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Site-Directed Mutagenesis of Pro327 in the lac Permease of Escherichia coli

Julius S. Lokkema, Irene B. Püttner, and H. Ronald Kaback*

Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

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ABSTRACT: By use of oligonucleotide-directed, site-specific mutagenesis, Pro327 in the lac permease of Escherichia coli has been replaced with Ala, Gly, or Leu. Permease with Ala at position 327 catalyzes lactose/H⁺ symport in a manner indistinguishable from wild-type permease. Permease with Gly at position 327, on the other hand, exhibits about one-tenth the activity of wild-type permease but catalyzes lactose accumulation to essentially the same steady-state level as wild-type permease. Finally, permease with Leu at position 327 is completely inactive. The results demonstrate that there is no relationship between permease activity and the helix-breaking (Pro and Gly) or helix-making (Ala and Leu) properties of the residue at position 327. It is suggested that it is primarily a chemical property of the side chain at position 327 (i.e., bulk, hydrophobicity, and/or ability to hydrogen bond) that is critical for activity and that neither cis/trans isomerization of Pro327 nor the presence of a kink at this position is important.

EXPERIMENTAL PROCEDURES

Materials

[1-14C]Lactose was synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group, Hoffmann-La Roche, Inc.) [125I]Protein A was purchased from Amersham/Searle and isopropyl-β-D-galactopyranoside from Bethesda Research Laboratories. All other materials were of reagent grade and were obtained from commercial sources as described (Sarkar et al., 1986).

Methods

Bacterial Strains. The following strains of E. coli K-12 were used: JM101, supE, thi, Δ(lac-proAB), [F′ traD36, proA⁺B⁺, lacFZΔM15] (Yanish-Perron et al., 1985); BMH7-18 mutL, Δ(lac-pro), supE, thi, proA⁺B⁺, lacFZΔM15/MutL::Tn10 (Kramer et al., 1984); T206 [lacB⁺O⁺Z⁺Y⁺(A), rpsL, met⁵, thr⁻, recA, hsdM, hsdR/F', lacIqZAT(A), rpsL, met⁻] harboring plasmid pGM21 [lacB⁺O⁺Z⁺Y⁺(A), recA(+)251] (Teather et al., 1980); T184 (T206 cured of plasmid pGM21) (Teather et al., 1980); HB101, hsdS20 (rB, mB), recA13, ara-14, proA2, lacY1, galK2, rpsL20(Sm²), xyl-5, mtl-1, supE44, λ⁺/F' (Boyer & Roulland-Dussoix, 1969).

Oligonucleotide-Directed, Site-Specific Mutagenesis. Site-directed mutagenesis using M13mp19 as a cloning vector was performed essentially as described (Sarkar et al., 1986) or with given modifications. The sequences of the mutagenic primers used to change codon 327 for Pro (CCG) into codons for Gly (GGG), Ala (GCC), or Leu (CTG) are given in Table I. Mismatch repair was minimized by transfecting the heteroduplex DNA into the mutator strain E. coli BMH71-18 mutL (Kramer et al., 1984). Mutations were verified by dyeodeoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) with a synthetic primer complementary to the region of lac Y 50-100 nucleotides downstream from codon 327 (cf. Padan et al., 1985). In addition, the entire lacY genes in M13mp19 single-stranded (ss) DNA encoding P327G and P327L were sequenced by using six synthetic oligonucleotide primers complementary to appropriate regions with regard to protein folding, we have now focused on Pro327 in putative helix X.

References

*To whom correspondence should be addressed.
of lac Y. Finally, all three mutations were confirmed by sequencing the region around codon 327 in plasmids pP327A, pP327G, and pP327L. With the exception of the base changes described, each gene has a nucleotide sequence identical with that reported by Büchel et al. (1980).

Cell Growth. E. coli T184 bearing a given plasmid was grown and induced with isopropyl 1-thio-β-D-galactopyranoside as described (Teather et al., 1980).

Transport Assays. Transport of [1-14C]lactose was assayed by rapid filtration as described (Trumble et al., 1984). The reaction mixtures contained cells at a concentration of 0.7 mg of protein/mL in 0.1 M potassium phosphate (pH 7.9), 10 mM magnesium sulfate, and 0.35 mM [1-14C]lactose.

Immunoblots. In order to assess the amount of permease in the membrane, E. coli T184 harboring a given plasmid was resuspended in distilled water to a protein concentration of 7 mg/mL, and aliquots (3 μL) were spotted in triplicate on a nitrocellulose filter (Schleicher & Schuell, BA 85/20). The cells were lysed by placing the filter in contact with Whatman filter paper soaked in 0.45 N NaOH containing 1% sodium dodecyl sulfate and incubating for 10 min at room temperature. The nitrocellulose was neutralized by placing it in contact with filter paper soaked in 1.0 M Tris-HCl (pH 7.4), followed by shaking for 10 min at room temperature in 10 mM Tris-HCl/150 mM NaCl (pH 7.4) (TBS) containing 0.1% Tween 20. Nonspecific binding sites were blocked by shaking for 20 min at room temperature in 10 mM Tris-HCl (pH 7.4) containing 5% bovine serum albumin (BSA) and 0.2% Nonidet P40. Four hundred microliters of ascites fluid containing monoclonal antibody (Mab) 4A10R (Herzlinger et al., 1984) was added to the filter, and incubation was carried out overnight at room temperature. The nitrocellulose was neutralized by placing it in contact with filter paper soaked in 1.0 M Tris-HCl (pH 7.4), followed by shaking for 20 min at room temperature in 10 mM Tris-HCl/150 mM NaCl (pH 7.4) (TBS) containing 0.1% Tween 20. Nonspecific binding sites were blocked by shaking for 1 h in 15 mL of TBS containing 5% bovine serum albumin (BSA) and 0.2% Nonidet P40. Four hundred microliters of ascites fluid containing monoclonal antibody (Mab) 4A10R (Herzlinger et al., 1984) was added to the filter, and incubation was carried out overnight at room temperature. The filter was then soaked sequentially in each of the following solutions for 20 min: (i) TBS/5% BSA/0.2% Nonidet P40; (ii) TBS/0.2% Nonidet P40; and (iii) TBS/0.1% Tween 20. The filter was blocked again as described above with TBS/BSA/Nonidet P40 and incubated for 2 h with [125I]protein A (25 μL of 3.7 MBq/mL; 1480 MBq/mg), followed by the same sequential washings as described after incubation with Mab 4A10R. Finally, the filter was air-dried, and the spots were excised and assayed for radioactivity in a γ counter. Control experiments demonstrated that the amount of cells spotted on the filter was within the linear range of the response curve. Cells harboring pACYC, which has no lac Y insert, were used as negative controls and yielded approximately 4% of the signal obtained with cells harboring pGM21.

RESULTS

Verification of Mutations by DNA Sequencing. The lac Y gene in each plasmid used was cloned initially from pGM21 into M13mp19 replicative form (RF) DNA, and ss phage DNA was isolated and used as template for site-directed mutagenesis. Subsequently, ss phage DNA containing mutated lac Y was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with an appropriate primer complementary to the region of lac Y 50–150 bases downstream from codon 327. The sequence analyses summarized in Table I demonstrate that the mutated lac Y genes contain changes in codon 327 such that Pro is replaced with Gly, Ala, or Leu as indicated. In addition, the entire nucleotide sequence of the lac Y genes encoding the P327G and P327L mutations was determined by using six synthetic sequencing primers. With the exception of the alterations given in Table I, the remainder of the sequences was identical with that reported by Büchel et al. (1980).

Colonial Morphology on Lactose/β-Eosin Methylen Blue (EMB). The lac Y insert was restricted from M13mp19 RF DNA with EcoRI and ligated into the EcoRI site of pACYC. The resulting plasmids pP327A, pP327G, and pP327L 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 (Padan et al., 1985). HB101 harboring pGM21, which encodes wild-type permease, grows as dark red colonies, indicative of lactose transport and subsequent metabolism at a high rate. In contrast, HB101 containing pACYC, the vector without a lac Y insert, grows as white colonies. HB101/pP327A is indistinguishable from HB101/pGM21, while HB101/pP327L is indistinguishable from HB101/pACYC. HB101/pP327G exhibits intermediate behavior; the colonies become red, but at a slower rate than observed with either HB101/pGM21 or HB101/pP327A. Qualitatively, therefore, it appears that P327A permease transports lactose like wild-type permease, while P327G permease is significantly less active and P327L permease is inactive.

Lactose Transport. The qualitative conclusions suggested by colony morphology are confirmed by transport studies with E. coli T184 (Z+Y) transformed with each plasmid (Figure 1). The initial rate of lactose transport and the steady-state level of accumulation are identical within experimental error in T184/pGM21 and T184/pP327A. On the other hand, the initial rate of transport in T184/pP327G is approximately 10% of that observed in T184/pGM21 or T184/pP327A, but the steady-state level of accumulation approaches that observed in the latter strains. Finally, T184/pP327L is completely

Table I: DNA Sequence Analysis of Codon 327 Mutants of the lac Y Gene

<table>
<thead>
<tr>
<th>plasmid</th>
<th>mutagenic primer</th>
<th>DNA sequence change</th>
<th>amino acid sequence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>pP327A</td>
<td>3'-AAACTTCATGCAAGGACGAC-5'</td>
<td>CCG → GCG</td>
<td>Pro → Ala</td>
</tr>
<tr>
<td>pP327G</td>
<td>3'-AAACTTCATGCAAGGACGAC-5'</td>
<td>CCG → GGG</td>
<td>Pro → Gly</td>
</tr>
<tr>
<td>pP327L</td>
<td>3'-AAACTTCATGCAAGGACGAC-5'</td>
<td>CCG → CTG</td>
<td>Pro → Leu</td>
</tr>
</tbody>
</table>

![FIGURE 1](image_url)
Table II: Specific Activity of the Pro327 Mutants and α-Helical Propensities of the Residues Involved

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Initial rate of uptake (nmol min⁻¹ mg⁻¹)</th>
<th>Expression (% w-t)</th>
<th>Specific activity (w-t) (%)</th>
<th>α-helical propensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro327 (w-t)</td>
<td>25.0</td>
<td>100</td>
<td>100</td>
<td>0.59</td>
</tr>
<tr>
<td>Ala327</td>
<td>25.4</td>
<td>105</td>
<td>97</td>
<td>1.45</td>
</tr>
<tr>
<td>Gly327</td>
<td>2.8</td>
<td>97</td>
<td>12</td>
<td>0.53</td>
</tr>
<tr>
<td>Leu327</td>
<td>0</td>
<td>72</td>
<td>0</td>
<td>1.34</td>
</tr>
</tbody>
</table>

The initial rate of uptake and the specific activity apply to the conditions in Figure 1. w-t = wild type. The α-helical propensities are taken from Chou and Fasman (1974). The scale ranges from 0.53 for Gly to 1.53 for Glu.

The specific activity of the strains described was estimated from the rate of transport at 30 s and the amount of permease present in the membrane, as determined by immunoblot analyses with Mab 4A10R and [125I]protein A (Table II). P327A permease has essentially the same activity as wild-type permease, P327G permease exhibits about 12% of wild-type activity, and P327L has no activity.

**DISCUSSION**

The side chain of a Pro 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.

Generally, the partially 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 found to be trans in the structures of folded proteins. Pro affects cis/trans configuration by two means. First, the energy barrier between cis and trans configurations is lowered due to ring puckering. Second, the sterically hindered nature of trans over cis is reduced considerably, since the hydrogen atom bonded to the peptide nitrogen is replaced with the side chain of Pro. Consistent with these considerations are the findings of cis configurations on the N-terminal side of Pro in a number of globular proteins (Schulz & Schirmer, 1978) and the role that cis/trans isomerization plays in protein folding (Brandts et al., 1975; Kelley & Richards, 1987). It is also noteworthy 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 $C_{\alpha}$, the N of the peptide bond, and the side chain of Pro severely restricts the dihedral angle between $C_{\alpha}$ and N, thereby limiting the degree of freedom for the 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 the hydrophobic proteins are based primarily on hydropathy analysis of 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 the 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 Pro residues.

The first aim of this study was to determine whether or not cis/trans isomerization of the peptide bond preceding Pro327 in the _lac_ permease might play a role in the transport mechanism (Dunker, 1982; Brandl & Deber, 1986). Since isomerization is a unique property of Pro residues, any replacement for Pro327 should yield inactive permease, which is clearly not the case. Thus, cis/trans isomerization of the peptide bond between Val326 and Pro327 cannot be important for lactose/H⁺ symport.

The second objective was to investigate the importance of a possible structural discontinuity at position 327 of the permease by replacing the Pro residue at this position with the wild-type molecule with Gly, another "helix-breaking" residue, and Ala or Leu, two "helix-making" residues (Chou & Fasman, 1974; cf. Table II). The results demonstrate clearly that there is no correlation between permease activity and tendency of the amino acid residue at position 327 to make or break helices. Thus, P327A permease catalyzes lactose/H⁺ symport as well as wild-type permease despite the contention that Ala is an amino acid with a high probability of being in a helix. In contrast, replacement of Pro327 with Gly, a residue even less likely than Pro to be found in α-helices, yields permease with about 10% of wild-type permease activity. Finally, replacement of Pro327 with Leu, another amino acid with a strong tendency to be found in α-helices, completely inactivates the permease.

Although it is impossible to provide a definitive interpretation for the role of Pro327 without a high-resolution structure of the permease, it is apparent that the effects of the replacements described are due to specific chemical properties of the side chains (i.e., bulk, hydropathy, and/or ability to hydrogen bond). In addition, the following possibilities merit consideration. (i) Pro327 may be in an α-helical domain (putative helix X), causing little or no structural discontinuity [cf. Menick et al. (1987b)], a conclusion consistent with the extremely high helical content of the permease and with the experiments of Bieseler et al. (1985). (ii) Pro327 may be in an α-helical domain and cause a structural discontinuity that is not important for activity. (iii) Pro327 may be in a non-helical domain. In any case, the results highlight one of the caveats inherent in applying principles derived from statistical studies on globular proteins to hydrophobic membrane proteins.

**References**


