Binding of Divalent Magnesium by *Escherichia coli* Phosphoribosyl Diphosphate Synthetase

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ABSTRACT: The mechanism of binding of the substrates Mg·ATP and ribose 5-phosphate as well as Mg^{2+} to the enzyme 5-phospho-D-ribosyl α-1-diphosphate synthetase from *Escherichia coli* has been analyzed. By use of the competive inhibitors of ATP and ribose 5-phosphate binding, α,*β*-methylene ATP and (+)-1-α,2-α,3-α-trihydroxy-4-*β*-cyclopentanemethanol 5-phosphate, respectively, the binding of Mg^{2+} and the substrates were determined to occur via a steady state ordered mechanism in which Mg^{2+} binds to the enzyme first and ribose 5-phosphate binds last. Mg^{2+} binding to the enzyme prior to the binding of substrates and products indicated a role of Mg^{2+} in preparing the active site of phosphoribosyl diphosphate synthetase for binding of the highly phosphorylated ligands Mg·ATP and phosphoribosyl diphosphate, as evaluated by analysis of the effects of the inhibitors adenosine and ribose 1,5-bisphosphate. Calcium ions, which inhibit the enzyme even in the presence of high concentrations of Mg^{2+}, appeared to compete with free Mg^{2+} for binding to its activator site on the enzyme. Analysis of the inhibition of Mg^{2+} binding by Mg·ADP indicated that Mg·ADP binding to the allosteric site may occur in competition with enzyme bound Mg^{2+}. Ligand binding studies showed that 1 mol of Mg·ATP was bound per mol of phosphoribosyl diphosphate synthetase subunit, which indicated that the allosteric sites of the multimeric enzyme were not made up by inactive catalytic sites.

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 + AMP, which proceeds by attack of the 1-hydroxyl of Rib-5-P on the *β*-phosphoryl of ATP resulting in the transfer of the *β*,*γ*-diphosphoryl moiety to Rib-5-P (Khorana et al., 1958; Miller et al., 1975). PRPP is a precursor of purine, pyrimidine, and pyridine nucleotides and in plants and microorganisms of the amino acids tryptophan and histidine (Jensen, 1983; Hove-Jensen, 1988, 1989). PRPP synthetase from the enteric bacteria *Escherichia coli* (Hove-Jensen, 1983, 1985; Hove-Jensen et al., 1986) and *Salmonella typhimurium* (Jochimsen et al., 1985; Bower et al., 1988) is encoded by the *prs* gene and have identical primary structures except for two conservative replacements (Bower et al., 1988, 1989). PRPP synthetase from enteric organisms as well as from human (Fox & Kelley, 1972; Nosal et al., 1993), rat (Roth et al., 1974), and *Bacillus subtilis* (Arnvig et al., 1990) have a requirement for Mg^{2+} to both form an Mg·ATP complex, and in addition free Mg^{2+} is an activator of the enzyme (Switzer, 1971; Bower et al., 1989). The *E. coli* and *S. typhimurium* enzymes have the highest activity in the presence of Mg^{2+}, but will accept other divalent metal ions (Switzer, 1969, 1971; Gibson & Switzer, 1980; Hove-Jensen et al., 1986; Willemoës et al., 1996). Calcium ions inhibit the enzyme even in the presence of excess Mg^{2+} (Switzer, 1971; Hove-Jensen et al., 1986). PRPP synthetase is also inhibited by Mg·ADP (Fox & Kelley, 1972; Switzer & Sogin, 1973; Roth & Deuel, 1974; Becker et al., 1975; Hove-Jensen et al., 1986; Ishijima et al., 1991; Nosal et al., 1993). For the *S. typhimurium* enzyme Mg·ADP inhibition occurs not only by competition with Mg·ATP for binding to the active site but also by binding to an allosteric site specific for Mg·ADP (Gibson et al., 1982). Although the mechanism of substrate binding has been studied for the human (Fox & Kelley, 1972) and the *S. typhimurium* enzymes (Switzer, 1971), no conclusive information on the mechanism of Mg^{2+} binding has been provided until now. In the present work we present a kinetic analysis of Mg^{2+} binding to PRPP synthetase from *E. coli* and suggest a mechanism for the binding of Mg^{2+}. We also propose a role for enzyme-bound Mg^{2+} in the binding of substrates, and we suggest that binding of Mg^{2+} plays a role in allosteric regulation of the enzyme by Mg·ADP.

EXPERIMENTAL PROCEDURES

Materials. Adenosine, Rib-5-P, Rib-1-P, and nucleotides were from Sigma, except ATP, which was from Boehringer. Rib-1,5-P_2 was a gift from H. Klenow (University of Copenhagen), and cRib-5-P (Parry et al., 1996) was a gift from R. J. Parry (Rice University, Texas). The concentration of nucleotides and adenosine at neutral pH was determined spectrophotometrically (Sambrook et al., 1989).

Protein Methods. PRPP synthetase was purified from the *E. coli* strain HO340/pHO11 (Hove-Jensen, 1985; Nilsson & Hove-Jensen, 1987). The purification procedure was essentially as described previously (Willemoës et al., 1996) except that between the two successive 20% ammonium sulfate precipitations the dissolved precipitate was stored overnight at −20 °C. This modification yielded a slightly purer enzyme and increased the specific activity. A similar effect has previously been observed with the *S. typhimurium* enzyme (Switzer & Gibson, 1978). The enzyme preparations

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[1] Abbreviations: PRPP, 5-phospho-D-ribosyl α-1-diphosphate; Rib-5-P, ribose 5-phosphate; cRib-5-P, (+)-1-α,2-α,3-α-trihydroxy-4-*β*-cyclopentanemethanol 5-phosphate; Rib-1-P, ribose 1-phosphate; Rib-1,5-P_2, ribose 1,5-bisphosphate; mATP, α,*β*-methylene ATP.

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were at least 98% pure as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Laemmli, 1970). The specific activity was approximately 170 μmol min^{-1} (mg of protein)$^{-1}$ when assayed with 2 mM ATP, 5 mM Rib-5-P, and 5 mM $MgCl_2$ as described below. 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. Amino acid analysis of PRPP synthetase used in ligand binding experiments was performed as described previously (Barkholt & Jensen, 1989).

Assay of PRPP Synthetase Activity. PRPP synthetase activity was assayed at 37 °C, pH 8.0, as previously described (Willemoës et al., 1996). The concentration of [γ-^{32}P]ATP, Rib-5-P, and $MgCl_2$ was varied as indicated, except that the concentration of nucleotides and free Mg^{2+} are presented after calculations performed as described below, unless otherwise noted.

Calculation of Concentrations of Magnesium Nucleotide Complexes, Free Mg^{2+}, and Maintenance of a Constant Concentration of Free Mg^{2+}. The concentrations of free Mg^{2+} and of the magnesium complexes of the assay components were calculated as described previously (Switzer, 1971; Switzer & Sogin, 1973; Nosal et al., 1993). The stability constants of the Mg·Rib-1,5-P_2, Mg·cRib-5-P, and Mg·Rib-1-P complexes were set to be equal to that of the Mg·Rib-5-P complex. In the case of Rib-1,5-P_2 equal and independent ability to bind magnesium ions by the two phosphoryls was assumed.

To vary the Mg·ATP concentration and at the same time maintain a constant free Mg^{2+} concentration within the range used in this work, we developed the following procedure in which UTP was used as an Mg^{2+} complexing analog of ATP: the total concentration of nucleotide (ATP + UTP) was held constant and equal to the concentration of nucleotide used to calculate the concentration of free Mg^{2+} under assay conditions. In the corresponding calculated concentration of Mg·nucleotide complex, the concentration of Mg·ATP was varied by varying the ATP content of total nucleotide. The concentration of mATP was included in these calculations, when present. When ADP was used, the Mg·ADP concentration was varied in a similar manner by the use of UDP. The choice of UTP or UDP for this purpose was justified by the observation that no significant inhibition of the reaction occurred when 2 mM UTP or 2 mM UDP (the highest concentrations used) was included in an assay containing 0.1 mM ATP, 2 mM Rib-5-P, and 5 mM $MgCl_2$ in excess over the nucleotide concentration. The stability constants of the Mg·UTP and Mg·UDP complexes were assumed to be equal to those of the corresponding adenine nucleotides, and the stability constant of Mg·mATP was assumed to be equal to that of Mg·ATP. In most experiments in which a saturating free Mg^{2+} concentration was used, UTP was omitted from the assay mix without serious effects on the calculated free magnesium ion concentration (±5−10%).

The computer program used for calculating the free Mg^{2+} concentration allows only for three potential magnesium ion ligands to be included (Switzer, 1971). Therefore, in Mg·ADP inhibition experiments the concentration of Rib-5-P was excluded from the calculations. In the absence of ADP, the effect on the calculated free Mg^{2+} concentration of the 0.5 mM Rib-5-P used in the experiments was negligible.

In binding studies the stability constant for Mg·ATP at 37 °C, pH 8.0 (Switzer, 1971), used for Mg·mATP was corrected to 25 °C, pH 8.3 (O'Sullivan & Smithers, 1979).

Ligand Binding Studies. [γ-^{32}P]mATP was prepared by the same procedure as [γ-^{32}P]ATP, except that ATP was replaced by mATP (Jensen et al., 1979). Ligand binding was performed by pressure dialysis (Paulus, 1969) at 25 °C as described previously (Levitzki & Koshland, 1972; Jensen, 1976). 80 μL of a 100 μL incubation containing 44 μM PRPP synthetase [subunit molecular mass 34 000 g/mol (Hove-Jensen et al., 1986)], 50 mM potassium phosphate, 25 mM triethanolamine buffer, pH 8.3, 3 mM $MgCl_2$, 0.5 mM Rib-5-P (when included), and 6.3−500 μM [γ-^{32}P]-mATP (approximately 2000 cpm) was applied to the pressure dialysis apparatus. Binding mixtures without enzyme served as background determinations. Under our conditions the maximum nucleotide concentration permitting a significant differentiation of radioactivity corresponding to enzyme bound nucleotide from the background determinations was 500 μM. The radioactivity retained on dialysis membranes was quantitated with a Packard 2000 Tri-Carb liquid scintillation analyzer. The scintillant was EcoscintA (National Diagnostics). For determination of radioactivity representing the total concentration of ligand, a 10 μL sample of the binding mixture together with 5 μL of 2 mM unlabeled mATP, added as a marker, was loaded on a polyethyleneimine−cellulose thin-layer chromatogram and developed in 0.3 M lithium chloride, 0.9 M acetic acid (Randerath & Randerath, 1965) to separate the nucleotide from $^{32}P_i$ remaining from the preparation of [γ-^{32}P]mATP. The mATP spot was identified under a UV mineral lamp (254 nm), cut out, and quantitated as above. In view of the high concentration of potassium phosphate during incubation, interference from binding of $^{32}P_i$ was considered negligible when determining the radioactivity of [γ-^{32}P]mATP bound to the enzyme.

Analysis of Data from Kinetic and Ligand Binding Studies. Results of the initial velocity experiments were analyzed by the use of a computer program, by fitting the data to one of the following equations (Cleland, 1979a). Equation 1 was used when varying two substrates in the presence of saturating concentrations of the third substrate, eq 2 for noncompetitive inhibition, eq 3 for competitive inhibition, and eq 4 for uncompetitive inhibition:

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = V_{app}S/[K_m(1 + I/K_{is}) + S(1 + I/K_{ii})] \quad (2)$$

$$v = V_{app}S/[K_m(1 + I/K_{is}) + S] \quad (3)$$

$$v = V_{app}S/[K_m + S(1 + I/K_{ii})] \quad (4)$$

where v is the initial velocity, V is the maximal velocity, K_{ia} is the dissociation constant for substrate A, and K_a and K_b are the Michaelis−Menten constants for substrate A or B, respectively. V_{app} is the apparent maximal velocity, and K_m is the apparent Michaelis−Menten constant for the varied substrate S. K_{ii} and K_{is} are the inhibitor constants for inhibitor (I) obtained from the effect on intercepts and slopes, respectively. In the experiments below data were analyzed with a computer program (UltraFit, version 2.11, BioSoft). When Mg^{2+} was varied in the presence of different concen-

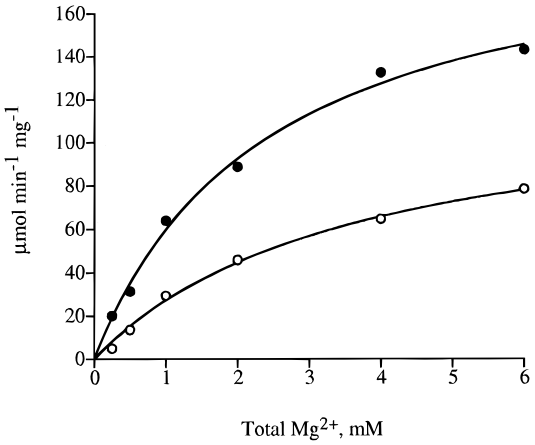

FIGURE 1: Activation of PRPP synthetase activity by Mg^{2+}. Assays were performed as described in Experimental Procedures. Total Mg^{2+} was varied as indicated in the presence of 10 mM Rib-5-P and either 0.1 mM ATP (open symbols) or 2 mM ATP (closed symbols).

trations of Mg·ADP a secondary plot was constructed with slopes and intercepts from straight lines obtained from double reciprocal plots. Reciprocal initial velocities were weighted assuming relative errors (Cleland, 1979a) and fitted to a straight line. The slopes versus Mg·ADP concentration were fitted without weighting to the equation

$$\text{slope} = Q[1 + (\text{I}/K'_{\text{is}})^n] \quad (5)$$

where Q is the intercept (here representing K_m/V_{app}), K'_{is} is the concentration of inhibitor that causes a doubling of the slope value and n is the apparent Hill coefficient. Intercepts versus Mg·ADP concentration were fitted without weighting to eq 5, keeping n fixed as 1 and replacing K'_{is} with K_{ii} (and Q representing $1/V_{app}$). All initial velocities are reported as μmol min^{-1} (mg of protein)$^{-1}$. Data from ligand binding studies were fitted without weighting to the equation

$$N = N_{max}L/(K_D + L) \quad (6)$$

where N is the concentration of ligand bound to the enzyme, N_{max} is the maximal concentration of binding sites, L is the concentration of unbound ligand, and K_D is the dissociation constant for the enzyme·ligand complex.

RESULTS

Kinetic Studies in the Absence of Inhibitors. Figure 1 shows the result of an experiment in which the total added Mg^{2+} was varied at two different ATP concentrations. At an ATP concentration of 0.1 mM excess Mg^{2+} appeared to be needed for full activity as proposed previously in addition to the formation of an Mg·ATP complex (Hove-Jensen et al., 1986). When total added Mg^{2+} was varied at 2 mM ATP, the enzyme activity responded to even small concentrations of this ion compared to the nucleotide concentration, so that inhibition by uncomplexed ATP was undetectable under normal assay conditions.

Considering Mg^{2+} a pseudo-substrate of the PRPP synthetase catalyzed reaction we performed two experiments in which either Mg·ATP and Rib-5-P or Mg·ATP and Mg^{2+} were varied in the presence of saturating concentrations of Mg^{2+} or Rib-5-P, respectively. Both experiments revealed a family of lines that intersected to the left of the ordinate in a double-reciprocal plot. The data were fitted to eq 1 for a sequential mechanism, and the calculated kinetic constants are given in Table 1. Unfortunately, the sensitivity of the assay prevented useful data from experiments in which Rib-5-P and Mg^{2+} were varied at a saturating Mg·ATP concentration (50−100 fold K_m). Therefore, it was not possible to determine the binding sequence from substrate saturation experiments alone (Viola & Cleland, 1982; Rudolph & Fromm, 1979). Assuming that Rib-5-P binding to the enzyme last is obligatory, as shown for the *S. typhimurium* enzyme (Switzer, 1971) a double-reciprocal plot of data, where Rib-5-P was varied in the presence of a fixed ratio of different concentrations of Mg·ATP and Mg^{2+} excluded an equilibrium random or an equilibrium ordered addition of the first two substrates as an intersection of the lines to the left of the ordinate was observed (Rudolph & Fromm, 1979).

Inhibition Studies with Mg·mATP, cRib-5-P, and Ca^{2+}. To study in more detail the mechanism of Mg^{2+} and substrate binding we employed Mg·mATP and cRib-5-P, which were shown to be competitive inhibitors of Mg·ATP and Rib-5-P binding, respectively. It appears that Rib-5-P binds after Mg·ATP binding, because cRib-5-P was an uncompetitive inhibitor with respect to Mg·ATP. Both Mg·mATP and cRib-5-P appeared to exhibit uncompetitive inhibition when Mg^{2+} was varied at different concentrations of the inhibitors. When Rib-5-P was varied (0.125−4 mM) in the presence of different concentrations of Mg·mATP (0−0.5 mM), substrate inhibition by Rib-5-P was observed that increased with the inhibitor concentration (data not shown). This observation is consistent with the order of binding suggested from the experiments above (Cleland, 1979b). The inhibition constants from experiments with Mg·mATP and cRib-5-P are given in Table 2. We conclude from these observations, that *E. coli* PRPP synthetase follows a steady state ordered sequential mechanism in which Mg^{2+} binds to the enzyme first followed by Mg·ATP and then Rib-5-P last (Fromm, 1979).

It was suggested that Ca^{2+} competes with free Mg^{2+} for the activator site, although this was not experimentally demonstrated (Switzer, 1971). Inhibition of PRPP synthetase by Ca^{2+} at various concentrations of Mg^{2+} is shown in Figure 2. It appears that Ca^{2+} is a competitive inhibitor of Mg^{2+} binding with a K_i of about 50 μM. Considering the relatively high total Mg^{2+} concentration, the formation of significant concentrations of inhibitory Ca·ATP is very unlikely.

Inhibition Studies with Adenosine, AMP, Rib-1,5-P_2, and Rib-1-P. Inhibition of Mg·ATP binding or free Mg^{2+} binding by adenosine was noncompetitive in both instances. Inhibition of free Mg^{2+} binding by AMP under the same experimental conditions was uncompetitive. The noncompetitive inhibition of Mg^{2+} binding by adenosine showed, in contrast to AMP that adenosine was not restricted to binding to the enzyme·Mg^{2+}·Mg·PRPP complex (Switzer, 1971), maybe due to the lack of a 5′-phosphoryl group. The inhibition constants are given in Table 2. Rib-1,5-P_2 was competitive versus Mg·ATP binding at a nonsaturating Rib-5-P concentration and demonstrated the importance of a phosphoryl at the 1-hydroxyl group of Rib-5-P for binding to the enzyme·Mg^{2+} complex. On the other hand, we did not detect inhibition by up to 20 mM Rib-1-P under the same experimental conditions as for Rib-1,5-P_2, demonstrating the importance of the 5-phosphoryl group in both PRPP and Rib-5-P, as previously suggested (Switzer & Simcox, 1974).

Table 1: Kinetic Constants for a Sequential Steady State Ordered Binding of Mg^{2+} and Substrates to *E. coli* PRPP Synthetase[a]

experiment	$K_{i(Mg^{2+})}$	$K_{Mg^{2+}}$	$K_{i(Mg\cdot ATP)}$	$K_{Mg\cdot ATP}$	$K_{Rib-5-P}$	V (μmol min^{-1} mg^{-1})
A	194 ± 56	85 ± 13		111 ± 17		168 ± 6.4
B			189 ± 70	113 ± 24	203 ± 46	181 ± 13

[a] In experiment A, Mg·ATP and free Mg^{2+} varied from 100 to 1000 μM and from 80 to 700 μM, respectively. The Rib-5-P concentration was 10 mM. In experiment B, Mg·ATP and Rib-5-P varied from 60 to 500 μM and from 130 to 1000 μM, respectively. The free Mg^{2+} concentration was 3.6 mM. All K values in μM.

Table 2: Inhibition Constants and Mode of Inhibition Determined for Various Inhibitors of PRPP Synthetase[a]

varied substrate	inhibitor[b]	mode of inhibition	K_{is}	K_{ii}	K'_{is}	n
Mg^{2+}	Mg·mATP[c]	uncompetitive		89 ± 6		
	cRib-5-P[d]	uncompetitive		266 ± 22		
	adenosine[e]	noncompetitive	6400 ± 2300	2100 ± 140		
	AMP[f]	uncompetitive		1790 ± 100		
	Mg·ADP (3A)	noncompetitive[g]		1400 ± 570	344 ± 16	3.4 ± 0.3
	Mg·ADP (3B)	noncompetitive[g]		130 ± 11	246 ± 3	3.11 ± 0.03
Mg·ATP	Mg·mATP[h]	competitive	77 ± 5			
	cRib-5-P[i]	uncompetitive		218 ± 11		
	adenosine[j]	noncompetitive	1400 ± 300	8000 ± 1900		
	Rib-1,5-P[k]	competitive	13700 ± 1700			
Rib-5-P	cRib-5-P[l]	competitive	237 ± 32			

[a] Inhibition constants were determined as described in Experimental Procedures. Standard errors are those given by the computer program. K values in μM. [b] Parentheses refer to the figure where assay conditions were described. [c] The concentration of free Mg^{2+} varied from 40 to 340 μM in the presence of 0−200 μM Mg·mATP, 100 μM Mg·ATP, and 1 mM Rib-5-P. [d] The concentration of free Mg^{2+} varied from 40 to 340 μM in the presence of 0−5 mM cRib-5-P, 900 μM Mg·ATP, and 200 μM Rib-5-P. [e] The concentration of Mg^{2+} varied from 78 to 780 μM in the presence of 0−8 mM adenosine, 100 μM Mg·ATP, and 1 mM Rib-5-P. [f] The concentration of Mg^{2+} varied from 78 to 780 μM in the presence of 0−2 mM AMP, 100 μM Mg·ATP, and 1 mM Rib-5-P. [g] Intercept-linear, slope-nonlinear noncompetitive. [h] The concentration of Mg·ATP varied from 60 to 500 μM in the presence of 0−200 μM Mg·mATP, 200 μM Rib-5-P, and 3.9 mM free Mg^{2+}. [i] The concentration of Mg·ATP varied from 30 to 250 μM in the presence of 0−5 mM cRib-5-P, 200 μM Rib-5-P, and 3.9 mM free Mg^{2+}. [j] The concentration of Mg·ATP varied from 0.125 to 1 mM in the presence of 0−4 mM adenosine, 2.6 mM Mg^{2+}, and 200 μM Rib-5-P. [k] The concentration of Mg·ATP varied from 32 to 250 μM in the presence of 0−6 mM Rib-1,5-P_2, 2.6 mM Mg^{2+}, and 200 μM Rib-5-P. [l] The concentration of Rib-5-P varied from 125 to 1000 μM in the presence of 0−800 μM cRib-5-P, 100 μM Mg·ATP, and 2.6 mM free Mg^{2+}.

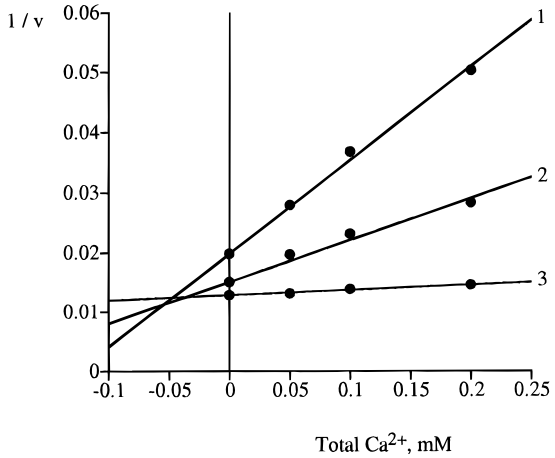

FIGURE 2: Inhibition of PRPP synthetase activity by Ca^{2+}. A Dixon plot (Dixon & Webb, 1964) of reciprocal initial velocities against total Ca^{2+} concentration is shown. Total Mg^{2+} concentrations were (1) 1.25, (2) 2.5, and (3) 5 mM. The concentrations of ATP and Rib-5-P were 0.1 and 1 mM, respectively.

Inhibition of Mg^{2+} Binding by Mg·ADP. Figure 3A,B shows the secondary plots of double-reciprocal plots of variation of Mg^{2+} at two different concentrations of Mg·ATP in the presence of different concentrations of Mg·ADP. In both experiments the inhibition by Mg·ADP was intercept-linear. Attempts to fit the slopes of both experiments as parabolic inhibition with the general equation (Cleland, 1963)

$$\text{slope} = A + BI + CI^2 \qquad (7)$$

failed to give a reasonable description of the data. Therefore the slopes in both cases were fitted to eq 5. The inhibition constants, which were obtained as described in Experimental Procedures, are given in Table 2.

Ligand Binding Studies. Results of ligand binding studies with Mg·mATP and PRPP synthetase in the absence or presence of Rib-5-P are shown in Figure 4. Apparently, Rib-5-P greatly decreased K_D for Mg·mATP. When data were fitted to eq 6 the K_D for Mg·mATP was calculated as 180 ± 30 and 5.8 ± 1.3 μM in the absence and presence of Rib-5-P, respectively, and N_{max} was calculated as 1.00 ± 0.08 and 1.00 ± 0.04 in the absence and presence of Rib-5-P. The concentration of PRPP synthetase subunit used in the binding experiments, determined by amino acid analysis, was 2.72 mg/mL, a value in excellent agreement with 2.85 mg/mL determined colorimetrically. The K_D for Mg·mATP in the absence of Rib-5-P agreed well with the value for $K_{i(Mg\cdot ATP)}$ determined from kinetic experiments at 37 °C (Table 1). We obtained no significant deviation from 1 for n, the apparent Hill coefficient when the data in Figure 4 were fitted to the Hill equation:

$$N = N_{max}L^n/(K_D + L^n) \qquad (8)$$

In addition the kinetic data obtained at 37 °C did not indicate any cooperativity of Mg·ATP binding.

DISCUSSION

The results presented in this report show that binding of free Mg^{2+} and the substrates Mg·ATP and Rib-5-P to *E. coli* PRPP synthetase most likely occurs via a steady state ordered sequential mechanism. As also found for PRPP synthetase from *S. typhimurium* (Switzer, 1971; Parry et al., 1996), we find that Mg·ATP binds before Rib-5-P. Earlier investiga-

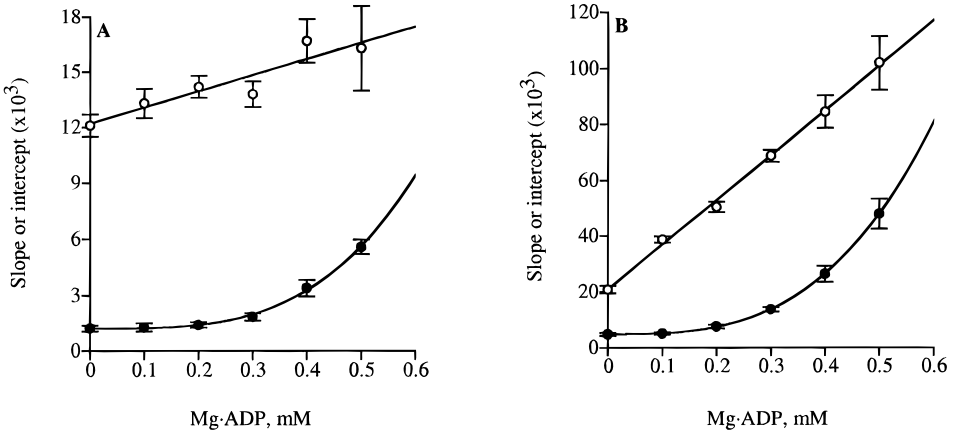

FIGURE 3: Inhibition of PRPP synthetase activity by Mg·ADP. Replots of slope values (closed symbols) and intercept values (open symbols) from double-reciprocal plots in which Mg^{2+} was varied at the indicated fixed concentrations of Mg·ADP are shown. The Mg·ATP concentration was 1.3 mM (A) or 0.1 mM (B). The free Mg^{2+} concentration varied from 0.08 to 1 mM, and the Rib-5-P concentration was 0.5 mM.

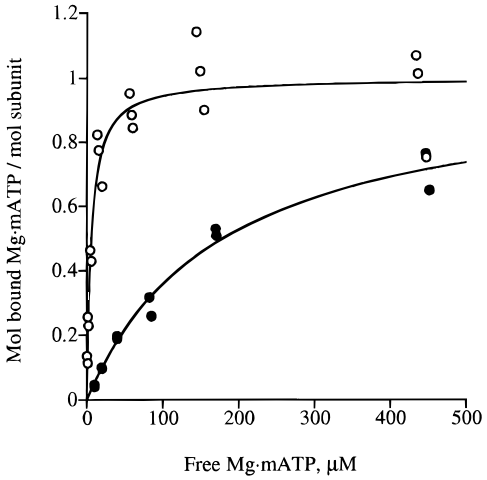

FIGURE 4: Binding of Mg·[γ-^{32}P]mATP to PRPP synthetase in the absence (closed symbols) and presence (open symbols) of 0.5 mM Rib-5-P. Ligand binding was performed as described in Experimental Procedures.

tions of Mg^{2+} binding to PRPP synthetase did not provide conclusive information on the kinetic mechanism, except that sequential patterns were observed (Switzer, 1971; Fox & Kelley, 1972). In the present work we have shown that the binding of Mg^{2+} to the free *E. coli* enzyme is obligatory prior to binding of the substrates.

The role of Mg^{2+} in the reaction once bound to the enzyme is still unclear. Evidence that a metal ion may coordinate to the α-phosphoryl of ATP has been provided by kinetic studies of the *S. typhimurium* enzyme employing the two enantiomers of adenosine 5′-*O*-(1-thiotriphosphate) (Gibson & Switzer, 1980). We have previously presented data that indicates a role for a divalent metal ion in the binding of Rib-5-P (Willemoës et al., 1996). The binding order of substrates together with the results of inhibition studies with adenosine and Rib-1,5-P_2 indicate a role of Mg^{2+} as a part of the binding site for Mg·ATP and PRPP. This might occur by interacting with the phosphoryls of these ligands and perhaps by inducing the proper conformation of the active site for the binding of substrates and products, as the metal ion appeared to add in steady state rather than rapid equilibrium.

For the *S. typhimurium* PRPP synthetase it was suggested from kinetic experiments that Mg·ADP binds to the active site in competition with Mg·ATP but that an observed nonlinearity of slopes and intercepts introduced by saturation with Rib-5-P was likely to be the consequence of Mg·ADP binding to an allosteric site (Switzer & Sogin, 1973). Later, equilibrium binding studies confirmed this mechanism of inhibition (Gibson et al., 1982). By inference, dependency on Rib-5-P is excluded for the observed slope effect of Mg·ADP on Mg^{2+} binding demonstrated in Figure 3, as our observations suggests that Rib-5-P binding occurs only after binding of Mg^{2+} to PRPP synthetase. The characteristics of the slope effect in Figure 3, indicate that Mg·ADP binding to the allosteric site of PRPP synthetase occurs in competition with binding of Mg^{2+} to the free enzyme. First, the calculated constants (K'_{is}) and Hill coefficients (n) for the slope effect given in Table 2 match the corresponding constants from binding studies of Mg·ADP to the allosteric site with an $S_{0.5}$ (the concentration of ligand corresponding to 50% saturation) of 150−300 μM and Hill coefficients between 3 and 4 (Gibson et al., 1982). Although this does not prove binding of Mg·ADP to the allosteric site in competition with Mg^{2+} binding, the similarity of the constants is striking. Second, the observed slope effect (Figure 3) is unlikely to be the consequence of Mg·ADP binding to the Mg·ATP site in a manner that would be competitive with Mg^{2+} binding, because both AMP and Mg·ATP apparently only bind to this site after binding of Mg^{2+} to the enzyme. Saturating Mg^{2+} levels, which correspond to the intercepts (Figure 3), apparently render Mg·ADP inhibition competitive with Mg·ATP binding, as judged from the change in the intercept effects with Mg·ATP concentration (Table 2). The linearity of the intercepts is probably due to the unsaturating Rib-5-P concentration used in both experiments. At saturating Rib-5-P nonlinearity of the intercepts would be expected as a result of Rib-5-P induced binding of Mg·ADP to the allosteric site. It is not clear why Mg·ADP binds both to the allosteric site of the free enzyme and to enzyme with Mg^{2+}, Mg·ATP (or Mg·ADP), and Rib-5-P bound to the active site. However additional experimental evidence indicate that Mg^{2+} binding may be the target of regulation of PRPP synthetase activity by Mg·ADP. It was shown for *S. typhimurium* PRPP synthetase that Ca^{2+} diminished the observed nonlinearity of slope and intercept effects seen for inhibition of Mg·ATP binding by Mg·ADP (Switzer & Sogin, 1973). Since Ca^{2+} apparently binds in competition with Mg^{2+} (Figure 2), it suggests a connection between Mg·ADP inhibition and Mg^{2+} binding. Future experiments

will have to focus on the mechanism of Mg·ADP inhibition and hopefully they will offer a rational explanation for the observed slope effect in Figure 3.

Ligand binding has been previously used in the analysis of *S. typhimurium* PRPP synthetase (Gibson et al., 1982). For both the *S. typhimurium* and the *E. coli* enzyme the K_D value for Mg·mATP decreased in the presence of Rib-5-P. Mg·mATP appeared to bind to *S. typhimurium* PRPP synthetase in a cooperative manner, in contrast to what was found in the present work. It is likely that this cooperative binding by the *S. typhimurium* enzyme was an effect of the temperature at which the experiments were performed, 0 °C for the *S. typhimurium* enzyme and 25 °C for the *E. coli* enzyme. Furthermore, the *S. typhimurium* enzyme revealed a saturating binding of 0.6−0.7 mol of Mg·ATP or Mg·ADP per mol of PRPP synthetase subunit in the absence of Rib-5-P and 1.2−1.4 mol of Mg·ADP per mol of subunit PRPP synthetase in the presence of Rib-5-P, which might indicate that the allosteric site for Mg·ADP binding is in fact inactive catalytic nucleotide binding sites, as the *S. typhimurium* enzyme is known to be a multimer consisting of five or six identical subunits (Schubert et al., 1975). Such a model implies that only half of the sites would bind substrates, giving a ratio of 0.5 mol of Mg·ATP bound per mol of subunit. However, our finding with the *E. coli* enzyme does not support this model and, rather, the allosteric sites probably are different from the catalytic sites not only kinetically but also physically. We can think of one plausible explanation for the difference in ligand to subunit stochiometry in the previous and the present work: the specific activity of *S. typhimurium* PRPP synthetase was 115 μmol min^{-1} mg^{-1} compared to 170 μmol min^{-1} mg^{-1} of the *E. coli* enzyme used in the present work. This gives a ratio of 0.68 active sites per monomer of *S. typhimurium* PRPP synthetase, which is close to the ligand to subunit stochiometry that was actually found.

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SUPPORTING INFORMATION AVAILABLE

Four figures: double-reciprocal plots of initial velocity of PRPP synthetase activity against Mg·ATP concentration at different fixed concentrations of free Mg^{2+} or Rib-5-P and saturating concentrations of Rib-5-P or free Mg^{2+}, respectively; double-reciprocal plot of initial velocity of PRPP synthetase activity against Rib-5-P concentration at different fixed concentrations of Mg·ATP and free Mg^{2+}; double-reciprocal plots of initial velocity against substrate concentration in the presence of Mg·mATP or cRib-5-P; double-reciprocal plots of initial velocity against free Mg^{2+} concentration in the presence of Mg·mATP or cRib-5-P (4 pages). Ordering information is given on any current masthead page.

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