Luria broth, and plasmid DNA was isolated by alkaline lysis. Orientation of *lacY* was assayed by restriction analysis with *Hin*cII (Sarkar et al., 1986), and only those plasmids containing mutated *lacY* genes with the same orientation as wild-type *lacY* in pGM21 were used for further study.

All mutations were verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) of single-stranded M13mp19 phage DNA containing the mutated *lacY* genes. Furthermore, with all of the mutants that exhibited defective transport activity, the entire *lacY* gene was sequenced in order to ensure the absence of other unwanted mutations; otherwise, only the region in the immediate vicinity of the mutation was sequenced. As a final check, the deleterious mutations were verified by sequencing double-stranded plasmid DNA in the region of the mutations.

Membrane Vesicles. Right-side-out membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975).

Transport Assays. Active transport of lactose was measured in *E. coli* T184 (Z^-Y^-) transformed with each of the plasmids described. Cells were grown as described (Teather et al., 1980) from overnight cultures to an OD_{420} of 0.5–0.75 (3–4 h). IPTG was added to a final concentration of 0.2 mM, and the cells were grown for an additional 90 min. Cells were harvested by centrifugation, washed in ice-cold 100 mM potassium phosphate (KP_i, pH 7.5)/10 mM magnesium sulfate, concentrated by centrifugation, diluted to an OD_{420} of 10.0 in the same solution (ca. 0.7 mg of protein/mL), and kept on ice until use. Individual aliquots (50 μL) of the cell suspension were used for each time point, and the reactions were carried out at room temperature in plastic tubes. The assay was initiated by addition of [1-^{14}C]lactose (10 mCi/mmol) to a final concentration of 0.4 mM and stopped at given times by addition of 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM lithium chloride, followed by rapid filtration through glass fiber filters (Whatman GF/F). The filters were washed once with stop solution, and radioactivity trapped on the filters was determined by liquid scintillation spectrometry. Nonspecific adsorption of radioactivity was determined by adding stop solution to a cell suspension prior to addition of radioactive lactose, followed by rapid filtration and washing as described. All transport assays with cells expressing mutant permeases were carried out in parallel with cells harboring pACYC (the plasmid expression vector with no *lacY* insert) and with cells harboring pGM21 (pACYC containing wild-type *lacY*) as negative and positive controls, respectively.

Immunological Analyses. An aliquot of the same cell suspension used for transport assays was subsequently used for analysis of the level of expression of *lac* permease by using either Western blots (Carrasco et al., 1982) or immunodot blots (Lolkema et al., 1988). For both procedures, cells were concentrated 10-fold by centrifugation to an OD_{420} of 100 (ca.

7 mg of protein/mL). For Western blot analysis, a 50-μL aliquot of the suspension was sonicated and solubilized in an equal volume of 2 times concentrated sample buffer (Laemmli, 1970) which contained 6% sodium dodecyl sulfate ($NaDodSO_4$) and 200 mM dithiothreitol instead of 2-mercaptoethanol, and incubated at 37 °C for 1–2 h. Equal volumes of each sample were electrophoresed through 12% polyacrylamide gels containing 0.1% $NaDodSO_4$, and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, BA85/20) by electroblotting. For immunodot-blots, various volumes (1–5 μL) of the cell suspensions were applied in triplicate to the nitrocellulose membrane and air-dried. The cells were then lysed by placing the membrane in contact (10 min) with filter paper presoaked in 0.5 N sodium hydroxide/1% $NaDodSO_4$. The membrane was neutralized by placing it in contact with filter paper soaked with 1.0 M Tris-HCl (pH 7.4) and air-dried. The nitrocellulose membranes were then treated similarly for either technique. They were first rinsed with 10 mM Tris-HCl/0.9% sodium chloride/0.2% Triton X-100 (TBST) for 20 min and then incubated for 1 h in TBST containing 5% bovine serum albumin to block nonspecific binding sites. Mouse ascites fluid (0.4 mL) containing monoclonal antibody 4A10R was added to the blocking solution; the membrane was incubated for 2 h, washed 3 times with TBST for 20 min each, and incubated again in TBST containing 5% bovine serum albumin for 1 h. ^{125}I-Labeled protein A (25 μL containing 3.7 MBq/mL) was added; the membrane was incubated for 2 h, washed as described above, air-dried, and subjected to autoradiography for visualization. In addition, spots detected by immunodot-blot were excised and assayed for radioactivity in a γ counter. In all cases, samples from cells harboring pACYC and pGM21 were used as negative and positive controls, respectively.

Flow Dialysis. Flow dialysis was carried out with right-side-out membrane vesicles under nonenergized conditions as described by Rudnick et al. (1976).

Protein Determinations. Protein was assayed either by the method of Schaffner and Weissmann (1973) or the method of Lowry et al. (1951).

Results

Verification of Mutations by DNA Sequencing. The *lacY* gene in each plasmid used was cloned initially from pGM21 into M13m19 replicative form DNA, and single-stranded phage DNA was isolated and used as template for site-directed mutagenesis. Subsequently, single-stranded phage DNA containing mutated *lacY* was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with appropriate primers complementary to regions of *lacY* 50–100 bases downstream from the mutations. In addition, each of the *lacY* mutations encoding Pro28, Pro31, Pro123, and Pro280 was verified by sequencing double-stranded plasmid DNA in the region of the mutation. The sequence analyses summarized in Table I demonstrate that the mutated *lacY* genes contain changes in given codons such that Pro is replaced with Gly, Ala, or Leu, as indicated. Finally, the entire nucleotide sequences of the *lacY* genes encoding each mutation in Pro28, Pro31, Pro123, and Pro280 were determined by using six synthetic sequencing primers complementary to appropriate regions of *lacY*. With the exception of P31G, the remainder of the sequences was identical with that reported by Büchel et al. (1980). In P31G, an additional mutation was identified in codon 231 resulting in a change from Gly to Asp. The unwanted mutation was removed by restricting plasmid pP31G/G231D with *Eco*RI and *Aval*, thereby yielding a fragment containing the *lac* promoter/operator region and the first 70 codons of *lacY*. The fragment was then ligated into pGM21 from which the corresponding fragment had been removed.

Table II: Colony Morphology[a] of *E. coli* HB101 Harboring *lacY* Pro Mutants Grown on Lactose/EMB Indicator Plates

proline	alanine	glycine	leucine
28	pink	pink	pink
31	red	red	white
61	red	red	red
89	red	red	red
97	pink	red	red
123	red	red	white
192	red	red	ND[b]
220	red	red	red
280	pink	red	white
327	red	red	white

[a] Colony morphology is based upon visual inspection of *E. coli* HB101 cells transformed with plasmids encoding given mutant permeases plated on EMB containing 25 mM lactose after 24-h growth at 37 °C. [b] Not determined.

Colony Morphology on Lactose/EMB. Mutated *lacY* inserts were restricted from M13mp19 replicative form DNA with *Eco*RI and ligated into the *Eco*RI site of pACYC. The resulting plasmids were used to transform the cryptic strain *E. coli* HB101 (Z^+Y^-), and the transformed cells were grown on EMB plates containing 25 mM lactose as a qualitative estimate of transport activity (Padan et al., 1985). *E. coli* HB101 harboring pGM21, which encodes wild-type permease, grows as dark red colonies, indicative of lactose transport and subsequent metabolism at a high rate (Table II). In contrast, HB101 harboring pACYC, the vector without a *lacY* insert, grows as white colonies. With the exception of Pro28, in each instance where Gly replaced a given prolyl residue, the cells grow as red colonies that are qualitatively indistinguishable from cells expressing wild-type *lac* permease. When Pro28 is replaced with Gly, the cells grow as faintly pink colonies. Alternatively, when Ala is used to replace Pro, permease mutated at positions 31, 61, 89, 97, 123, 192, 220, or 327 confers the wild-type phenotype on HB101, while cells expressing permease mutated at position 280 grow as less red colonies and cells expressing permease mutated at position 28 grow as faintly pink colonies. Finally, when the prolyl residues are replaced with Leu, HB101/pP61L, pP89L, pP97L, or pP220L grows as red colonies, HB101/pP28L grows as pink colonies, and HB101/pP31L, pP123L, pP280L, or pP327L grows as white colonies. Therefore, with the possible exception of Pro28, it appears that none of the Pro residues in the permease is absolutely required for transport, since each Pro residue can be repaced with either Gly or Ala with little or no qualitative effect on colony morphology.

Lactose Transport. To a large extent, the qualitative conclusions suggested by colony morphology are confirmed by transport studies with *E. coli* T184 (Z^-Y^-) transformed with each plasmid (Figures 2–4). Rates of lactose transport and steady-state levels of accumulation in T184 harboring pP31G, pP61G, pP89G, pP97G, pP123G, P220G, and pP280G fall within a range that varies from 60 to 110% of T184/pGM21 (Figure 2 and Table III). T184/pP192G transports lactose at a rate approximating T184/pGM21, but the steady-state level of accumulation is about 45%. In contrast, T184/pP28G exhibits little or no activity, as judged by the observation that lactose transport in this strain is similar to T184 harboring pACYC which has no *lacY* gene.

Rates of transport and steady-state levels of lactose accumulation are essentially identical within experimental error in T184/pGM21 and T184/pP89A, pP123A and pP220A (Figure 3 and Table III). In T184/pP97A and pP61A, rates

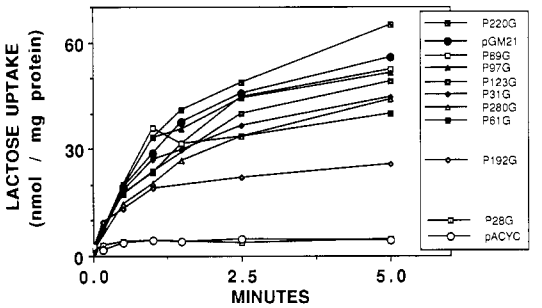

FIGURE 2: Active transport of lactose in *E. coli* T184 harboring plasmid pGM21 encoding wild-type *lac* permease or given Pro → Gly mutants. Aliquots (50 μL) of cells at an OD_{420} of 10 in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were equilibrated at 23 °C, and transport was initiated by addition of [1-^{14}C]lactose (10mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at given times by addition of 3.0 mL of 100 mM KP_i (pH 5.5)/100 mM LiCl and rapidly filtered through Whatman GF/F filters as described under Methods.

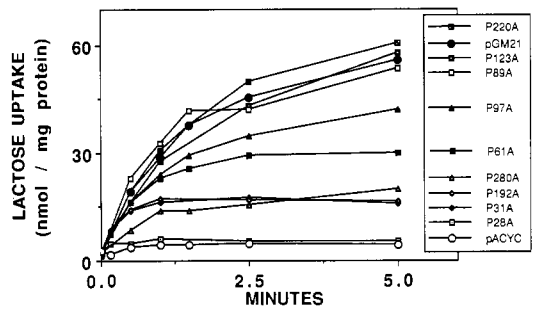

FIGURE 3: Active transport of lactose in *E. coli* T184 harboring plasmid pGM21 encoding wild-type *lac* permease or given Pro → Ala mutants. Experimental conditions were as described in Figure 2.

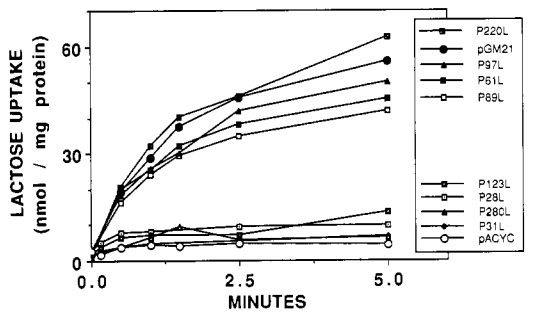

FIGURE 4: Active transport of lactose in *E. coli* T184 harboring plasmid pGM21 encoding wild-type *lac* permease or given Pro → Leu mutants. Experimental conditions were as in Figure 2.

of transport are similar to T184/pGM21, but steady-state levels of accumulation are about 55% and 70%, respectively. T184/pP31A and pP192A transport at rates that are approximately 70% of wild type and accumulate lactose to about 30% of the steady-state level observed in T184/pGM21, while T184/pP280A exhibits 30% of both the rate and steady-state level of accumulation of T184/pGM21. Transport activity in T184/pP28A is essentially the same as observed in T184/pACYC, indicating that permease with Ala in place of Pro28 is inactive.

Finally, the transport activity of T184 harboring pP61L, pP89L, pP97L, or pP220L falls within a range that varies from about 80% to 120% of T184/pGM21 (Figure 4 and Table III). On the other hand, T184/pP28L, pP31L, pP123L, and pP280L exhibit activities that approximate or slightly exceed the negative control T184/pACYC.

Importantly, none of the differences in transport activity described can be attributed to a decrease in the concentration of the altered permease molecules in the cell membrane.

Table III: Relative Transport Activity of Pro Mutants[a]

mutant	initial rate (% wt)	steady state (% wt)	mutant	initial rate (% wt)	steady state (% wt)
28 A	15	7	123 A	103	83
28 G	15	6	123 G	111	76
28 L	26	14	123 L	17	30
31 A	64	29	192 A	80	44
31 G	90	87	192 G	86	67
31 L	14	12	220 A	93	119
61 A	73	55	220 G	94	127
61 G	82	76	220 L	103	123
61 L	97	88	280 A	32	36
89 A	112	104	280 G	68	86
89 G	96	96	280 L	12	10
89 L	75	80	327 A[b]	97	100
97 A	98	69	327 G[b]	12	100
97 G	90	99	327 L[b]	0	0
97 L	84	95			

[a] Data are presented as percentages of the initial rate and steady-state level of lactose accumulation relative to *E. coli* strain T184 pGM21 which expresses wild-type permease. Experiments were performed as described under Methods, and the data were taken from Figures 2–4. [b] Data from Lolkema et al. (1988).

Table IV: Expression of *lac* Permease Pro Mutants Relative to Wild Type[a]

residue	alanine	glycine	leucine
28	100[b]	107	100[b]
31	100[b]	100[b]	100[b]
61	83	115	122
89	108	120	98
97	96	102	98
123	73	112	71
192	109	118	ND[c]
220	155	145	140
280	103	96	105
327	105	97	72

[a] Values shown are derived from immunodot-blot experiments (Lolkema et al., 1988) and are expressed as a percentage of the immunoreactivity of wild-type *lac* permease in T184/pGM21. Background immunoreactivity in T184/pACYC was 10–15% of T184/pGM21. [b] Values based upon visual inspection of Western blots. [c] Not determined.

Western blot (Carrasco et al., 1982) and/or immunodot-blot (Lolkema et al., 1988) analyses carried out with the same samples on which transport was assayed demonstrate that the specific permease content of *E. coli* T184 membranes harboring each of the mutated *lacY* genes approximates that of T184/pGM21 (i.e., approximately 3% of the membrane protein) (Table IV).

P28S Permease. In contradistinction to each of the other prolyl residues in *lac* permease, when Pro28 is replaced with Ala, Gly, or Leu, lactose transport is almost completely abolished. Therefore, out of the total of 12 prolyl residues, only Pro28 appears to be essential for activity. Overath et al. (1987) reported that a randomly selected *lac* permease mutant with Ser at position 28 retains the ability to accumulate TDG to about 50% of the wild-type level, suggesting that a prolyl residue at this position per se is not critical for transport. Therefore, P28S permease was constructed by site-directed mutagenesis. Like the other mutants at this position, P28S permease is severely defective in lactose accumulation relative to the wild type (data not shown). However, as reported by Overath et al. (1987), P28S permease catalyzes TDG accumulation about 50% as well as the wild type, and P28L permease also exhibits significant activity toward the β-galactoside analogue (Figure 5). Although data are not shown, it is noteworthy that preliminary experiments indicate that P28S permease catalyzes melibiose transport as well, but is unable

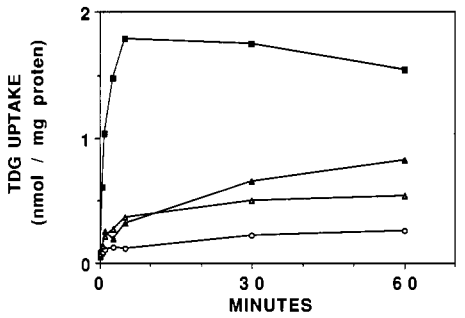

FIGURE 5: Active transport of TDG in *E. coli* T184 harboring plasmid pGM21 encoding (■) wild type, (▲) P28S, (△) P28L *lac* permease, or (○) the vector alone. Experiments were carried out as described in Figure 2 except that [^{3}H]TDG (95 mCi/mmol) was added to a final concentration of 10 μM.

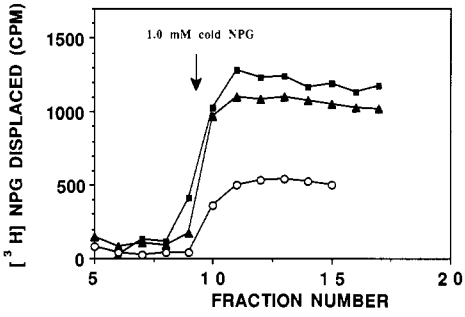

FIGURE 6: Binding of [^{3}H]NPG to P28S *lac* permease in right-side-out membrane vesicles. Flow dialysis was performed with 0.2 mL of right-side-out membrane vesicles (3.0 mg of protein) in 100 mM KP_i (pH 6.6)/10 mM $MgSO_4$ as described under Methods. [^{3}H]NPG (27.2 Ci/mmol) was added at fraction 1 to final concentrations of (○) 0.2, (▲) 1.0, or (■) 5.0 μM. Unlabeled NPG was added at fraction 9 to 1.0 mM, final concentration, as indicated by the arrow. The data are presented as displaced counts per minute to emphasize the extent of binding.

to catalyze transport of methyl 1-thio-β-D-galactopyranoside.

In addition, right-side-out membrane vesicles containing P28S permease were assayed for binding of the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside (NPG) by flow dialysis under nonenergized conditions (Figure 6). As indicated by the increase in the dialyzable concentration of [^{3}H]NPG after addition of excess unlabeled NPG, P28S binds significant amounts of ligand at concentrations of 0.2, 1.0, and 5.0 μM, a finding consistent with the conclusion (Overath et al., 1987) that the mutant has a higher affinity for NPG than the wild type.

Discussion

The side chain of a prolyl residue curls back in covalent linkage to the nitrogen of the peptide bond forming a pyrrolidine ring, which makes both the peptide bond and the side chain rigid. These unique features of Pro and the prolyl peptide bond account for certain aspects of polypeptide folding at positions where Pro is found.

In general, the partial double-bond character of the peptide bond inhibits free rotation about the bond, and steric hindrance caused by the two neighboring side chains favors the trans over the cis configuration. Therefore, the peptide bond, which is synthesized stereospecifically in the trans configuration at the ribosome, is also trans in the structures of folded proteins. Pro affects cis/trans configuration in two ways: (i) the energy barrier between cis and trans configurations is lowered secondary to ring puckering; and (ii) the steric advantage of trans over cis is reduced considerably, since the H atom bonded to the peptide nitrogen is replaced with the side chain of Pro. These considerations are consistent with the finding of cis configurations on the N-terminal side of Pro in a number of globular proteins (Schulz & Schirmer, 1979) and the role that cis/trans isomerization plays in protein folding (Brandts et al., 1975; Kelley & Richards, 1987). It is also interesting that cis/trans isomerization of the peptide bond preceding Pro has been proposed to play a role in the mechanism of active transport (Dunker, 1982; Brandl & Deber, 1986).

In addition, the ring formed by C_α, the N of the peptide bond, and the side chain of Pro severely restricts the dihedral angle between C_α and N, thereby limiting the degree of freedom for folding of the polypeptide backbone. Using data based on globular proteins of known structure, Chou and Fasman (1974) demonstrated initially that Pro is found with high frequency in β-turns and in low frequency in α-helices or β-sheets. Similar statistics do not exist for membrane proteins because of insufficient structural data, and secondary-structure predictions for these hydrophobic proteins are based primarily on hydropathy analysis of the amino acid sequence (Kyte & Doolittle, 1982) and on inferences derived from globular proteins. For many membrane proteins, *lac* permease in particular (Foster et al., 1983), the hydropathy profiles exhibit multiple stretches of hydrophobic residues long enough to traverse the membrane in α-helical conformation separated by shorter, hydrophilic regions. In many instances, however, the putative helical domains contain Pro residues, in apparent contradiction to statistical analyses of globular proteins. Thus, it has been suggested that structural discontinuities (i.e., kinks) may be present in the transmembrane α-helical domains at prolyl residues. Furthermore, Brandl and Deber (1986) and von Heijne (1986) have noted that there is a statistically significant abundance of prolyl residues in the putative transmembrane helices of membrane transport proteins, 9 out of 12 in the case of *lac* permease. Thus, prolyl residues in polytopic membrane proteins may contribute to folding, membrane insertion, and/or stability, and they may be directly involved in transport.

The first aim of this study was to determine whether cis/trans isomerization of the peptide bonds preceding any of the remaining nine prolyl residues in the permease might play a role in the transport mechanism (Dunker, 1982; Brandl & Deber, 1986). Since isomerization is a unique property of prolyl residues, any replacement for Pro should yield inactive permease, which is clearly not the case (Figure 2). With the sole exception of P28G permease which will be discussed below, replacement of each of the other prolyl residues with Gly, including Pro327 (Lolkema et al., 1988), yields permease with significant ability to catalyze lactose accumulation against a concentration gradient. It is significant, however, that permease with either Gly or Ala in place of Pro192 transports lactose to significantly lower steady-state levels of accumulation than the wild type. That is, although both mutants retain significant activity, they may be partially uncoupled. In any event, it is evident that cis/trans isomerization of peptide bonds preceding the prolyl residues in the permease cannot be absolutely required for lactose/H^+ symport.

The second objective was to investigate the importance of possible structural discontinuities by replacing the prolyl residues in the permease with Gly, another helix-breaking residue, and Ala or Leu, two helix-making residues (Chou & Fasman, 1974). The results demonstrate clearly that there is no correlation between permease activity and the tendency of the amino acid replacement to make or break helices. Except for Pro28, each of the prolyl residues in the permease can be replaced with Ala, and significant activity is retained (Figure 3). As mentioned above, some of the mutants (P31A, P61A, P192A, and P280A) exhibit significantly reduced

steady-state levels of accumulation, suggesting that Ala replacement at these positions may lead to partial uncoupling. In any event, it is particularly striking that Pro61, -89, -97, or -220 can be replaced with Leu with little or no effect on permease activity (Figure 4).

Although it is not possible to provide a definitive interpretation for the role of prolyl residues in *lac* permease without a high-resolution structure, it seems apparent that the effects of the replacements described are due to specific chemical properties of the side chains (i.e., bulk, hydrophathy, and/or ability to H-bond). In an effort to simplistically describe and summarize the results presented herein, the effects of the various replacements for Pro have been examined by averaging the data obtained for each type of amino acid substitution. In general, there is an approximate inverse correlation between the hydrophobicity (or size) of the replacement amino acid and both the rate and steady-state level of lactose accumulation. That is, the greater the hydrophobicity (or size) of the residue used to replace Pro, the lower the transport activity. Since 9 of the 12 prolyl residues in the permease are in putative transmembrane helices, the result may seem contradictory. However, theoretical analysis of helix orientation as described by Rees et al. (1989) is consistent with the idea that many of the helices in the permease are amphipathic (T. G. Consler, M. Wesson, H. R. Kaback, and D. Eisenberg, unpublished information). Moreover, in most instances where replacement of Pro with Leu leads to inactivation, the prolyl residue is predicted to be either on the hydrophilic face of the helix or at the boundry between the hydrophilic and hydrophobic faces. Thus, it is possible that a hydrophobic amino acid substitution may interfere with transport activity when placed on the hydrophilic face of an amphipathic helix.

Of the 12 prolyl residues in *lac* permease, replacement of Pro28 alone with Gly, Ala, or Leu leads to inactivation of lactose transport. However, in confirmation of the studies of Overath et al. (1987), permease with Ser in place of Pro28 catalyzes accumulation of TDG and binds the high-affinity ligand NPG. Moreover, P28L permease catalyzes significant TDG accumulation. Taken as a whole, the data are consistent with the notion that Pro28 is important for substrate binding and recognition. Interestingly, Tyr26, a nearby residue in putative helix I, also appears to be involved in binding, as replacement with Phe causes a marked decrease in affinity for NPG (Roepe & Kaback, 1989). In any case, since replacement of Pro28 with Ser does not abolish the general property of substrate accumulation, it is unlikely that the residue plays a central role in the translocation mechanism.

As judged by immunological criteria, each of the Pro mutants in *lac* permease described here is present in the membrane at approximately the same concentration as wild-type permease expressed from plasmid pACYC184. Therefore, it is unlikely that the prolyl residues are critical for the process of membrane insertion. Furthermore, as shown by Roepe et al. (1989), unstable truncation mutants in the permease are absent from the membrane when expressed from pACYC184 because the rate of proteolysis exceeds the rate of synthesis. Since the Pro mutants are found at normal concentrations when expressed under the same conditions, it is highly unlikely that prolyl residues in *lac* permease are particularly important for stability after insertion into the membrane.

In conclusion, the following possibilities deserve consideration: (i) The prolyl residues in *lac* permease may be in α-helical domains, but cause little or no structural discontinuity [cf. Menick et al. (1987)], a possibility consistent with the extremely high helical content of the permease (Foster et al., 1983; Vogel et al., 1985)[1] and the conclusions of Calamia and Manoil (1990) derived from *lac* permease–alkaline phosphatase fusions. (ii) The prolyl residues may be in α-helical domains and cause structural discontinuities that are not important for activity, a possibility that cannot be assessed without a high-resolution structure. (iii) The prolyl residues may be in nonhelical domains, a possibility that seems relatively unlikely in view of the high helix content of the permease and the experiments of Calamia and Manoil (1990).

Registry No. Pro, 147-85-3; *lac* permease, 9033-40-3; lactose, 63-42-3; hydrogen ion, 12408-02-5.

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Involvement of the Protein–Protein Interactions in the Thermodynamics of the Electron-Transfer Process in the Reaction Centers from *Rhodopseudomonas viridis*

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ABSTRACT: Reaction centers from *Rhodopseudomonas viridis* were reconstituted into dimyristoylphosphatidylcholine (DMPC) and dielaidoylphosphatidylcholine (DEPC) liposomes. Freeze–fracture electron micrographs were performed on the samples frozen from temperatures above and below the phase transition temperatures of those lipids (T_c = 23 and 9.5 °C, in DMPC and DEPC, respectively). Above T_c, in the fluid conformation of the lipids, the reaction centers are randomly distributed in the vesicle membranes. Below T_c, aggregation of the proteins occurs. The Arrhenius plots of the rate constants of the charge recombination between P^+ and Q_A^- display a break at about 24 °C in DMPC vesicles and about 10 °C in DEPC vesicles (P represents the primary electron donor, a dimer of bacteriochlorophyll, and Q_A the primary quinone electron acceptor). This is in contrast to what was previously observed for the proteoliposomes of egg yolk phosphatidylcholine and for chromatophores [Baciou, L., Rivas, E., & Sebban, P. (1990) *Biochemistry 29*, 2966–2976], for which Arrhenius plots were linear. In DMPC and DEPC proteoliposomes, the activation parameters were very different on the two sides of T_c ($\Delta H°$ for $T < T_c$ = 2.5 times $\Delta H°$ for $T > T_c$), leading however, to the same $\Delta G°$ values. Taking into account the structural and thermodynamic data, we suggest that, in vivo, protein–protein interactions play a role in the thermodynamic parameters associated with the energy stabilization process within the reaction centers.

The light excitation energy harvested by the antenna of the photosynthetic organisms is converted at the level of the reaction centers into chemical free energy. This occurs via a transmembrane charge separation. In bacteria, the chromatophore membrane is mainly composed by phospholipids, 25% of total lipids being phosphatidylcholine (Niederman & Gibson, 1978; Rivas et al., 1987). The different kinetic steps of the electron transfer within the reaction centers as well as the prosthetic groups involved in these processes have been known for about 15 years. However, a main step for a better understanding of the energy stabilization in the reaction centers

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