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Carbamyl Phosphate Synthetase of *Escherichia coli* Uses the Same Diastereomer of Adenosine-5′-[2-thiotriphosphate] at Both ATP Sites*

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Frank M. Raushel, Paul M. Anderson, and Joseph J. Villafranca‡

From the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802 and the Department of Biochemistry, School of Medicine, University of Minnesota, Duluth, Duluth, Minnesota 55812

SUMMARY

Carbamyl phosphate synthetase from *Escherichia coli* has been shown to use only the A isomer of adenosine-5′-[2-thiotriphosphate] in both the ATPase reaction (MgATP $\xrightarrow{HCO_3^-}$ MgADP + P_i) and the carbamyl phosphate synthesis reaction (2MgATP + HCO_3^- + L-glutamine → 2MgADP + P_i + carbamyl-P + L-glutamate). The B isomer was less than 5% as reactive. In the reverse reaction, only the A isomer of adenosine-5′-[2-thiotriphosphate] is synthesized from adenosine-5′-[2-thiodiphosphate] and carbamyl-P as determined by ^{31}P NMR and a coupled enzymatic assay with Cd^{2+}-hexokinase. It is therefore proposed that carbamyl phosphate synthetase uses the same diastereomer of MgATP at both ATP sites.

Carbamyl phosphate synthetase from *Escherichia coli* catalyzes the following reaction:

$$2MgATP + HCO_3^- + \text{L-Gln} \rightarrow 2MgADP + P_i + \text{carbamyl-P} + \text{L-Glu} \quad (1)$$

The enzyme also catalyzes two other partial reactions:

$$MgATP \xrightarrow{HCO_3^-} MgADP + P_i \quad (2)$$

$$MgADP + \text{carbamyl-P} \rightarrow MgATP + NH_3 + HCO_3^- \quad (3)$$

From the strong inhibition of this enzyme by diadenosine pentaphosphate (1) and recent kinetic studies (2), it appears that there are two separate ATP sites instead of one common site for both molecules of ATP used in the reaction.

Recently Cornelius and Cleland (3) have determined the absolute stereochemistry of the β,γ-Mg^{2+} complex of ATP that is active with yeast hexokinase by using the substitution inert Co(III)$(NH_3)_4$ATP. Using this information, Jaffe and Cohn (4) have assigned the absolute stereochemistry to the two diastereomers of adenosine-5′-[2-thiotriphosphate] since yeast hexokinase was shown by them to use only the B isomer[1] in the presence of Mg^{2+}.

Since carbamyl phosphate synthetase appears to have two ATP sites, it is of interest to determine whether both sites have the same stereochemistry for MgATP. Therefore, in this report, both isomers of Ado-5′-[2-thioPPP][2] were tested as substrates for carbamyl phosphate synthetase and the stereochemistry of the reactive species was determined for each site. The results in this communication demonstrate that both sites use the A isomer.

EXPERIMENTAL PROCEDURES

Materials and Methods—Carbamyl phosphate synthetase was isolated from *E. coli* according to the method of Matthews and Anderson (6). All other reagents were obtained from Boehringer Mannheim or Sigma. Isomers A and B of Ado-5′-[2-thioPPP] were synthesized enzymatically as previously described by Eckstein and Goody (5).

For the measurement of the apparent K_m values and maximal velocities, the ATPase and carbamyl phosphate synthesis reactions were assayed by analyzing the inorganic phosphate that was produced during the reaction. Each 1.0-ml reaction vial contained: 20 mM $MgCl_2$, 20 mM HCO_3^-, 50 mM Hepes, pH 7.5, 100 mM KCl, and various amounts of ATP or Ado-5′-[2-thioPPP]. When glutamine was used, its concentration was 10 mM. Each solution was incubated at 37°C and the reaction was initiated by addition of carbamyl phosphate synthetase. At equal time intervals, the reaction was stopped and the inorganic phosphate was assayed (7). The carbamyl phosphate synthesis reaction was also coupled with ornithine transcarbamylase and the citrulline produced was determined colorimetrically at 430 nm (8).

The kinetic constants for the reverse reaction in Equation 3 were measured using a hexokinase and glucose-6-P dehydrogenase coupling system. Each 1.0-ml cuvette contained: 50 mM Hepes, 100 mM KCl, 10 mM $MgCl_2$, 1 mM $Cd(CH_3COO)_2$, 50 units of hexokinase, 10 units of glucose-6-P dehydrogenase, 0.5 mM NADP, 20 mM carbamyl phosphate, and various levels of ADP or Ado-5′-[2-thioPP]. The cuvettes were incubated at 37°C and the reaction was initiated with carbamyl phosphate synthetase and monitered at 340 nm.

^{31}P NMR spectra were recorded at 40.3 MHz on a JEOL NMR spectrometer. The field was locked on deuterium and all spectra were recorded with broad band proton decoupling. Spectra were recorded on 1.5-ml samples (25% D_2O) at pH 7.8 in 10-mm tubes. Chemical shifts are relative to an internal reference of inorganic phosphate. Positive values are assigned to chemical shifts at higher field than the reference.

Data Processing—The kinetic data were fitted to the equation:

$$v = \frac{VA}{K + A} \quad (4)$$

and analyzed using the FORTRAN programs of Cleland (9).

RESULTS AND DISCUSSION

Both the A and B isomers of Ado-5′-[2-thioPPP] were tested as substrates for the ATPase and carbamyl phosphate synthesis reactions of carbamyl phosphate synthetase. Only

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‡ To whom correspondence should be addressed at the Department of Chemistry, The Pennsylvania State University, University Park, Penn. 16802.

[1] The A and B designations are those suggested by Eckstein and Goody (5) to distinguish the diastereomers differing in configuration at P_β.

[2] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ado-5′-[2-thioPPP], adenosine-5′-[2-thiotriphosphate]; Ado-5′-[2-thioPP], adensoine-5′-[2-thiodiphosphate].

TABLE I

Kinetic constants for Ado-5'-[2-thioPPP] and Ado-5'-[2-thioPP]

This information was taken from fits to Equation 4 of the data at pH 7.5, 37°C. Other assay conditions are listed under "Materials and Methods."

Reaction	Nucleotide substrate	K_m	Relative V_{max}
		mM	
1. 2MgATP + HCO_3^- + L-Gln → 2MgADP + L-Glu + carbamyl-P + P_i	ATP	0.90 ± 0.06	100[a]
	Ado-5'-[2-thioPPP][b]	2.1 ± 0.3	8
2. MgATP $\xrightarrow{HCO_3^-}$ MgADP + P_i	ATP	0.16 ± 0.01	12
	Ado-5'-[2-thioPPP][b]	1.3 ± 0.1	2
3. MgADP + carbamyl-P → MgATP + NH_3 + HCO_3^-	ADP	0.08 ± 0.01	10
	Ado-5'-[2-thioPP]	0.51 ± 0.16	0.2

[a] Corresponds to a specific activity of 174 μmol h^{-1} (mg of enzyme)$^{-1}$.
[b] Isomer A of Ado-5'-[2-thioPPP].

the A isomer was a substrate for both reactions. The B isomer was less than 5% as reactive as the A isomer. When a mixture of A and B isomers was tested in the overall reaction, only about half of the Ado-5'-[2-thioPPP] was used, indicating that one isomer was used at both sites. The relative V_{max} and K_m values are listed in Table I for ATP and the A isomer of Ado-5'-[2-thioPPP].

Ado-5'-[2-thioPP] was also tested as a substrate in the back reaction. Carbamyl phosphate synthetase (2 mg) was incubated with 10 mM dithiothreitol, 50 mM Hepes, 100 mM KCl, 25 mM carbamyl phosphate, 20 mM $MgCl_2$, 15 mM Ado-5'-[2-thioPP] at 37°C in a volume of 1.25 ml. The reaction was monitored by spotting aliquots of the reaction mixture on thin layer plates of polyethyleneimine cellulose and developing with 2 M LiCl. To determine which isomer of Ado-5'-[2-thioPPP] was formed, the reaction was stopped, after ~50% completion, with a few drops of CCl_4 and 40 mM EDTA. The mixture was centrifuged and the product was examined by ^{31}P NMR. Jaffe and Cohn (10) have recently shown that the two diastereomers of Ado-5'-[2-thioPPP] can be distinguished by their ^{31}P NMR spectra.

Shown in Fig. 1 is the ^{31}P NMR spectrum of the β-P region. The top spectrum (*A*) shows the doublet for the β-P of unreacted Ado-5'-[2-thioPP] at ~31 ppm from inorganic phosphate. At ~27 ppm is the triplet for the β-P of the newly synthesized Ado-5'-[2-thioPPP]. *Spectrum B* shows the result when the product from carbamyl phosphate synthetase was mixed with an authentic sample of the A isomer and *Spectrum C* shows the result when mixed with isomer B. Clearly the Ado-5'-[2-thioPPP] from carbamyl phosphate synthetase is identical with the A isomer. The bottom spectrum (*D*) shows the result when authentic A is added to *Spectrum C*. The two triplets are separated by about 0.13 ppm with the more downfield triplet assigned to isomer A and the more upfield triplet to B under the experimental conditions described.

The K_m and relative V_{max} values for ADP and Ado-5'-[2-thioPP] in the back reaction of carbamyl phosphate synthetase are listed in Table I. The coupled assay system included hexokinase. Jaffe and Cohn (4) have shown that when Cd^{2+} is used instead of Mg^{2+}, hexokinase changes specificity from the B to the A isomer of Ado-5'-[2-thioPPP]. This is due to the preference of Cd^{2+} for sulfur ligands. Thus, in our kinetic experiments, both Mg^{2+} and Cd^{2+} were included in the assay solution. Since we have shown by NMR analysis that carbamyl phosphate synthetase produces the A isomer from carbamyl-P and Ado-5'-[2-thioPP], the inclusion of Cd^{2+} in the assay permitted the use of a simple spectrophotometric assay for the determination of the apparent kinetic constants listed in Table I (Reaction 3). In a control experiment, if Mg^{2+} was

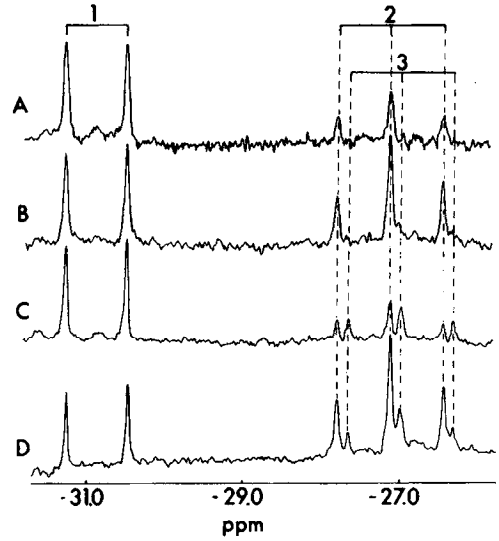

FIG. 1. ^{31}P NMR spectra of the β-P region of Ado-5'-[2-thioPP] and Ado-5'-[2-thioPPP] at 40.3 MHz. Chemical shifts are relative to an internal reference of inorganic phosphate. The magnetic field increases from the *left* to *right*. Initial concentrations: 15 mM adenosine, 20 mM $MgCl_2$, 40 mM EDTA, 25% D_2O, pH 7.8. NMR parameters: pulse width, 45°; spectral width, 3,000 Hz; repetition rate, 3.0 s; 2048 transients. *A*, Ado-5'-[2-thioPPP] made from carbamyl phosphate synthetase; *B*, the product in *Spectrum A* mixed with an authentic sample of isomer A of Ado-5'-[2-thioPPP]; *C*, the product in *Spectrum A* mixed with an authentic sample of isomer B of Ado-5'-[2thioPPP]; *D*, the sample in *C* mixed with isomer A of Ado-5'-[2-thioPPP]. Resonance assignments: *1*, doublet for the β-P of Ado-5'-[2-thioPP]; *2*, triplet for the β-P of the A isomer of Ado-5'-[2-thioPPP]; *3*, triplet for the β-P of the B isomer of Ado-5'-[2-thioPPP]. Additional information is given in the text.

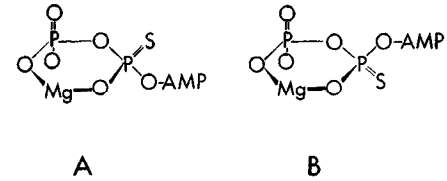

FIG. 2. Absolute stereochemistry of isomers A and B of Ado-5'-[2-thioPPP] as determined by Jaffe and Cohn (4).

the only divalent cation used in the coupled assay, the Ado-5'-[2-thioPPP] produced (detected by thin layer chromatography as described earlier) did not react with hexokinase.

Shown in Fig. 2 are the structures of the Mg^{2+}-β,γ complexes of the two isomers of Ado-5'-[2-thioPPP] as recently assigned

by Jaffe and Cohn (4) based on the active isomer of $Co(NH_3)_4$ ATP used by yeast hexokinase (3). In this paper, we have shown that carbamyl phosphate synthetase use only the A isomer of Ado-5′-[2-thioPPP] in both the ATPase and carbamyl-P synthesis reactions. In the back reaction, only the A isomer of Ado-5′-[2-thioPPP] was detected when synthesized from Ado-5′-[2-thioPP] and carbamyl phosphate. Thus, both ATP sites of carbamyl phosphate synthetase use the same isomer of MgATP. It is interesting to note that a closely related enzyme, carbamate kinase, has been shown to use the B isomer of Ado-5′-[2-thioPPP].[3]

[3] R. P. Pillai, M. Marshall, and J. J. Villafranca, manuscript to be submitted.

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