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Stereochemistry of Binding of Thiophosphate Analogs of ATP and ADP to Carbamate Kinase, Glutamine Synthetase, and Carbamoyl-Phosphate Synthetase[1]

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Thiophosphate analogs of adenine nucleotides were used to establish the absolute stereochemistry of nucleotide substrates in the reactions of carbamate kinase (*Streptococcus faecalis*), unadenylylated glutamine synthetase (*Escherichia coli*), and carbamoyl-phosphate synthetase (*E. coli*). ^{31}P NMR was used to determine that carbamate kinase uses the B isomer of Ado-5′-(2-thioPPP) in the presence of Mg^{2+}. The stereospecificity of the reaction with carbamate kinase was not reversed by Cd^{2+} suggesting that the metal ion does not bind to the β-phosphoryl group or that both Mg^{2+} and Cd^{2+} bind to the sulfur atom. Carbamate kinase uses both A and B isomers of Ado-5′-(1-thioPP) with Mg^{2+} and Cd^{2+}. We have previously reported that carbamoyl-phosphate synthetase uses the A isomer of Ado-5′-(2-thioPPP) at both ATP sites with Mg^{2+} (Raushel *et al.*, 1978 *J. Biol. Chem.* **253,** 6627). Current experiments show that the stereospecificity is reversed by Cd^{2+} and that both A and B isomers are used when Zn^{2+} is present. With Ado-5′-(1-thioPPP), the B isomer is used with Mg^{2+}, the A isomer with Cd^{2+}, and both isomers with Zn^{2+}. Neither carbamate kinase nor carbamoyl-phosphate synthetase utilized Co(III)$(NH_3)_4$ATP as a substrate and thus we can only speculate that the Δ chelate ring configuration is the chelate structure utilized by carbamoyl-phosphate synthetase (based on the analogy between thiophosphate-ATP analogs and Co^{3+}-ATP analogs utilized by hexokinase (E. K. Jaffe, and M. Cohn, 1978 *Biochemistry* **17,** 652). If the sulfur of the β-phosphoryl of Ado-5′-(2-thioPPP) binds to the metal ion with carbamate kinase, then the Δ chelate ring is also used in this enzyme that catalyzes one of the steps in the overall reaction catalyzed by carbamoyl-phosphate synthetase. Glutamine synthetase reacts with the B isomer of both Ado-5′-(2-thioPPP) and Ado-5′-(1-thioPPP) in the presence of Mg^{2+}. When Co^{2+} is used with this enzyme the A and B isomers of both thio-ATP compounds are substrates. Co(III)$(NH_3)_4$ATP is not a substrate for glutamine synthetase. Glutamine synthetase is therefore different from the two previously mentioned enzymes in that it used the opposite Λ ring configuration for the metal-ATP chelate.

Recently substantial experimentation has been focused on the enzymatic mechanisms of phosphoryl transfer reactions of adenine nucleotides. The stereochemistry of metal–nucleotide binding to enzymes has been approached by using (i) various substitution-inert analogs (Co^{3+}-ATP and Cr^{3+}-ATP, (1–4)) and (ii) thiophosphate analogs of adenine nucleotides (5–9). The stereochemistry of phosphoryl transfer reactions has been studied with thio-ATP analogs as well as with specific ^{18}O and ^{17}O substitution at phosphorus (10–12).

Adenosine 5′-(2-thiotriphosphate) (Ado-5′-(2-thioPPP)) and adenosine 5′-(1-thiotriphosphate (Ado-5′-(1-thioPPP)) exist as pairs of diastereomers, A and B, as defined by Eckstein and Goody (6). They showed that pyruvate kinase and acetate kinase react with the A form of Ado-5′-(1-thioPP) to produce the A isomer of Ado-5′-(1-

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thioPPP). Arginine kinase produces the B isomer by reaction with the B isomer of Ado-5′-(1-thioPP). In the case of Ado-5′-(2-thioPPP), isomer A is produced upon reaction of pyruvate kinase with Ado-5′-(2-thioPP), while the B isomer is produced by acetate kinase.

Sulfur substitution causes a considerable downfield shift of the phosphorus resonance signal in the NMR spectrum of the nucleotides (7, 13). The chemical shift is extremely sensitive to pH, ionic strength, metal ion binding, concentration, temperature, and the orientation of the P–S bond with respect to the phosphorus chain. With the diastereomers of Ado-5′-(1-thioPP), Ado-5′-(1-thioPPP), and Ado-5′-(2-thioPPP), there is sufficient difference in geometry so that small differences in chemical shifts are observable between the A and B forms. Sheu and Frey (7) and Jaffe and Cohn (13) have demonstrated the use of ^{31}P NMR to characterize the A and B forms of Ado-5′-(1-thioPPP) and Ado-5′-(2-thioPPP), respectively. The enzymatic specificity of the various isomers may also be used to identify them (6.)

The stereospecificity of thio-ATP isomers used in an enzymatic reaction is sometimes dependent on the metal ion that is employed. Inversion of stereospecificity is observed with yeast hexokinase and rabbit muscle pyruvate kinase (8) when Cd^{2+} is substituted for Mg^{2+}. This results because Mg^{2+} is binding to oxygen ligands and Cd^{2+} to sulfur ligands. The metal chelate ring is the same stereochemistry, however. The absolute stereochemistry of ATP in the hexokinase reaction was established by Cornelius and Cleland (2) using Co(III)-$(NH_3)_4$ATP. The active form of Co(III)-$(NH_3)_4$ATP in the hexokinase reaction has the Λ screw sense (1). The enzyme uses the B isomer of Ado-5′-(2-thioPPP) when Mg^{2+} is used as the metal ion. Using this information, Jaffe and Cohn (8) assigned the absolute stereochemistry of the A and B diastereomers of Ado-5′-(2-thioPPP), given in Fig. 1. Burgers and Eckstein (14) and Bryant and Benkovic (15) have determined the absolute stereochemistry of the A and B isomers of Ado-5′-(1-thioPPP) (Fig. 2).

In this report we outline our studies on the stereochemistry of nucleotide analogs in the reactions of carbamate kinase (Eq. [1]) from *Streptococcus faecalis*, glutamine synthetase (Eq. [2]) from *Escherichia coli*, and carbamoyl-phosphate synthetase (Eq. [3]) from *E. coli*.

$$NH_2—\overset{\overset{O}{\|}}{C}—O—PO_3^{2-} + ADP \rightleftarrows NH_2—\overset{\overset{O}{\|}}{C}—O^- + ATP \quad [1]$$

$$ATP + \text{L-glutamate} + NH_4^+ \rightarrow \text{L-glutamine} + ADP + P_i \quad [2]$$

$$2ATP + HCO_3^- + \text{L-glutamine} \rightarrow 2ADP + P_i + \text{carbamoyl-P} + \text{L-glutamate} \quad [3]$$

MATERIALS AND METHODS

Materials

Ado-5′-thioP and Ado-5′-(2-thioPP) were purchased from Boehringer-Mannheim. Phosphoenol pyruvate (monocyclohexylammonium salt) (PEP),[4] lithium potassium acetyl phosphate, carbamyl phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), L-glutamate, dithiothreitol, glucose, glucose 6-phosphate dehydrogenase, hexokinase, adenylate kinase, pyruvate kinase, and acetate kinase were purchased from Sigma. Polyethyleneimine(PEI)-cellulose sheets for thin layer chromatography were obtained from Scientific Products. Ammonium carbamate was supplied by ICN. Carbamate kinase (16) and glutamine synthetase (17) were isolated as previously described.

Methods

Preparation of thio-ATP analogs. Ado-5′-(1-thioPP) and Ado-5′-(1-thioPPP) were a gift from Mr. Floyd Bryant of The Pennsylvania State University Chemistry Department, who prepared them chemically. The Ado-5′-(1-thioPPP) isomers were separated on a DEAE Sephadex column and obtained in essentially pure form as shown by their NMR spectra. The A isomer of Ado-5′-(1-thioPPP) was also enzymatically

[4] Abbreviations used: PEP, phosphoenol pyruvate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI, polyethyleneimine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

prepared by the coupled reactions of adenylate and pyruvate kinases, as described by Jaffe and Cohn (13). The reaction mixture contained 20 mM Ado-(5′-thioP), 25 mM $MgCl_2$, 0.5 mM ATP, 45 mM PEP, 100 mM KCl, 1 mM dithiothreitol, 100 mM Hepes buffer (pH = 8.0), 0.2 mg adenylate kinase, and 0.1 mg pyruvate kinase in a total volume of 1.0 ml. The reaction mixture was incubated at 37°C for 6 h and terminated by addition of 25 mM EDTA or by passing the mixture through Chelex.

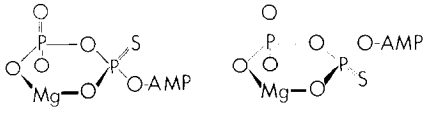
Ado-5′-(2-thioPPP) (A isomer) was prepared enzymatically by the method of Eckstein and Goody (6). The reaction mixture contained 20 mM Ado-5′-(2-thioPP), 25 mM PEP, 100 mM Hepes buffer (pH = 8.0), 25 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and 1 mg of pyruvate kinase in a total volume of 1 ml. The reaction was run for 2 h and followed by thin layer chromatography as described below.

The B isomer of Ado-5′-(2-thioPPP) was prepared by the acetate kinase reaction (6). The reaction mixture contained 20 mM Ado-5′-(2-thioPP), 25 mM acetyl phosphate, 100 mM Hepes buffer (pH = 7.4), 100 mM KCl, 25 mM $MgCl_2$, and 0.3 mg of acetate kinase in a total volume of 1 ml. The progress of the reaction was monitored by thin layer chromatography.

Metal ion specificity. The reaction of Ado-5′-(2-thioPP) with carbamate kinase was conducted using Cd^{2+} or Mg^{2+} as the divalent metal ion. The reaction mixture contained 10 mM Ado-5′-(2-thioPP), 10 mM carbamyl phosphate, 100 mM Hepes buffer (pH = 7.5), 500 units of carbamate kinase, and 1 mM $Cd(CH_3CO_2)_2$ or 10 mM $MgCl_2$ in 1 ml. The reaction was allowed to proceed for 30 min and was terminated by the addition of EDTA.

Thin layer chromatography. Aliquots, 5 μl, of the various reaction mixtures described above were withdrawn at 30-min intervals and spotted on strips of PEI-cellulose. The strips were eluted for 45 min with 2 M LiCl or 0.75 M KH_2PO_4 (pH = 3.5). The components were visualized under a uv light and identified by their R_f values (5). The reactions were terminated when the spots corresponding to the reactants were no longer visible. The reactions of carbamate kinase and glutamate synthetase with the various isomers were also monitored by the same method.

Kinetic assays. The reactions of the various isomers



A B

FIG. 1. Absolute stereochemistry of the A and B isomers of Ado-5′-(2-thioPPP). A isomer (left) = *S*, B isomer (right) = *R*.

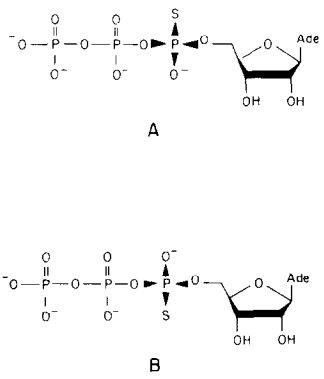


FIG. 2. Absolute stereochemistry of the A and B isomers of Ado-5′-(1-thioPPP). A isomer (top) = *S*, B isomer (bottom) = *R*. Ade = adenine.

with carbamate kinase were followed spectrophotometrically in a Beckman Model DU spectrometer equipped with a Gilford photomultiplier and a Fisher recorder. The reverse reaction of Eq. [1] was followed as previously described (16). The reaction mixture contained the following: 10 mM $MgCl_2$, 1 mM PEP, 100 mM ammonium carbamate, 100 μg of pyruvate kinase, 50 μg of lactate dehydrogenase, 0.1 mM NADH, and varying amounts of Ado-5-(2-thioPPP) in a volume of 1.0 ml. The mixture was incubated at 37°C and then 1 unit of carbamate kinase was added to initiate the reaction.

The reaction mixture for following the forward reaction (Eq. [1]) contained the following in 1 ml: 10 mM $MgCl_2$, 5 mM glucose, 20 μg each of hexokinase and glucose 6-phosphate dehydrogenase, 10 mM carbamyl phosphate, 0.5 mM NADP and varying amounts of the nucleotide diphosphate. Carbamate kinase was added to the reaction mixture, incubated at 37°C, and the reaction followed by the change in the absorbance at 340 nm. The kinetic data were analyzed using the FORTRAN programs of Cleland (18). The K_m and V for ATP and ADP and the corresponding thio analogs were compared.

Glutamine synthetase was assayed by the procedure of Woolfolk *et al.* (19), which monitors the formation of inorganic phosphate in the reaction. The reaction mixture contained 50 mM glutamate, 50 mM $MgCl_2$, 100 mM NH_4Cl, 100 mM KCl, and the thiotriphosphate under study in a total volume of 1 ml at pH 7.5. The above mixture was incubated at 37°C and the reaction initiated by the addition of glutamine synthetase. Aliquots of the mixture were withdrawn at various intervals and the reaction stopped by the addition of ~1% $FeSO_4$ in dilute H_2SO_4. The concentration of phosphate was determined by adding a 7% solution of ammonium molybdate and measuring the absorbance at 660 nm. The data were analyzed as in the case of carbamate kinase and compared with the reaction of ATP.

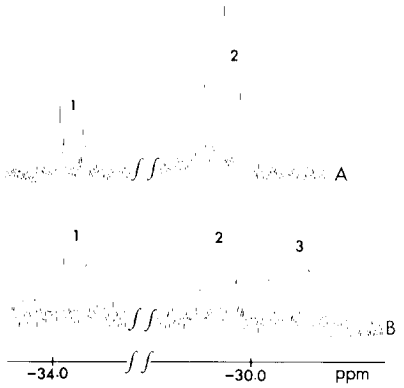


FIG. 3. The β-phosphoryl region of the ^{31}P NMR spectra of Ado-5′-(2-thioPPP) (B isomer) produced by carbamate kinase (A), and its mixture with authentic A isomer (B) at 145.6 MHz. NMR parameters: pulse width = 45°, pulse delay = 6 s, number of scans = 100. The numbered peaks are: 1, the β-phosphoryl of Ado-5′-(2-thioPP); 2, the β-phosphoryl of Ado-5′-(2-thioPPP) (B isomer); 3, the β-phosphoryl of Ado-5′-(2-thioPPP) (A isomer).

To test for substrate activity of thio analogs of ATP with carbamoyl-phosphate synthetase (9), enzyme (0.1 mg) was incubated in a volume of 0.051 ml with 10 mM HCO_3^-, 50 mM Hepes, pH 7.5, 100 mM KCl, 10 mM L-ornithine, 2–3 mM Ado-5′-(2-thioPPP) or Ado-5′-(1-thioPPP), and 10 mM glutamine. When Mg^{2+} was used as the activating divalent cation it was used at a concentration of 10 mM in excess of the nucleotide concentration. Zn^{2+} and Cd^{2+} were used at a concentration equal to that of the nucleotide concentration. Substrate activity was determined by spotting aliquots at various times on plates of PEI-cellulose as described before.

NMR spectra. ^{31}P NMR spectra were recorded at 40 MHz on a JEOL PS-100 spectrometer operating in the Fourier transform mode. A few spectra were also recorded at 81 MHz on a Bruker WP 200 NMR and at 145 MHz on a Bruker WH 360 at the Regional NMR facility of the University of Pennsylvania. All samples contained EDTA and 20% D_2O. All spectra were recorded at pH 8 in 10-mm tubes with the field locked on deuterium, and with broad band proton decoupling. For a solution containing 20 mM total adenine concentration, the following conditions were used at 40 MHz: 45° pulse width, 3-s pulse delay, 16K data points, and 2000–4000 transients. The sweep width employed was 3000 Hz for the 2-thiophosphates and 4000 Hz for the 1-thiophosphates.

Co(III)$(NH_3)_4$ATP experiments. The method of Cornelius and Cleland (2) was used to prepare and characterize Co(III)$(NH_3)_4$ATP. These authors have shown that the two isomers of this ATP analog differ in the sign of their CD spectra at ~550 nm. To test whether either or both of the isomers were substrates for the three enzymes studied herein, the following experimental protocol was used: 5 ml of a mixture containing 80 mM Co(III)$(NH_3)_4$ATP, 100 mM L-glutamate, 150 mM NH_4Cl, 100 mM KCl 4 mg/ml unadenylylated glutamine synthetase, and 5 mM Pipes at pH 7.0 was incubated at 25 or 37°C. The CD spectrum was recorded in a JASCO Model J-20 spectropolarimeter after 1–8 h. A control experiment was conducted with hexokinase under the conditions described by Cornelius and Cleland (2) and we quantitatively reproduced their results. For reaction with carbamate kinase, 80 mM Co(III)$(NH_3)_4$ATP, 100 mM ammonium carbamate, 5 mM Pipes, pH 7.5, and 3 mg/ml enzyme were incubated at 25 or 37°C and the CD spectra recorded as described above. For reaction with carbamoyl-phosphate synthetase, 80 mM Co(III)-$(NH_3)_4$ATP, 20 mM HCO_3^-, 20 mM L-glutamine, 10 mM $MgCl_2$, 10 mM ornithine, 100 mM KCl, 10 mM Pipes, pH 7.5, and 1 mg/ml enzyme were incubated at 25 or 37°C and CD spectra recorded as above.

RESULTS

Carbamate Kinase

The ^{31}P NMR spectra of authentic samples of the A and B isomers of Ado-5′-(2-thioPPP) have been reported (7, 13). Figure 3A shows the β phosphoryl region of the NMR spectrum (~30 ppm) of Ado-5′-(2-thioPPP) (unpurified reaction mixture) produced by carbamate kinase (Eq. [1]) while Fig. 3B shows the spectrum of the product

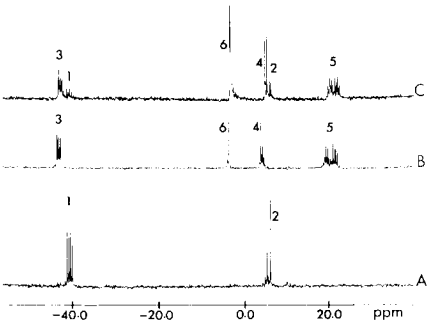


FIG. 4. ^{31}P NMR spectra of the mixture of isomers of Ado-5′-(1-thioPP) (A), the product of their reaction with carbamate kinase (B), and a mixture of the two (C) at 40.3 MHz. NMR parameters: 3-s delay, 4000-Hz sweep width, 45° pulse width, 4000 scans. Total thiophosphate concentration = 10 mM. The numbered peaks are: 1, α-phosphoryl, 2, β-phosphoryl of Ado-5′-(1-thioPP) (A and B isomers); 3, α-phosphoryl, 4, γ-phosphoryl, 5, β-phosphoryl of Ado-5′-(1-thioPPP) (A and B isomers); 6, inorganic phosphate.

mixed with an authentic sample of the A isomer. Comparison of the spectra demonstrates that carbamate kinase produces the B isomer of Ado-5′-(2-thioPPP). The limit of detection of the A isomer is ~5% under the experimental conditions employed for the reaction in Fig. 3A. The two triplets in the β region are separated by about 0.1 ppm, which is the reported chemical shift difference between the two isomers (13). When the two pure isomers of Ado-5′-(2-thioPPP) were incubated separately with carbamate and the enzyme for various periods of time and the reaction mixtures then spotted on PEI-cellulose thin layer strips, only the B isomer was a substrate as judged by disappearance of ATP and appearance of ADP. This supports the conclusion reached from the NMR experiment.

As discussed by Jaffe and Cohn (13) with the appropriate criteria presented, Cd^{2+} should preferentially bind to the sulfur atom of Ado-5′-(2-thioPPP) while Mg^{2+} shows a preference for binding to oxygen ligands. When Cd^{2+} was used as the divalent cation with carbamate kinase, only the B isomer of Ado-5′-(2-thioPPP) was made from Ado-5′-(2-thioPP) and carbamoyl phosphate. This is unlike hexokinase (8) where inversion of stereochemistry was observed when Cd^{2+} replaced Mg^{2+}.

Figure 4A shows the NMR spectrum of the mixture of isomers of Ado-5′-(1-thioPP), which is characterized by the two sets of doublets around 42 ppm, separated by 0.4 ppm. When carbamate kinase reacts with the above mixture, the product yields the spectrum given in Fig. 4B, the α region (~44 ppm) of which is similar to 4A. When more Ado-5′-(1-thioPP) was added to the above solution, the spectrum of Fig. 4C was obtained. The presence of four doublets in the α region leads to the conclusion that carbamate kinase reacts with both isomers of Ado-5′-(1-thioPP). However, less than 10% reaction was detected when carbamate kinase (1 mg) was incubated with 10 mM solutions of the isomers of Ado-5′-(1-thioPP) at 37°C for 4 h. It was also concluded from thin layer chromatography that carbamate kinase reacts with both isomers of Ado-5′-(1-thioPP) with Mg^{2+}.

When Co(III)$(NH_3)_4$ATP was tested as a substrate as described under Methods, no reaction could be detected as judged by the lack of change in the CD spectrum (2).

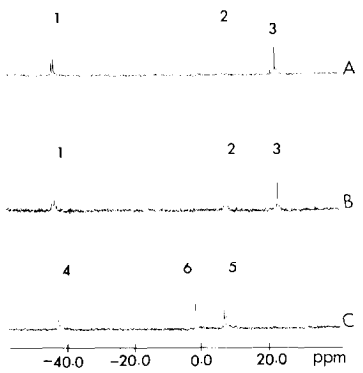


FIG. 5. ^{31}P NMR spectra of the A isomer (A) and the B isomer (B), of Ado-5′-(1-thioPPP) and the product of the reaction of the B isomer with glutamine synthetase (C) at 40.3 MHz. NMR parameters: 45° pulse width, 3-s pulse delay, 4000-Hz sweep width, 3000 scans. The numbered peaks are: 1, α-phosphoryl, 2, γ-phosphoryl and 3, β-phosphoryl of Ado-5′-(1-thioPPP); 4, α-phosphoryl, 5, β-phosphoryl of Ado-5′-(1-thioPP); 6, inorganic phosphate.

Glutamine Synthetase

When the A and B isomers of Ado-5′-(2-thioPPP) were tested by thin layer chromatography as substrates for unadenylylated glutamine synthetase of *E. coli* with Mg^{2+} as metal ion cofactor, the reaction was found to proceed only with the B isomer. The same conclusion was reached when the reaction was followed by ^{31}P NMR.

Figures 5A and B show the ^{31}P NMR spectra of the A and B isomers of Ado-5′-(1-thioPPP). After incubation with glutamine synthetase, the spectrum of the A isomer was unchanged, while that of the B isomer showed complete conversion to the diphosphate (Fig. 5C). The stereospecificity of glutamine synthetase for the B isomer of Ado-5′-(1-thioPPP) was confirmed by thin layer chromatography. When Cd^{2+} was tried as the divalent cation with glutamine synthetase none of the thio analogs of ATP were substrates. The normal catalytic activity with Cd^{2+} and ATP is $<1\%$ of that with Mg^{2+} (20) so it is not surprising that activity was not observed under our experimental conditions. Also the thio-ATP analogs usually

have lower V values than ATP which also vitiates the reaction of glutamine synthetase with these thio analogs.

Unadenylylated glutamine synthetase can use Co^{2+} as a substitute for Mg^{2+}. With Co^{2+}, the enzyme uses both A and B isomers of Ado-5′-(1-thioPPP) and Ado-5′-(2-thioPPP) as determined by following the reaction chromatographically.

The kinetic parameters obtained from carbamate kinase and glutamine synthetase assays are summarized in Table I. The velocity of the reaction of the B isomer of Ado-5′-(2-thioPPP) with glutamine synthetase was found to be less than 1% of the velocity of the ATP reaction. K_m for this substrate could not be accurately determined. All the thio analogs were found to have low relative velocities for their reactions with carbamate kinase and glutamine synthetase, except for the reaction of the B isomer of Ado-5′-(1-thioPPP) with glutamine synthetase. In this case the relative velocity was 55%. An apparent K_m (Table I) was obtained for the reaction of carbamate kinase with a mixture of isomers of Ado-5′-(1-thioPP), since the coupling enzyme, viz. hexokinase, reacts with only the A isomer of the Ado-5′-(1-thioPPP) produced in the reaction.

$Co(III)(NH_3)_4ATP$ was tested as a substrate (see Methods) and no reaction could be detected.

Carbamoyl-Phosphate Synthetase

When *E. coli* carbamoyl-phosphate synthetase was incubated with Mg^{2+} and HCO_3^- in the presence or absence of glutamine only the A isomer of Ado-5′-(2-thioPPP) was found to be a substrate (9). In this paper we report similar experiments with Cd^{2+} as the activating cation. Only the B isomer of Ado-5′-(2-thioPPP) was utilized with Cd^{2+}. The A isomer reacted at less than 10% of the rate of the B isomer as determined by PEI-cellulose chromatography. Both isomers were substrates when Zn^{2+} was used as the divalent cation and were utilized at approximately equal rates.

With Ado-5′-(1-thioPPP), carbamoyl-phosphate synthetase uses only the B isomer in the presence of Mg^{2+}, and only the A isomer in the presence of Cd^{2+}. The other isomer in each case was used at less than 10% of the rate of the active isomer with the appropriate metal ion. However, both isomers were substrates in the presence of Zn^{2+}.

$Co(III)(NH_3)_4ATP$ was tested as a substrate as described under Methods and was found not to react with this enzyme under the experimental conditions employed.

DISCUSSION

The objective of the studies reported in this paper was to establish the absolute stereochemistry of nucleotide binding to three ATP utilizing enzymes, carbamate kinase, glutamine synthetase, and carbamoyl-phosphate synthetase. These data provide information on coordination geometry of the metal ion ligand interaction and the stereochemistry of the phosphoryl transfer reactions catalyzed by these enzymes. $Co(III)(NH_3)_4ATP$ or bidentate Cr(III)ATP may be used to obtain the same information. The analogs used in the present study closely resemble the parent nucleotides and the structural information thus obtained is directly applicable to the parent nucleotide system.

Jaffe and Cohn have shown using NMR that Mg^{2+} binds predominately to oxygen ligands and Cd^{2+} to sulfur in these thiophosphate nucleotides (8). This result readily explains the reversal of specificity by yeast hexokinase toward the isomers of Ado-5′-(2-thiotriphosphate) when Cd^{2+} replaces Mg^{2+} as the activating divalent cation. The proposed active metal–nucleotide complex still retains the same geometrical arrangement about the β-phosphoryl. They have also shown that metals which will readily bind either to oxygen or sulfur (Mn^{2+}, Co^{2+}, Zn^{2+}) are nonspecific for the isomers of Ado-5′-(2-thiotriphosphate). It is assumed in this discussion that for enzymes which show a large preference for one isomer of either Ado-5′-(1-thioPPP) or Ado-5′-(2-thioPPP), which can also be reversed on changing from Mg^{2+} to Cd^{2+}, that it can be stated that sometime during the reaction sequence the metal ion is complexed to that phosphoryl group.

Carbamate Kinase

Carbamate kinase was found to use or synthesize only the B isomer of Ado-5′-(2-

TABLE I

KINETIC PARAMETERS FOR Ado-5′-(1-thioPP), Ado-5′-(1-thioPPP), Ado-5′-(2-thioPP), AND Ado-5′-(2-thioPPP)

Enzyme	Nucleotide substrate	K_m	Relative V
Carbamate kinase	ATP	8 μM	100
	Ado-5′-(2-thioPPP) (B)	11 μM	3
	ADP	50 μM	100
	Ado-5′-(2-thioPP)	30 μM	11
	Ado-5′-(1-thioPP)	42 μM[a]	18
Glutamine synthetase	ATP	0.2 mM	100
	Ado-5′-(2-thioPPP) (B)	—	<1[b]
	Ado-5′-(1-thioPPP) (B)	8 mM	55

[a] Only an apparent K_m since only the A isomer reacts with the coupling enzyme used.
[b] Relative V, since K_m is unknown.

thioPPP) with either Mg^{2+} or Cd^{2+} as the divalent cation. Both isomers of Ado-5′-(1-thioPP) were substrates. Since no inversion of stereochemistry is observed for Mg^{2+} and Cd^{2+} with the isomers of Ado-5′-(2-thioPPP) it cannot be stated unequivocally whether the β-P is complexed by the metal ion during the reaction. The observed preference for the B isomer may be determined by groups on the enzyme that are binding either the oxygen or sulfur. The metal ion could still be binding to the β-P but then both the Mg^{2+} and Cd^{2+} must be bound to the same atom on the enzyme-bound nucleotide (S or O). In the B isomer, if S is bound to the metal ion, the chelate ring formed has the Δ configuration (Table II). The present study cannot distinguish between these two cases and other techniques may be necessary to resolve the issue. However, since oxygen has a greater tendency for hydrogen bonding than sulfur, and chemical modification studies suggest that there are residues at the active site capable of strong hydrogen bonding (arginine) (21), it is likely that the favored configuration has the metal ion coordinated to the sulfur. Since carbamate kinase shows no preference for the isomers of Ado-5′-(1-thioPP) then either the metal ion does not bind to the α-P at the active site or the enzyme does not have a preference for metal binding to either the pro-R or pro-S oxygen of the α-P of ADP or ATP.

Glutamine Synthetase

Glutamine synthetase is highly specific for the B isomer of both Ado-5′-(1-thioPPP) and Ado-5′-(2-thioPPP) when Mg^{2+} is the activating divalent cation. No reaction could be detected with these analogs when Cd^{2+} was the activating cation probably because

TABLE II

STEREOSPECIFICITY OF THIO-ATP ANALOGS WITH CARBAMATE KINASE, GLUTAMINE SYNTHETASE, AND CARBAMOYL-PHOSPHATE SYNTHETASE AND DIFFERENT METAL IONS[a]

	Mg^{2+}		Cd^{2+}		Zn^{2+}		Co^{2+}	
Enzyme	ATP(βS)	ATP(αS)	ATP(βS)	ATP(αS)	ATP(βS)	ATP(αS)	ATP(βS)	ATP(αS)
Carbamate kinase	B	AB[b]	B	AB[b]	—	—	—	—
Glutamine synthetase	B	B	—	—	—	—	AB	AB
Carbamoyl-phosphate synthetase	A	B	B	A	AB	AB	—	—

[a] The simplified nomenclature for the thio phosphate analogs is used with ATP(βS) = ado-5-(2-thioPPP) and ATP(αS) = ado-5′-(1-thioPPP).
[b] For carbamate kinase ADP (αS) was used as the substrate in the reverse direction.

Cd^{2+} is such a poor activator of glutamine synthetase. Both pairs of isomers were substrates when Co^{2+} was the activating divalent cation. This shows that metals which can complex with the sulfur will allow the A isomers to be substrates. This suggests that at some point in the reaction sequence that the metal ion in the glutamine synthetase reaction binds to both the α- and β-phosphoryl groups of the nucleotide. Both phosphoryl groups need not be bound at the same time, however. The preferred oxygens that are complexed by metal are the pro-S oxygen of the α-phosphoryl and the pro-S oxygen of the β-phosphoryl. Nothing can be said about binding to the γ-phosphoryl, however.

Carbamoyl-Phosphate Synthetase

With Mg^{2+}, carbamoyl-phosphate synthetase is specific for the A isomer of Ado-5′-(2-thioPPP) and the B isomer of Ado-5′-(1-thioPPP). Both specificities are reversed when Cd^{2+} is the activating cation suggesting that in the active site of this enzyme both the α- and β-phosphoryl groups are complexed to the metal ion. The preference is for the pro-S oxygen of the α-phosphoryl and the pro-R oxygen of the β-phosphoryl of ATP.

The stereochemistry of the carbamate kinase, glutamine synthetase, and carbamoyl-phosphate synthetase reactions established above could not be correlated to their stereochemistry in the reaction with Co(III)$(NH_3)_4$ATP, since circular dichroism spectra showed that none of the enzymes react with this ATP analog.

The geometry of substrates at the active site may be determined from distance measurements using NMR relaxation rates, but the distances of several nuclei from the same reference point must be determined before a model of the active site can be constructed. Since the distance determinations are based solely on binding, there is no assurance that the configuration obtained is kinetically active. The present study, however, uses the chemical reactivity of the various substrate analogs as the criterion for the determination of the stereochemistry. Hence the configuration obtained must be the active configuration. Moreover, the results may be confirmed by varying the metal ion used in the reaction. Thus this technique yields internally consistent information about the active configurations of nucleotides in their reactions and is therefore most useful in the study of kinases.

It is not clear at this time why one configuration is more reactive than another. It is likely that the differences are caused by a combination of the differences in the nature of the interaction between the nucleotide analog and the metal ion or the ligands on the enzyme. X-ray crystallographic studies will further elicit information about the active site of these three enzymes which may be used to explain the differences in stereochemistry of binding the phosphoryl groups of ATP to the metal ion and enzyme amino acid residues.

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