Characterization of the *Escherichia coli* prsA1-encoded Mutant Phosphoribosylpyrophosphate Synthetase Identifies a Divalent Cation-Nucleotide Binding Site*

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The *prsA1* allele, specifying a mutant *Escherichia coli* phosphoribosylpyrophosphate (PRPP) synthetase, has been cloned. The mutation was shown by nucleotide sequence analysis to result from substitution of Asp-128 (GAT) in the wild type by Ala (GCT) in *prsA1*. This alteration was confirmed by chemical determination of the amino acid sequence of a tryptic peptide derived from the purified mutant enzyme. The mutation lies at the N-terminal end of a 16 residue sequence that is highly conserved in *E. coli*, *Bacillus subtilis*, and rat PRPP synthetases and has the following consensus sequence: D L H A X Q I Q G F F D' / V P' / V D. There was little alteration in the *Km* for ribose 5-phosphate. The *Km* for ATP of the mutant enzyme was increased 27-fold when Mg2+ was the activating cation but only 5-fold when Mn2+ was used. Maximal velocities of the wild type and mutant enzymes were the same. The mutant enzyme has a 6-fold lower affinity for Ca2+, as judged by the ability of Ca2+ to inhibit the reaction in the presence of 10 mM Mg2+. Wild type PRPP synthetase is subject to product inhibition by AMP, but AMP inhibition of the *prsA1* mutant enzyme could not be detected. It has been previously proposed that a divergent cation binds to PRPP synthetase and serves as a bridge to the α-phosphate of ATP and AMP at the active site. The *prsA1* mutation appears to alter this divergent cation site.

Phosphoribosylpyrophosphate (PRPP) synthetase catalyzes a pyrophosphoryl transfer from ATP to Rib-5-P (Khorana et al., 1958; Miller et al., 1975).

\[ \text{ATP} + \text{Rib-5-P} \rightarrow \text{PRPP} + \text{AMP} \]

The PRPP synthetase from *Salmonella typhimurium* has been the subject of extensive mechanistic study Switzer, 1971; Li et al., 1978; Gibson and Switzer, 1980; Gibson et al., 1982), the results of which are also valid for the *Escherichia coli* enzyme because these two enzymes differ by only two conservative amino acid substitutions (Hove-Jensen et al., 1986; Bower et al., 1988). Little is known about the roles of specific amino acid residues in PRPP synthetase catalysis. However, molecular cloning of the genes encoding the *E. coli* and *S. typhimurium* PRPP synthetases (Hove-Jensen et al., 1986; Bower et al., 1988) has enabled structure-function relationships to be examined by characterization of mutant enzymes. Bacterial PRPP synthetase mutants have been isolated in *S. typhimurium* by Jochimsen et al. (1980, 1985) and in *E. coli* by Hove-Jensen and Nygaard (1982). These mutants were inadvertently discovered during a search for mutants derived from purine auxotrophs that were able to grow on exogenous guanosine. It is not clear why reduced PRPP synthetase activity confers this phenotype. The *E. coli* mutant allele, *prsA1*, encodes a PRPP synthetase that in crude extracts appeared to be defective in the *Km* values for ATP and Rib-5-P and was modified in its allosteric inhibition by ADP. This paper reports the molecular cloning of the mutant *E. coli prsA1* allele, characterization of the mutation, and purification and kinetic characterization of the homogeneous mutant enzyme. The *prsA1* mutation results in replacement of an Asp residue by Ala in a highly conserved sequence of PRPP synthetase. The properties of the mutant enzyme indicate that this sequence functions as a divalent cation binding site and that the enzyme-bound divalent cation is involved in binding of adenine nucleotides to the enzyme.

**MATERIALS AND METHODS**

*Genetic Methods*—The strains and plasmids used in this study are described in Table I. Plasmid pH028 was constructed as described in Fig. 1. Bacteriophage P1-mediated transductions were performed as described previously by Hove-Jensen (1988). Techniques for manipulation of DNA have been described previously (Bower et al., 1988; Hove-Jensen, 1985). DNA nucleotide sequence determination was by a dideoxy chain termination method from double-stranded plasmid templates (Chen and Seeburg, 1985). Ampicillin and kanamycin were used at concentrations of 50 and 30 μg/ml, respectively.

*Purification and Assay of PRPP Synthetase*—Wild type PRPP synthetase was purified from plasmid-bearing strains as previously described by Hove-Jensen et al. (1986). Mutant PRPP synthetase was purified from strain H0376 containing plasmid pH0126 as follows. All steps were done at 4 °C. Cell paste (330 g) was suspended in buffer (50 mM potassium phosphate, pH 7.5) in a total volume of 2000 ml. Cells were broken by ultrasonic treatment and centrifuged at 11,000 rpm in a Sorvall GSA rotor. A solution of 19 g of streptomycin sulfate in 190 ml of buffer was added to the clear supernatant.
Mutant PRPP Synthetase

Table I

Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/construction</th>
</tr>
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<tbody>
<tr>
<td>E. coli BOE71</td>
<td>lysA polA1</td>
<td>L. Boe</td>
</tr>
<tr>
<td>H072</td>
<td>prsA1</td>
<td>Hove-Jensen (1983)</td>
</tr>
<tr>
<td>H0376</td>
<td>prsA1</td>
<td>Hem' transductant of H0371 with P1(H072)</td>
</tr>
<tr>
<td>H0483</td>
<td>lysA polA1 xcg-2402::Tn10 hemA prsA1</td>
<td>Tet^R transductant of BOE71 with P1(H0371)</td>
</tr>
<tr>
<td>H0484</td>
<td>lysA polA1 prsA1</td>
<td>Hem' transductant of H0483 with P1(H0376)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH05</td>
<td>1.8-kb prsA^+ insert in pBR322</td>
<td>Hove-Jensen (1985)</td>
</tr>
<tr>
<td>pH011</td>
<td>1.7-kb prsA^+ insert in pBR322</td>
<td>Hove-Jensen (1985)</td>
</tr>
<tr>
<td>pH028</td>
<td>KanR insert into prsA</td>
<td>This work, Fig. 1</td>
</tr>
<tr>
<td>pH0126</td>
<td>1.8-ab prsA^+ insert in pBR322</td>
<td>This work, Fig. 2</td>
</tr>
<tr>
<td>pHUC4-K</td>
<td>KanR encoding DNA cassette</td>
<td>Vieira and Messing (1982)</td>
</tr>
</tbody>
</table>

Fig. 1. Inactivation of a plasmid-borne prsA gene. Heavy open lines indicate E. coli chromosomal DNA, heavy closed lines indicate the kanamycin resistance (Kan^R)-encoding DNA fragment, and light lines indicate vector DNA. The plasmid pH05 was digested with BssHII and NsiI, followed by S1 nuclease treatment to generate blunt ends. This DNA was then ligated to the KanR-encoding gene, harbored in a HincII generated DNA fragment of pH028. A 333-base pair BssHII to NsiI fragment of prsA^+ has been deleted and replaced by the KanR-encoding fragment.

RESULTS

Cloning the prsA1 Allele—The prsA1 allele was cloned by a P1-mediated marker rescue procedure similar to that described by Ljestrom et al. (1985) and Saarilahti and Palva (1985), which is shown in Fig. 2. A plasmid-borne, inactive prsA gene, prsA3, was constructed as described under "Materials and Methods." The plasmid (pH028) was introduced into E. coli strain H0484 (polA1 prsA1) by transformation and selection for ampicillin resistance. Since ColEl-derived plasmids require DNA polymerase I for replication, the ampicillin-resistant colonies represent those in which the plasmid has entered the chromosome by homologous recombination. The bacteriophage P1 was grown on a kanamycin-resistant colony and used to transduce E. coli strain H0376 (prsA^+ prsA1) to ampicillin resistance. In the polA^+ background, the plasmid can be excised from the chromosome and be replicated. Plasmid excision yielded, as expected, plasmids bearing either the null prsA3 allele or the prsA1 allele. Selection on LB medium gave only plasmids bearing the null prsA3 allele, whereas selection on minimal medium gave plasmids bearing either the prsA1 allele or the prsA3 allele. The reason for this fortuitous selectivity in LB is not known. A plasmid bearing the prsA1 allele, pH0126, was selected for further characterization.

Localization of the prsA1 Mutation—The observation that E. coli strains containing the prsA1 allele on a high copy
used to transduce strain H0376 (pol+prsAI) to ampicillin resistance. This caused excision of the plasmid by homologous recombination. Vector DNA flanked by the prsA3 and prsAl alleles.

A, the prsA3::KanR-harboring plasmid, pH028, was in combination at position 1 regenerated pH028, harboring the prsA3::Kan". D, recombination at position 2 generated the prsA1 harboring plasmid, pH0126.

Identification of the mutation in prsA1. Equivalent portions of the dideoxy chain termination sequencing ladders of the same fragment of the wild type (prsA+) and mutant (prsA1) alleles are shown. The wild type template was pH05, and the mutant template was pH0126 cloned into pH05. The primer was a synthetic oligonucleotide that was complementary to nucleotides 801 to 817 of the plasmid carrying the prsA1 allele, whereas recombination at position 2 generated the prsA1 harboring plasmid, pH0126.

acid sequence of the wild type and mutant proteins (see below).

Purification of prsA1-encoded Mutant Enzyme—The mutant protein was isolated from strain H0376 bearing the plasmid pH0126 as described under "Materials and Methods." This plasmid-harboring strain contained about 60-fold higher levels of PRPP synthetase activity than did the haploid prsA1 strain. The heat step used in the standard procedure was omitted, as this treatment inactivated 90% of the mutant PRPP synthetase activity. The mutant enzyme was isolated in gram quantities with greater than 98% purity as judged by Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Amino Acid Altered in the prsA1 Mutant—DNA nucleotide sequence analysis predicted that the mutation in the prsA1 allele should convert Asp-128 of the wild type enzyme to Ala. This residue lies near the N terminus of the largest peptide (59 residues) obtained from tryptic digestion of PRPP synthetase. The peptide, which was the last tryptic peptide to elute from a reverse phase HPLC column, was isolated from both the prsA1 mutant and wild type enzymes. Each peptide was subjected to 10 cycles of automated Edman degradation. Identification of the phenylthiohydantoin-amino acids agreed

Failure of ColEl-derived plasmids carrying prsA1 to grow on LB medium served to indicate which restriction fragments contained the mutation. Each isolated restriction fragment was used to replace the analogous restriction fragment from a plasmid with the wild type gene. E. coli strain H0376 (prsA1) was transformed with the generated plasmids, and identical portions of the transformation mixture were plated on minimal medium + 0.2% glucose + 50 μg/ml ampicillin (MM) or LB medium + 50 μg/ml ampicillin (LB).

Identification of the phenylthiohydantoin-amino acids agreed

number plasmid would grow on minimal medium, but not on LB medium, provided a rapid screen for the presence of the mutation. Restriction fragments isolated from pH0126, a plasmid carrying the prsA1 allele, were substituted for analogous fragments from pH011 (the wild type allele). The ligation mixture was used to transform the prsA1 E. coli strain H0376. Samples of the transformation mixture were spread on minimal ampicillin or LB-ampicillin plates to determine whether the wild type or mutant phenotype was expressed. As shown in Fig. 3, fragments ending at or upstream of the BssHII site (797 bp) generated the wild type phenotype, whereas those ending at or downstream from the PstI site (987 bp) generated the mutant phenotype. This result located the mutation between 797 and 987 bp in the previously published prsA sequence (Hove-Jensen et al., 1986). Determination of the nucleotide sequence from 790 to 1050 bp revealed the single change of nucleotide 900 from A to C, resulting in the amino acid residue 128 change from Asp to Ala (Fig. 4). The assignment of this change was confirmed by direct chemical determination of the corresponding amino

FIG. 4. Identification of the mutation in prsA1. Equivalent portions of the dideoxy chain termination sequencing ladders of the same fragment of the wild type (prsA+) and mutant (prsA1) alleles are shown. The wild type template was pH05, and the mutant template was an isolate, described in Fig. 3, with the EcoRI to PstI fragment of pH0126 cloned into pH05. The primer was a synthetic oligonucleotide that was complementary to nucleotides 801 to 817 of the transcribed strand of prsA.
exactly with the sequences predicted from nucleotide sequence analysis and confirmed the conversion of wild type Asp-128 to Ala in the mutant. The nucleotide and amino acid sequence also corrected an error in the previously published sequence of *E. coli* PRPP synthetase (Hove-Jensen et al., 1986). Codon 126 was shown to be ACA (not ATA), and residue 126 was identified as Thr (not Ile) in both wild type and mutant enzymes.

**Kinetic Studies of Purified prsAl Mutant PRPP Synthetase**—Michaelis constants for ATP and Rib-5-P were determined for the purified mutant and wild type *E. coli* PRPP synthetases. With Mg\(^{2+}\) (4 mM excess) as the added divalent cation, the *Kₘ* for ATP for the mutant enzyme (6.2 ± 1.3 mM) was 27-fold larger than for the wild type enzyme (0.23 ± 0.03 mM). With 2 mM ATP, the *Kₘ* for Rib-5-P was 0.88 ± 0.1 mM for the mutant enzyme, but with 20 mM ATP, which is saturating for both enzymes, the *Kₘ* values for Rib-5-P were the same (0.3 ± 0.1 mM) for the mutant and wild type enzymes. There was significant substrate inhibition of the wild type enzyme at 20 mM ATP, but it was clear that the *Kₘ* for Rib-5-P of the prsAl PRPP synthetase approached that for the wild type enzyme as the mutant enzyme was saturated with ATP. The maximal velocities of the wild type and mutant enzymes were equal. Thus, the most obvious defect of the mutant enzyme is in saturation with MgATP.

Pronounced differences between the divalent cation activation profiles of the mutant and the wild type enzymes, especially with Mg\(^{2+}\) (Fig. 5), indicated that the mutant enzyme is defective in the binding of divalent cations. The difference in *Kₘ* for ATP between mutant and wild type enzymes was 27-fold with Mg\(^{2+}\), but half-saturation values differed by only 5-fold with Mn\(^{2+}\) (0.15 ± 0.05 mM for wild type enzyme and 0.05 ± 0.01 mM for the mutant enzyme). Since Mg\(^{2+}\) and Mn\(^{2+}\) bind equally well to ATP, we conclude that the mutant enzyme is defective in the binding of divalent cations, especially Mg\(^{2+}\), which in turn leads to reduced affinity for ATP.

Ca\(^{2+}\) is a potent inhibitor of PRPP synthetase, even in the presence of excess Mg\(^{2+}\) ions, presumably because Ca\(^{2+}\) displaces Mg\(^{2+}\) from its enzyme-bound site (Switzer, 1971). The *Kₘ* for inhibition of the mutant PRPP synthetase was 120 ± 50 μM, 6 times the value of 22 ± 4 μM observed under the same conditions with the wild type enzyme (data not shown). This result is also consistent with the conclusion that the mutant enzyme has an alteration leading to reduced affinity for divalent cations.

Product inhibition by AMP was examined with Mg\(^{2+}\) as the activating cation. No significant inhibition by concentrations of AMP up to 10 mM could be detected for the mutant enzyme. Under the same experimental conditions, AMP inhibition of the wild type enzyme was readily observed; when fit to the equation for noncompetitive inhibition with respect to ATP, a slope inhibition constant (*Kₘ*) for AMP of 1.9 ± 0.8 mM was determined. In view of previous evidence that divalent cations form a bridge between PRPP synthetase and the α-phosphate residue of ATP and AMP (Gibson and Switzer, 1980), the insensitivity of the mutant enzyme to product inhibition by AMP supports the view that the prsAl enzyme is defective in binding Mg\(^{2+}\) at this enzyme-nucleotide bridging site.

**DISCUSSION**

The prsAl allele encoding a mutant PRPP synthetase from *E. coli* was cloned by a procedure which is both direct and general for cases in which a null mutation can be constructed on a plasmid derived from ColEl and the mutant allele permits bacterial growth. The reason for the failure of cells bearing the prsAl allele on a plasmid to grow on LB medium is not known, but this fortuitous observation provided an extremely rapid means of locating plasmids that carry the prsAl mutation and hence of locating the mutation on a small restriction fragment (Fig. 3).

Previous kinetic studies (Switzer, 1971) led to the conclusion that PRPP synthetase requires divalent cations in two roles: to form MgATP complex, which is the true substrate, and as a direct activator of the enzyme. Subsequent studies on the divalent cation dependence of stereoselectivity of the enzyme for isomers of ATPαS and of product inhibition by AMPαS (Gibson and Switzer, 1980) provided evidence that the divalent cation activator serves as a bridge between PRPP synthetase and the α-phosphate residue of ATP and AMP. The exact roles played by divalent cations in the PRPP synthetase remain unclear. On the basis of studies with the hidenate β\(_γ\)-Co(III)(NH\(_3\))\(_n\)ATP complex and EPR studies on Mn\(^{2+}\) binding, Li et al. (1978) concluded that the true substrate for PRPP synthetase is the β\(_γ\) complex with ATP and that 1 atom of Mn\(^{2+}\) per subunit binds to the free enzyme as a putative “activator” site. Unfortunately, in very recent studies with the wild type PRPP synthetase, we have been unable to confirm that β\(_γ\)-Co(III)(NH\(_3\))\(_n\)ATP is a substrate or that Mn\(^{2+}\) binding can be detected by EPR line-broadening techniques. Thus, it is not clear whether one or more cations are ligated to the ATP molecule at the active site of the enzyme.

All of the results of kinetic studies of the purified prsAl mutant PRPP synthetase are consistent with the conclusion that this mutant has reduced ability to bind divalent cations, especially Mg\(^{2+}\), and that this defect results in an apparently

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Reduced affinity for ATP and AMP. Much higher concentrations of Mg$^{2+}$ are needed to activate the mutant enzyme than are needed for the wild type enzyme (Fig. 5). The $K_a$ for MgATP in the Mg$^{2+}$-supported reaction, which is approximately equal to the dissociation constant for MgATP for the wild type enzyme (Gibson et al., 1982), is 27-fold larger for the mutant enzyme. Similar but smaller defects were noted for the Mn$^{2+}$-supported reaction. The decreased sensitivity of the mutant enzyme to inhibition by Ca$^{2+}$ in the presence of excess Mg$^{2+}$ and the failure to detect product inhibition by AMP with the mutant enzyme are also consistent with the conclusion that the altered cation-binding site normally contains a cation that ligates to the $\alpha$-phosphate residue of adenine nucleotides. Whether this cation also is ligated to the $\beta$- or $\gamma$-phosphate residues is not clear. Once saturated with MgATP, the mutant enzyme is a fully competent catalyst; maximal velocities were not reduced. $K_v$ values were observed if subsaturating MgATP was used. This is expected as the consequence of an ordered kinetic mechanism in which MgATP binds first (Switzer, 1971).

It is of considerable interest that the site of the prsA1 mutation lies at the N-terminal end of a region of PRPP synthetase in which the sequence is very highly conserved over a great phylogenetic distance (Fig. 6). A striking feature of this sequence is the presence of four potential ligands to divalent cations: Asp-128, His-130, Asp-139, and Asp-143. Such ligands would bind especially well to a "hard" cation such as M$^+$, which supports the highest PRPP synthetase activity of any cation tested. The properties of the Asp-128 to Ala mutation in the prsA1 PRPP synthetase implicate this residue as part of the cation-binding site. The involvement of the other amino acid residues in cation binding is conjectural at this point. However, it is noteworthy that His-130 has been identified as the site of labeling of PRPP synthetase by the active site-directed reagent PSBA. In the absence of a three-dimensional structure of PRPP synthetase, site-directed mutagenesis provides an approach to testing this conjecture. Such studies are in progress in our laboratory.

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