Effects of Mutagenesis of Aspartic Acid Residues in the Putative Phosphoribosyl Diphosphate Binding Site of *Escherichia coli* Phosphoribosyl Diphosphate Synthetase on Metal Ion Specificity and Ribose 5-Phosphate Binding†

Martin Willemoës,‡ Dan Nilsson,§ and Bjarne Hove-Jensen*,‡,§

Center for Enzyme Research and Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT: The three conserved aspartic acid residues of the 5-phospho-D-ribosyl α-1-diphosphate binding site (213-GRDCVLV**DD**MI**D**TGGT-228) of *Escherichia coli* phosphoribosyl diphosphate synthetase were studied by analysis of the mutant enzymes D220E, D220F, D221A, D224A, and D224S. The mutant enzymes showed an increase in K_M for ribose 5-phosphate in the presence of at least one of the divalent metal ions Mg^{2+}, Mn^{2+}, Co^{2+}, or Cd^{2+}, with the most dramatic changes revealed by the D220E and D220F enzymes in the presence of Co^{2+} and the D221A enzyme in the presence of Mn^{2+} or Co^{2+}. The D220F and D221A enzymes both showed large decreases in V_{app} in the presence of the various divalent metal ions, except for the D221A enzyme in the presence of Co^{2+}. V_{app} of the D220E enzyme was similar to that of the wild-type enzyme in the presence of Mg^{2+}, Mn^{2+}, or Cd^{2+}, whereas the V_{app} was increased in the presence of Co^{2+}. V_{app} values of the D224A and D224S enzymes were lowered to 10−15-fold and 3−4-fold in the presence of Mg^{2+} or Mn^{2+}, respectively, whereas V_{app} was similar to that of the wild-type and K_M for Rib-5-P was increased 4-fold in the presence of Cd^{2+}. The changes in K_M for ribose 5-phosphate and V_{app} of the mutant enzymes were dependent on the metal ion present, suggesting a function of the investigated aspartic acid residues both in the binding of ribose 5-phosphate, possibly via a divalent metal ion, and in the interaction with a divalent metal ion during catalysis.

The enzyme 5-phospho-D-ribosyl α-1-diphosphate (PRPP)[1] synthetase (EC 2.7.6.1) catalyzes the reaction Rib-5-P + ATP → PRPP + 5′-AMP by attack of the 1-hydroxyl of Rib-5-P on the β-phosphate of ATP, resulting in transfer of the β,γ-diphosphoryl moiety to Rib-5-P (Khorana et al., 1958). The *Salmonella typhimurium* PRPP synthetase reaction occurs by a sequential, ordered mechanism with ATP binding to the enzyme first and PRPP dissociating last (Switzer, 1971). A divalent metal ion−ATP complex is the true substrate, and in addition, a free divalent metal ion is needed for activity (Switzer, 1969, 1971). *Escherichia coli* PRPP synthetase has similar kinetic properties (Hove-Jensen et al., 1986). PRPP is a precursor of purine, pyrimidine, and pyridine nucleotides and in plants and microorganisms the amino acids tryptophan and histidine (Hove-Jensen, 1988). PRPP synthetase is encoded by the *prs* gene in the enteric organisms *E. coli* (Hove-Jensen, 1983, 1985; Hove-Jensen et al., 1986) and *S. typhimurium* (Bower et al., 1988; Jochimsen et al., 1985), in the Gram-positive organisms *Bacillus subtilis* (Nilsson & Hove-Jensen, 1987; Nilsson et al., 1989) and *Bacillus caldolyticus* (Krath & Hove-Jensen, 1995, 1996).

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A GRDCVLVDDMIDTGGT 228
B GERVVIVDDLLATGGT 135
C GEGFIVIDDLVDTGGT  96
D GR-VMLVDDVI-TAGT 131
E GLAD-AICDLVSTGAT 176
F DKNVLLVDDSIVRGTT 374
G PRPDYLFADIVGTGGD 282
H ERMALIVDPMLATGGS 138
I GKNVLIVEDIIDTGKT 141
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FIGURE 1: Comparison of PRPP binding site sequences. Gaps in the sequence alignment are indicated by hyphenation. Numbers at the right indicate amino acid positions: (*A*) *E. coli* PRPP synthetase (Hove-Jensen et al., 1986), (*B*) *E. coli* adenine phosphoribosyltransferase (Hershey & Taylor, 1986), (*C*) *E. coli* xanthine-guanine phosphoribosyltransferase (Pratt & Subramani, 1983), (*D*) *E. coli* (Poulsen et al., 1983) or *S. typhimurium* (Scapin et al., 1993) orotate phosporibosyltransferase, (*E*) *S. typhimurium* ATP phosphoribosyltransferase (Piszkiewicz et al., 1979), (*F*) *E. coli* glutamine PRPP amidotransferase (Tso et al., 1982), (*G*) *E. coli* anthranilate phosphoribosyltransferase (Horowitz et al., 1982), (*H*) *E. coli* uracil phosphoribosyltransferase (Andersen et al., 1992), and (*I*) human hypoxanthine-guanine phosphoribosyltransferase (Wilson et al., 1982).

The primary structures of PRPP synthetase from a number of organisms across a wide phylogenetic range have been previously compared (Nilsson et al., 1989). A stretch of 16 amino acid residues of PRPP synthetase shows a high degree of identity to a sequence of most phosphoribosyltransferases, enzymes that utilize PRPP as substrate (Hershey & Taylor, 1986; Hove-Jensen et al., 1986; Boer & Glickmann, 1991) (Figure 1). Despite the difference in the chemistry of diphosphoryl transfer and phosphoribosylation, the sequence shown has been designated a PRPP binding motif on the basis of the sequence similarity and the fact that all the compared enzymes bind PRPP (Hershey & Taylor, 1986; Hove-Jensen et al., 1986). In the present article, we have tested the function of this sequence and present a kinetic

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* Author to whom correspondence should be addressed at Center for Enzyme Research, Institute of Molecular Biology, University of Copenhagen, 83H Sølvgade, DK-1307 Copenhagen K, Denmark. Telephone: +45 3532 2027. Fax: +45 3532 2040. E-mail: hove@mermaid.molbio.ku.dk.

‡ Center for Enzyme Research.

§ Department of Biological Chemistry.

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[1] Abbreviations: PRPP, 5-phospho-D-ribosyl α-1-diphosphate; Rib-5-P, ribose 5-phosphate.

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analysis of *E. coli* PRPP synthetase variants altered in the conserved aspartic acid residues Asp-220, Asp-221, and Asp-224, and we provide evidence that these amino acid residues are involved in catalysis and in the binding of Rib-5-P possibly via the binding of a divalent metal ion.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media. Strain HO773 [*araC*$_{am}$ *araD* Δ(*lac*)*U169 trp*$_{am}$ *mal*$_{am}$ *rpsL relA thi deoD gsk-3 udp supF* Δ*prs-4*::Kan] is deleted for the chromosomal *prs* gene and was used as a host strain for the various *prs* alleles. The construction and properties of the strain were described before (Post et al., 1996). Strain MC1061 [*araD139* Δ(*ara-leu*) Δ(*lac*)*X74 galE15 galK16 rpsL*] (Casadaban et al., 1983) was used to propagate plasmid DNA. A *dam* derivative was used to provide plasmid DNA, which was unmethylated at GATC DNA sequences. Strain NM522 (Gough & Murray, 1983) was used to propagate bacteriophage M13 derivatives. Cells were grown in NZY broth (Hove-Jensen & Maigaard, 1993). Cell growth was followed in an Eppendorf 6121 spectrophotometer as A_{436}. An A_{436} of 1 (1 cm path length) corresponds to approximately 3×10^8 cells/mL.

DNA Methodology and the Construction of Plasmids. Plasmid DNA and the replicative form of bacteriophage M13 or derivatives thereof were isolated as previously described (Birnboim & Doly, 1979). Restriction endonucleases (Boehringer Mannheim, Promega, Amersham, or New England Biolabs), S1 nuclease (Amersham), the large fragment of *E. coli* DNA polymerase I, and T4 DNA ligase (Boehringer Mannheim) were incubated under the conditions specified by the vendors. DNA fragments were isolated from agarose gels with the Gene Clean kit (Bio-101, Vista, CA). Nucleotide sequences were determined by the chain termination method (Sanger et al., 1977; Sambrook et al., 1989). The vectors used were pBR322 (Bolivar et al., 1977), pSELECT (Promega), and bacteriophage M13mp19 (Yanisch-Perron et al., 1985). In addition, we used DNA of pHO5, which contains the wild-type *prs* allele in a 1785 bp *Bam*HI−*Pvu*II DNA fragment, DNA of pHO11, which contains the wild-type *prs* allele in a 1658 bp *Eco*RI−*Pvu*II DNA fragment, and DNA of pHO2, which contains the wild-type *prs* allele in a 5.6 kbp *Bam*HI-generated DNA fragment in pBR322 (Hove-Jensen, 1985). DNA of pHO2 contains two *Cla*I restriction endonuclease recognition sites, one of which is within the *prs* coding sequence, the other in the vector sequence. The former site is protected from *Cla*I digestion by methylation. The latter site was removed by *Cla*I restriction endonuclease digestion and S1 nuclease treatment followed by ligation to generate pHO125. In addition, we constructed pHO18, which was identical to pHO5, except that the *prs* gene was harbored in a 1790 bp *Bam*HI DNA fragment, due to the insertion of a *Bam*HI linker (5′-CCGGATCCGG-3′, Boehringer Mannheim) at a *Pvu*II site. This 1790 bp *Bam*HI DNA fragment was inserted into the unique *Bam*HI site of pSELECT to generate pMW529 and into the unique site of the replicative form of bacteriophage M13mp19 DNA to generate M13DA755. Procedures for rescue of the *prs* template strand of pMW529 and M13DA755 have been described previously (Dente et al., 1983). We constructed five mutant *prs* alleles. *prs-51*, specifying the D221A mutant PRPP synthetase, was constructed with single-stranded DNA of M13DA755, by using a mismatch oligodeoxyribonucleotide 5′-GGTCGATGCTATGAT-3′, with the underlining indicating the mismatch nucleotide, together with the oligodeoxyribonucleotide-directed *in vitro* Mutagenesis System (version 2, Amersham). Sequencing of the mutated DNA revealed that no further mutations had been introduced within a 314 bp *Nsi*I-*Cla*I DNA fragment, which was then ligated to *Nsi*I-*Cla*I-digested pHO125 DNA, followed by digestion with *Ava*I to obtain pDA791, which contained the *prs-51* allele in a 2.3 kbp *Bam*HI-*Ava*I DNA fragment of pBR322. The same procedure was used to construct the *prs-52* allele, specifying the D224A PRPP synthetase, except that the mismatch oligodeoxyribonucleotide was 5′-TATGATCGCCACTGGC-3′. Sequencing and recloning resulted in pDA794, which contained the *prs-52* allele in a 2.3 kbp *Bam*HI-*Ava*I DNA fragment in pBR322. The *prs-53* and *prs-54* mutant alleles, specifying the D220E and D220F mutant PRPP synthetases, respectively, were constructed with single-stranded DNA of pMW529 as the template, the mismatch oligodeoxyribonucleotide 5′-GACTGCGTACTGGTCNNNGATATGATCGACACT-3′, with N indicating A, G, C, or T, and the Altered Sites *in vitro* Mutagenesis System (Promega). The mutations were verified by sequencing, and a 330 bp *Nsi*I-*Sfu*I DNA fragment containing the mutations was used to replace the wild-type *Nsi*I-*Sfu*I DNA fragment of pHO11. This treatment resulted in pBR322 derivatives pMW547 (*prs-53*) and pMW546 (*prs-54*). The *prs-55* allele, specifying the D224S PRPP synthetase, was constructed with the mismatch oligodeoxyribonucleotide 5′-GATATGATCTCCACTGGCGG-3′, single-stranded pMW529 DNA, and the Altered Sites *in vitro* Mutagenesis System. The mutation was verified and recloned as described for *prs-53* and *prs-54* to yield pMW545.

Purification of PRPP Synthetase and Protein Methods. The purification of PRPP synthetase was based on the protocol described before for the purification of the *prs-1*-encoded mutant enzyme (Bower et al., 1989). Culture flasks containing 1 L of NZY broth supplemented with ampicillin (100 mg/L), kanamycin (30 mg/L), and NAD (25 mg/L) were inoculated with 5 mL overnight cultures of strain HO773 harboring the various plasmid-borne *prs* alleles. Cells were grown with shaking for 24 h and yielded 2−5 g of cell paste. All subsequent steps were carried out at 4 °C. Cells were harvested by centrifugation, washed in 50 mM potassium phosphate buffer (pH 7.5), resuspended in 20 mL of the same buffer, and sonicated (Branson Sonic Power) at maximum power for 6×0.5 min pulses. Cell debris was removed by centrifugation at 14 000 rpm for 20 min in a Sorvall SS-34 rotor. The volume of the supernatant fluid was adjusted to 27 mL with 50 mM potassium phosphate buffer (pH 7.5). Three milliliters of 10% (w/v) streptomycin sulfate in 50 mM potassium phosphate buffer (pH 7.5) was added, and the precipitate was removed by centrifugation as above. While gently stirred over a period of 30 min, the supernatant fluid was made 20% saturated with ammonium sulfate by the addition of a solution of 40% saturated ammonium sulfate in 50 mM potassium phosphate buffer with the pH adjusted to 7.5 with potassium hydroxide. The precipitate was left to stand for an additional 30 min, and the precipitated protein was recovered by centrifugation as above and dissolved in 10 mL of 50 mM potassium phosphate buffer (pH 7.5). The ammonium sulfate precipitation was repeated as above, and precipitated protein was dissolved in 50 mM potassium

Table 1: Apparent Kinetic Constants[a]

		V_{app} (μmol min^{-1} mg^{-1})		K_M (μM)	
metal ion	enzyme	Rib-5-P	ATP	Rib-5-P	ATP
Mg^{2+}	wild-type	129 ± 5	143 ± 8	280 ± 40	97 ± 19
	D220E	78 ± 10	88 ± 9	1300 ± 300	181 ± 18
	D220F	0.0157 ± 0.0006	0.0138 ± 0.0002	160 ± 30	78 ±5
	D221A	0.0307 ± 0.0008	0.036 ± 0.002	500 ± 50	150 ± 30
	D224A	7.4 ± 0.5	10.6 ± 0.9	180 ± 40	60 ± 20
	D224S	10.2 ± 0.6	15.9 ± 0.7	210 ± 40	61 ± 11
Co^{2+}	wild-type	11.7 ± 0.3	13.5 ± 0.2	37 ± 3	41.2 ± 1.3
	D220E	110 ± 5	104 ± 5	600 ± 80	77 ± 12
	D220F	0.0038 ± 0.0001	0.0053 ± 0.0003	670 ± 80	19.5 ± 0.3
	D221A	1.49 ± 0.05	1.49 ± 0.03	2600 ± 200	51 ± 3
Mn^{2+}	wild-type	114 ± 10	92 ± 3	200 ± 30	78 ± 6
	D220E	86 ± 7	113 ± 3	200 ± 50	89 ± 10
	D220F	0.0142 ± 0.0004	0.0136 ± 0.0005	120 ± 30	54 ± 8
	D221A	0.15 ± 0.03	0.115 ± 0.006	7000 ± 3000	280 ± 30
	D224A	34.0 ± 1.4	38.4 ± 1.8	86 ± 13	63 ± 12
	D224S	40 ± 3	44.7 ± 1.5	150 ± 30	70 ± 9
Cd^{2+}	wild-type	14.5 ± 0.4	18.7 ± 0.9	70 ± 9	20 ± 4
	D220E	12.0 ± 0.4	14.9 ± 0.6	48 ± 9	17 ± 3
	D224A	20.1 ± 0.1	20.3 ± 1.1	263 ± 26	20 ± 5
	D224S	19.8 ± 0.5	21 ± 1	263 ± 31	21 ± 4

[a] Depending on the enzyme assayed, the Rib-5-P concentration varied from 39 μM to 10 mM in the presence of 2 mM ATP and 5 mM $MgCl_2$, 1 mM ATP and 2 mM $MnCl_2$ or 2 mM $CdCl_2$, or 0.5 mM ATP and 2 mM $CoCl_2$. Depending on the enzyme assayed, the ATP concentration varied from 7.8 μM to 1 mM in the presence of 10 mM Rib-5-P and 5 mM $MgCl_2$ or 5 mM Rib-5-P and 2 mM $MnCl_2$, 2 mM $CoCl_2$, or 2 mM $CdCl_2$, except for the D221A enzyme which was assayed at 10 mM Rib-5-P. Standard errors were calculated by the computer program (Experimental Procedures).

phosphate buffer (pH 7.5) and dialyzed against the same buffer. Yields were 2−3 mg of protein per gram of cell paste. The purity of the enzymes was approximately 98% as evaluated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Laemmli, 1970). The enzymes were stable when stored in 50 mM potassium phosphate buffer (pH 7.5) at −20 °C. The protein concentration was determined by the bicinchoninic acid procedure with reagents provided by Pierce (Smith et al., 1985) and with bovine serum albumin as a standard.

Assay of PRPP Synthetase Activity and Determination of Apparent Kinetic Constants. PRPP synthetase activity was assayed at 37 °C as previously described (Jensen et al., 1979). The amounts of radioactivity in ATP and PRPP spots on thin-layer chromatograms were quantitated with a Packard Instant Imager 2024. Reactions were initiated by the addition of Rib-5-P to a mixture containing a final volume of 100 μL assay buffer (50 mM potassium phosphate−50 mM triethanolamine buffer, pH 8.5), ATP, divalent metal ions (chloride salts), and diluted enzyme. Concentrations of Rib-5-P, ATP, and divalent metal ion varied as described in Results and Discussion. The concentration of Mg^{2+} was kept at 10 mM or lower, whereas the concentrations of Mn^{2+}, Co^{2+}, and Cd^{2+} were kept at 2 mM or lower to prevent the formation of divalent metal ion−phosphate precipitates. Enzyme was diluted appropriately in assay buffer containing bovine serum albumin (1 mg/mL). Initial velocities were determined at least twice at different enzyme concentrations.

For the determination of optimum pH, the assay conditions were 1.3 mM ATP, 10 mM Mg^{2+}, 10 mM Rib-5-P, 55 mM potassium phosphate, and either 50 mM triethanolamine (pH range 7.0−8.5) or 50 mM glycine (pH range 8.5−9.5). The determination of activities at pH values around 10 was prevented by the rapid formation of a precipitate during incubation, presumably magnesium phosphate.

Determinations of initial velocity of the wild-type and mutant enzymes were analyzed for each experiment by fitting the data to eq A or B by the use of a computer program (Cleland, 1979). (A) $v = V_{app}S/(K_M + S)$, and (B) $v = V_{app}S/(K_M + S + S^2/K_i)$, where v is the initial velocity, V_{app} is the apparent maximal velocity, S is the concentration of the varied substrate, K_M is the apparent Michaelis-Menten constant, and K_i is the apparent inhibition constant of the substrate. For the Mg^{2+} activation experiments, the apparent activation constant K_{act} was substituted for K_M in eq A. Data were fitted to eq A except where apparent substrate inhibition occurred when data were fitted to eq B: for the wild-type enzyme in the presence of Mn^{2+} and Co^{2+} when ATP or Rib-5-P is varied and for D220F and D221A enzymes when ATP is varied in the presence of Co^{2+}. The concentration of free Mg^{2+} in the assay incubations was calculated as previously described (Switzer, 1971; Nosal & Switzer, 1993).

RESULTS AND DISCUSSION

We chose to investigate the function of the PRPP binding site by analyzing the mutant enzymes described below. The amino acid replacements were chosen with the aid of Figure 1. Thus, the D220E, D224A, and D224S substitutions all occur within the compared sequences. The D220F replacement was expected to block binding of substrate to the PRPP binding site. We also wanted to investigate the effect of deleting the carboxylate of Asp-221 and chose the D221A replacement for this purpose.

Effects of Substitution of Asp-221 with Alanine. Comparison of the apparent kinetic constants of the D221A enzyme with those of the wild-type enzyme revealed decreases in V_{app} of the D221A enzyme with each of the divalent metal ions. However, with Co^{2+}, this decrease was moderate, about 9-fold, compared to about 3 orders of magnitude or more in the presence of Mn^{2+} or Mg^{2+} (Table 1). The K_M for ATP was comparable to those of the wild-type enzyme, apart from a slight increase in the presence of Mn^{2+} (Table 1). The K_M for Rib-5-P was greatly increased

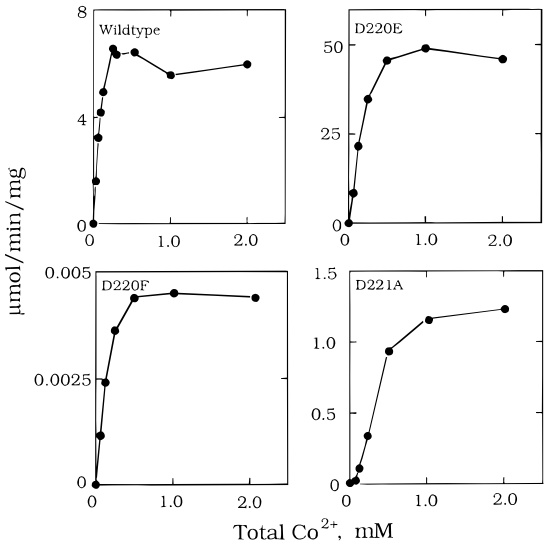

FIGURE 2: Effect of total Co^{2+} concentration on the activity of wild-type and mutant PRPP synthetases. Assays were performed as described in Experimental Procedures with 0.1 mM ATP and 10 mM Rib-5-P.

Table 2: Activation of Wild-Type and Mutant Enzymes by Free Mg^{2+} [a]

enzyme	K_{act} (mM)	enzyme	K_{act} (mM)
wild-type	0.37 ± 0.3	D221A	0.46 ± 0.05
D220E	0.76 ± 0.04	D224S	0.31 ± 0.01
D220F	0.31 ± 0.03		

[a] Assays were performed as described in Experimental Procedures with 0.1 mM ATP and 10 mM Rib-5-P. $MgCl_2$ varied from 0.1 to 10 mM, resulting in calculated free Mg^{2+} concentrations of 21 μM to 3.4 mM (Experimental Procedures).

in the presence of both Mn^{2+} and Co^{2+} but only about 2-fold in the presence of Mg^{2+} (Table 1). The relatively higher V_{app} determined for the D221A enzyme in the presence of Mn^{2+}, and especially Co^{2+}, when compared to that with Mg^{2+} seemed to be associated with increases in K_M for Rib-5-P. No substrate inhibition was observed for the D221A enzyme in the presence of Mn^{2+}, which for the wild-type enzyme gave K_i values for ATP and Rib-5-P of 2.7 ± 0.3 and 2.5 ± 0.8 mM, respectively. The wild-type enzyme also showed substrate inhibition by both ATP and Rib-5-P in the presence of Co^{2+} and K_i values of 0.91 ± 0.04 and 3.6 ± 0.5 mM, respectively. Co^{2+} itself also seemed to inhibit the enzyme at higher concentrations (Figure 2). However, we were unable to further test the significance of this observation by increasing the concentration of the metal ion due to the limited solubility of divalent ions in the assay incubation (see Experimental Procedures). In the presence of Co^{2+}, substrate inhibition of the D221A enzyme was only observed with ATP (K_i = 0.85 ± 0.06 mM). The K_{act} for Mg^{2+} of the D221A enzyme was similar to that of the wild-type enzyme (Table 2), but the D221A enzyme showed a decrease in the apparent affinity for Co^{2+}, evaluated by the dependence of activity on the concentration of Co^{2+} (Figure 2). Inhibition by excess Co^{2+} was not observed with the D221A enzyme. The D221A enzyme revealed a pH profile with a maximum of about pH 9 (data not shown).

The observed effects on K_M for Rib-5-P and V_{app} caused by the change of the side chain in the D221A enzyme depended greatly on the divalent cation present. This suggests that the Asp-221 was involved in the binding of Rib-5-P possibly via a divalent metal ion as indicated by the metal ion dependent changes in K_M for this substrate. Also, Asp-221 may occupy a key role in transition state formation, maybe by interacting with the same metal ion as suggested by the altered preference for divalent metal ions, with respect to V_{app}, compared to the preference of the wild-type enzyme (Table 1).

Effects of Substitution of Asp-220 with Glutamate or Phenylalanine. Apart from a slightly lower V_{app} and an increase in K_M for Rib-5-P of about 4-fold in the presence of Mg^{2+}, there were only minor differences between the D220E and the wild-type enzyme in the presence of Mg^{2+}, Mn^{2+}, or Cd^{2+} (Tables 1 and 2). However, in the presence of Co^{2+}, the D220E enzyme showed an 8-fold higher V_{app} and a 15-fold increase in K_M for Rib-5-P compared to the wild-type enzyme (Table 1). As a result, the kinetic properties of the D220E enzyme appeared almost independent of whether Mg^{2+}, Mn^{2+}, or Co^{2+} was present (Table 1). No substrate inhibition in the presence of Co^{2+} or Mn^{2+} was observed with the D220E enzyme, but as with wild-type enzyme, Co^{2+} seemed inhibitory, although at relatively higher concentrations of the cation than seen for the wild-type enzyme (Figure 2). The pH profile of the D220E enzyme revealed a pH optimum of about pH 9 (data not shown).

With Mg^{2+} or Mn^{2+}, the K_M values for ATP and Rib-5-P of the D220F enzyme were comparable to those of the wild-type enzyme but no substrate inhibition in the presence of Mn^{2+} was observed for the D220F enzyme. However, we found a decrease in V_{app} of about 4 orders of magnitude with either Mg^{2+} or Mn^{2+} present. The value of K_{act} for Mg^{2+} was comparable to that of the wild-type enzyme (Table 2). When the D220F enzyme was assayed in the presence of Co^{2+}, the K_M for Rib-5-P was increased and was similar to that of the D220E enzyme, and the V_{app} was decreased approximately 2500-fold compared to that of the wild-type enzyme (Table 1). The D220F enzyme only displayed substrate inhibition by ATP (K_i = 0.84 ± 0.03 mM) in the presence of Co^{2+}, and inhibition by this metal ion was not detectable (Figure 2). The pH optimum for D220F was about pH 9 (data not shown).

The results of analysis of the D220E enzyme indicated that Asp-220 may play a role in catalysis similar to that of Asp-221. In comparison to those of the wild-type enzyme, the changes in V_{app} and K_M for Rib-5-P in the presence of Mg^{2+} or Co^{2+} were less dramatic than those found for the D221A enzyme as could be expected from the relatively conservative substitution in the D220E enzyme. Apparently, the mere presence of the bulky aromatic side chain did not seriously affect the binding of substrates to the D220F enzyme. The observation that the D220F enzyme in comparison with the wild-type enzyme showed large decreases in V_{app} with all of the divalent metal ions and relatively small changes in K_M suggested that the Asp-220 residue might function primarily in the formation of the transition state. The only large increase in K_M relative to the wild-type enzyme was that for Rib-5-P in the presence of Co^{2+}, but this was also observed for the D220E enzyme.

Effects of Substitution of Asp-224 with Alanine or Serine. The D224A and D224S enzymes showed very similar kinetic properties (Table 1). In comparison with that of the wild-type enzyme under the same conditions, the V_{app} values for the D224A and D224S enzymes were reduced about 10−

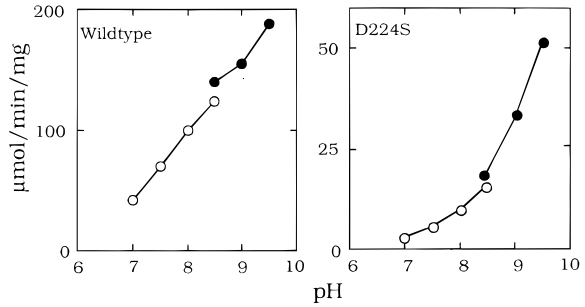

FIGURE 3: Effect of pH on the activity of wild-type and D224S PRPP synthetase. Assays were performed as described in Experimental Procedures: open symbols, triethanolamine buffer (pH range 7.0−8.5); and closed symbols, glycine buffer (pH range 8.5−9.5).

15-fold in the presence of Mg^{2+} and only about 2−3-fold in the presence of Mn^{2+}. The K_M values for ATP and Rib-5-P were comparable to those of the wild-type enzyme in the presence of either metal (Table 1). When the enzymes were assayed with Cd^{2+} present, the observed difference was a 4-fold increase in K_M for Rib-5-P for the D224A and D224S enzymes (Table 1). No substrate inhibition was observed in the presence of Mn^{2+}. The D224S enzyme also displayed substrate inhibition by both Rib-5-P and ATP like the wild-type enzyme in the presence of Co^{2+} (data not shown). The K_{act} for Mg^{2+} of the D224S enzyme was similar to that of the wild-type enzyme (Table 2). The activity of the D224S enzyme increased 17-fold by increasing in pH from 7 to 9.5 compard to only about 4-fold for the wild-type enzyme (Figure 3).

In contrast to the wild-type enzyme, the D224A and D224S enzymes were more active with Mn^{2+} than with Mg^{2+} at pH 8.5. This might indicate altered binding properties of a divalent metal ion as discussed above for the D221A, D220E, and D220F enzymes. The increase in K_M for Rib-5-P in the presence of Cd^{2+} might also indicate that the Asp-224 forms weak contacts with this substrate at least in the presence of this cation. However, the pH dependence of the D224S enzyme showed that the decrease in activity of about 10-fold at pH 8.5, compared to that of the wild-type enzyme, was partially overcome by increasing the pH and indicates that the major role of Asp-224 might be to increase the pH at the active site. The decrease in V_{app} of the D224A and D224S enzymes was much too low for Asp-224 to act as a catalytic base, but the residue might play a role in creating a basic environment in the active site maybe around the 1-hydroxyl of Rib-5-P as discussed below.

All the enzymes bound Mg^{2+} with about equal affinity (Table 2) so that if a metal ion binding site had been altered it was still able to bind this ion with unchanged affinity. It is possible that the altered preference for metal ions displayed by the mutant enzymes reflects the dynamics of a catalytically important metal ion and that the amino acid changes disrupt or interfere with a possible movement of metal ion during catalysis. This would apply to the D221A and D220F enzymes which exhibited very low V_{app} values with almost all the metal ions tested. The observation that Cd^{2+} and Co^{2+} changed the kinetic constants of the wild-type enzyme does not necessarily mean that they align fundamentally different from Mg^{2+} in the active site, but the effect could be attributed to the size of the ion. It is noteworthy that the D220E enzyme appeared to approach wild-type properties with the increase in radius of the metal ion going from Mg^{2+} to Cd^{2+} in the order $Mg^{2+} < Co^{2+} < Mn^{2+} < Cd^{2+}$. This was also true for the D224A and D224S enzymes with respect to V_{app} (Table 1).

The inhibition of the wild-type enzyme by the substrates in the presence of Co^{2+} and the apparent inhibition by the cation itself complicate the interpretation of the results obtained with the mutant enzymes, but the following three findings support the suggestion of a possible interaction of Asp-220 and Asp-221 with a complex of Rib-5-P and a divalent metal ion. (i) The D220E, D220F, and D221A enzymes did not show substrate inhibition or only showed inhibition at high concentrations of ATP. (ii) They showed little or no apparent inhibition by Co^{2+} itself, under the conditions examined. (iii) They all had increased K_M values for Rib-5-P in the presence of Co^{2+}.

Implications for the Function of Asp-220, Asp-221, and Asp-224 from Structures of Phosphoribosyltransferases. High-resolution structures of *S. typhimurium* orotate phosphoribosyltransferase complexed with PRPP and orotate (Scapin et al., 1995), human hypoxanthine-guanine phosphoribosyltransferase complexed with 5′-GMP (Eads et al., 1994), and *B. subtilis* galutamine PRPP amidotransferase complexed with the inhibitor 5′-AMP (Smith et al., 1994) have been published. These structures show that the PRPP binding site is involved in the binding of the 5-phosphoribosyl moiety of the ligands. The acidic residue equivalent to Asp-220 is postulated to interact with the 3-hydroxyl of the bound ligands. In orotate phosphoribosyltransferase, this residue also is postulated to interact with Mg^{2+} bound by the diphosphoryl group of PRPP via a bridging water molecule (Scapin et al., 1995). By analogy, the results obtained with the D220E and D220F enzymes might be explained by altered interactions with the 3-hydroxyl of Rib-5-P and also a divalent metal ion coordinated to what would be the β- and γ-phosphoryls of ATP. These interactions might play an important role in the stabilization of the transition state rather than binding to the ground state because the D220F enzyme was primarily decreased in V_{app}.

None of the high-resolution structures implied an interaction of the bound ligand with the residue equivalent to Asp-221, which is the most conserved aspartic acid residue of the PRPP binding site (Boer & Glickman, 1991). The Asp-221 side chain appears to be situated close to the 5-phosphoribosyl moiety of the bound ligands in orotate phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, and glutamine PRPP amidotransferase. As discussed above, it is possible that this residue plays a role in metal ion binding. Mutagenesis studies have revealed an importance in catalysis of the equivalent residues in orotate phosphoribosyltransferase (Asp-125) (Ozturk et al., 1995) and glutamine PRPP amidotransferase (Asp-346) (P. Hu and R. L. Switzer, personal communication). The PRPP binding site of human hypoxanthine-guanine phosphoribosyltransferase has an acidic residue equivalent to Asp-224. This residue (Asp-137) is suggested to interact with the ligand 5′-GMP via the phosphoryl group, but it is also close to N-7 of the guanine ring structure. The latter would be close to where the diphosphoryl group transfer takes place in PRPP synthetase.

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