Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., & Smith, I. C. P. (1974) *Biochem. Biophys. Res. Commun. 60*, 844–850.
Suckling, K. E., Blair, H. A. F., Boyd, G. S., Craig, I. F., & Malcolm, B. R. (1979) *Biochim. Biophys. Acta 551*, 10–21.
Taylor, M. G., Akiyama, T., & Smith, I. C. P. (1981) *Chem. Phys. Lipids 29*, 327–339.
Taylor, M. G., Akiyama, T., Saito, H., & Smith, I. C. P. (1982) *Chem. Phys. Lipids 31*, 359–379.
Verma, S. P., & Wallach, D. F. H. (1973) *Biochim. Biophys. Acta 330*, 122–131.
Worcester, D. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359–378.
Yeagle, P. L. (1978) *Acc. Chem. Res. 11*, 321–327.
Yeagle, P. L., & Martin, R. B. (1976) *Biochem. Biophys. Res. Commun. 69*, 775–780.
Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3477–3481.
Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1977) *Biochemistry 16*, 4344–4349.

Substrate and Inhibitor Activities of the Screw Sense Isomers of Metal–Nucleotide Complexes in the Formyltetrahydrofolate Synthetase Reaction†

Magdalena R. Mejillano,‡ Michael F. Wendland,‡,§ Grover W. Everett,‖ Jesse C. Rabinowitz,⊥ and Richard H. Himes*,‡

Departments of Biochemistry and Chemistry, University of Kansas, Lawrence, Kansas 66045, and Department of Biochemistry, University of California, Berkeley, California 94720

Received August 13, 1985

ABSTRACT: Phosphorothioate analogues of ATP and isomers of CrATP and CrADP were used to examine the nucleotide stereoselectivity of formyltetrahydrofolate synthetase from procaryotic and eucaryotic sources. Substrate activity of the thio-ATP analogues increased as the site of sulfur substitution was changed from the γ to the α position. Thus, adenine nucleotide analogues substituted with sulfur at an α nonbridging position (ATPαS isomers) were the most active, and ATPγS was inactive. When Mg^{2+} was used as the divalent cation, both enzymes showed a clear preference (higher V/K_m value) for the S_P isomer of ATPβS although the magnitude of the preference was greater with the bacterial enzyme. With Cd^{2+} as the divalent cation the R_P isomer was preferred, but the difference was greater with the yeast enzyme. Both (S_P)-MgATPβS and (R_P)-CdATPβS have the Δ or right-hand screw sense configuration of the metal chelate ring. The reversal of stereoselectivity when the cation was changed indicates that the metal ion is coordinated to the β-phosphate group. No stereoselectivity was observed when ATPαS isomers were used in the presence of Mg^{2+} or Cd^{2+}, suggesting that the metals are not coordinated to the α-phosphate. ATPβS was also found to be a competitive inhibitor of MgATP and CdATP, and the lowest K_i values were obtained with the Λ screw sense isomers. The screw sense isomers of bidentate CrATP exhibited no detectable substrate activity but were competitive inhibitors of MgATP. The left-hand (Λ) screw sense isomer of bidentate CrATP and bidentate CrADP bound with greater affinity (lower K_i values) to both enzymes than did the Δ isomer. Although the Λ metal–ATP isomers bind with equal or better affinity than the Δ isomers, they are not as effective as substrates, indicating that geometric constraints imposed by the enzyme are different for binding and catalysis. The bacterial and yeast enzymes have similar stereochemical requirements for the metal nucleotide substrate but apparently differ in the degree of stereoselectivity.

Stereochemical and structural aspects of metal–nucleotide–protein interactions have been extensively studied for a number of enzymes with the use of phosphorothioate analogues of the nucleotide (Eckstein, 1983; Cohn, 1982) and inert chromium– and cobalt–nucleotide complexes (Dunaway-Mariano & Cleland, 1980b; Cleland, 1982). Substitution of a nonbridging oxygen by a sulfur atom in the α- and β-phosphoryl groups generates chirality at the phosphorus center, giving rise to a pair of diastereomers, R_P and S_P. Absolute configurations have been established for ATPαS[1] (Burgers et al., 1979; Burgers & Eckstein, 1978) and ATPβS (Jaffe & Cohn, 1978b). Coordination of the β- and γ-phosphates in the bidentate complex of CrATP creates a chiral center at the β-phosphorus, and two screw sense isomers[2] exist, Λ, or left-handed, and Δ, or right-handed (Cleland, 1982) (Figure 1). In addition, two isomers each of the Λ and Δ forms exist. These have been postulated to result from strong hydrogen bonding between the H_2O ligands and the pseudoaxial oxygen on the γ-phosphate and another H_2O and either the α- or

† This investigation was supported by National Institutes of Health Research Grant AM 07140 to R.H.H.
‡ Department of Biochemistry, University of Kansas.
§ Present address: Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143.
‖ Department of Chemistry, University of Kansas.
⊥ Department of Biochemistry, University of California.

[1] Abbreviations: H_4folate, tetrahydrofolate; 10-CHO-H_4folate, N^{10}-formyltetrahydrofolate; ATPαS, ATPβS, ATPγS, ADPαS, and ADPβS, adenine nucleotide analogues substituted with sulfur on the indicated phosphate in the nonbridging position; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; EPR, electron paramagnetic resonance.

[2] The screw sense nomenclature is that of Cornelius and Cleland (1978).

0006-2960/86/0425-1067$01.50/0

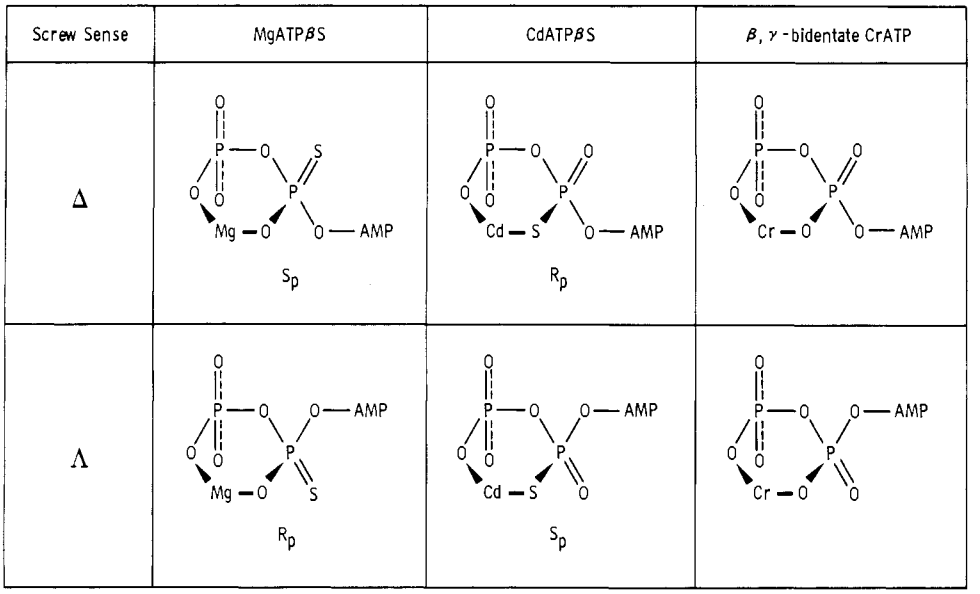

FIGURE 1: Structure of β,γ-bidentate metal–ATP analogues.

β-phosphate oxygen (Dunaway-Mariano & Cleland, 1980a).

Phosphorothioate analogues are substrates for many phosphoryl and nucleotidyl transfer reactions while inert nucleotide complexes are excellent inhibitors of enzymes using Mg–nucleotide complexes as substrates. Another interesting property of phosphorothioate analogues is that, for a given diastereomer, the metal chelates differ in their geometric configuration about the phosphorus, depending on the metal ion's preference for oxygen or sulfur ligands. From ^{31}P NMR studies, Mg^{2+} was shown to coordinate preferentially to the phosphoryl oxygen whereas Cd^{2+} prefers to bind to sulfur, and a number of divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} coordinate to both oxygen and sulfur (Jaffe & Cohn, 1978b). As a consequence, any given diastereomer of ATPβS or ATPαS complexed with Mg^{2+} will have the opposite stereochemical configuration of the metal chelate, Λ or Δ, compared to that with Cd^{2+} (Figure 1). Thus, the reversal of isomer selectivity by an enzyme when Mg^{2+} is replaced by Cd^{2+} indicates that the metal ion is bound to that particular phosphorothioate group (Cohn, 1982; Jaffe & Cohn, 1979).

In this study, we examined the preference of the enzyme formyltetrahydrofolate synthetase for sulfur-substituted ATP diastereomers as substrates and inhibition of the enzyme by the phosphorothioate analogues as well as by the inert β,γ-CrATP and α,β-CrADP complexes. The enzyme catalyzes the formylation of H_4folate at the N^{10} position (eq 1). Both

$$\text{MgATP} + \text{HCOO}^- + \text{H}_4\text{folate} \overset{M^+}{\rightleftharpoons} \text{MgADP} + \text{P}_i + \text{10-CHO-H}_4\text{folate} \quad (1)$$

the bacterial enzyme from *Clostridium cylindrosporum* and the eucaryotic enzyme from *Saccharomyces cerevisiae* were used. The bacterial enzyme is a tetramer of four identical subunits while the yeast protein is a trifunctional dimeric protein in which formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase, and methylenetetrahydrofolate dehydrogenase activities are in the same polypeptide chains (Paukert et al., 1976).

Experimental Procedures

Materials

Formyltetrahydrofolate synthetase was purified from *C. cylindrosporum* according to the method of Rabinowitz and Pricer (1962). The yeast enzyme, C_1-tetrahydrofolate synthase, was a gift from Dr. Charles Staben, University of California, and was also purified by using a modification of the heparin–agarose affinity chromatography procedure devised by Staben (1985) and described by Whitehead et al. (1985). The strain of *S. cerevisiae* used (also provided by Dr. Staben) had been transformed with the YEpADE3 plasmid. Cells were suspended at a ratio of 1 g of cell paste per 2 mL of 25 mM Tris–SO_4, 10 mM KCl, and 10 mM 2-mercaptoethanol, pH 7.5, and broken in a Braun homogenizer. After centrifugation, the extract was filtered through glass wool, and phenylmethanesulfonyl fluoride was added to a final concentration of 1 mM. The cell extract was applied to a heparin–agarose column prepared by the method of Davison et al. (1979) and washed with the same buffer, followed by buffer containing 0.1 M KCl. Finally, the enzyme was eluted with a 0.1–0.8 M KCl gradient in the same buffer. On SDS–PAGE only one major band ($M_r \sim 100000$) was present, which accounted for at least 95% of the protein.

6(*RS*)-H_4folate was prepared by the catalytic hydrogenation of folic acid in neutral aqueous solution and purified by chromatography on DEAE-cellulose (Samuel et al., 1970). In this procedure the H_4folate is obtained in 0.5 M 2-mercaptoethanol, which had to be removed for kinetic experiments in which Cd^{2+} was used. To remove the 2-mercaptoethanol, the H_4folate solution was lyophilized, dissolved in 0.1 M sodium ascorbate, lyophilized again, and dissolved in H_2O. Lyophilization and dissolution in H_2O was repeated 3 times. The absence of 2-mercaptoethanol was verified with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

AMPS, ADPβS, ATPγS, phosphoenolpyruvate, acetyl phosphate, and phospho-L-arginine were purchased from Boehringer Mannheim. Pyruvate kinase, acetate kinase, adenylate kinase, and arginine kinase were obtained from Sigma. Diphenyl phosphochloridate, tributylamine, and trioctylamine were purchased from Aldrich.

Methods

Preparation of Phosphorothioate Analogues of ATP and ADP. (S_P)-ATPβS and (R_P)-ATPβS were synthesized enzymatically as described by Pillai et al. (1980) using phosphoenolpyruvate, ADPβS, and pyruvate kinase for the S_P isomer and acetyl phosphate, ADPβS, and acetate kinase for the R_P isomer. (S_P)-ATPαS was prepared from AMPS by

using a coupled reaction of adenylate kinase and pyruvate kinase (Jaffe & Cohn, 1978a; Pillai et al., 1980). The R_P isomer of ATPαS was synthesized enzymatically with arginine kinase and adenylate kinase from a mixture of the R_P and S_P isomers of ADPαS, prepared chemically by the method of Eckstein and Goody (1976).

The phosphorothioate analogues were purified by DEAE-Sephadex chromatography using an NH_4HCO_3 gradient (Eckstein & Goody, 1976). Purity was verified by TLC on PEI–cellulose plates developed in 0.75 M KH_2PO_4, pH 3.5. The purity of the ATPβS analogues was also examined by determining the K_m and relative V values for both isomers with hexokinase under the conditions described by Jaffe and Cohn (1979). The V/K_m values obtained (1.11×10^{-5} and 4.64×10^{-2} min^{-1} mg^{-1}) were in close agreement with the reported values for (S_P)-ATPβS and (R_P)-ATPβS (1.83×10^{-5} and 4.95×10^{-2} min^{-1} mg^{-1}), respectively. The possibility that the substrate activity of the ATP analogues could be due to a small contamination by ATP was tested with formyltetrahydrofolate synthetase. This enzyme can be used for an assay of ATP because at subsaturating concentrations of the nucleotide and at high enzyme concentration, the reaction goes to completion very rapidly (within 1 min). Therefore, if the activity of the analogue were due to a contamination by ATP, under these conditions there would be a small, almost instantaneous formation of 10-CHO-H_4folate not equal to the amount of analogue present. If the substrate activity were due only to the analogue, the formation of product would be slow but the total amount formed would be equal to the amount of analogue present. The reaction mixtures consisted of 0.1 mM ATP or the analogues, 5 mM $MgCl_2$, 2 mM 6(*RS*)-H_4folate, 100 mM mercaptoethanol, 40 mM sodium formate, 50 mM KCl, 100 mM triethanolamine hydrochloride, pH 8, and 35 μg of clostridial enzyme in a volume of 0.25 mL. Samples were removed after different time periods up to 30 min at 37 °C and assayed for the presence of 10-CHO-H_4folate. As expected, when ATP was used, the formation of product was complete in less than 1 min. When any of the phosphorothioate analogues were substituted for ATP, the concentration of product increased gradually during the time period studied, indicating no significant amounts of ATP contamination.

Preparation and Purification of CrATP and CrADP. β,γ-Bidentate CrATP and α,β-bidentate CrADP were prepared by heating solutions containing equimolar concentrations of the sodium salt of the nucleotides and $CrCl_3$ at 80 °C for 10 min as described by Dunaway-Mariano and Cleland (1980a). Purification was accomplished on Dowex-50-H^+ (Cleland, 1982; Dunaway-Mariano & Cleland, 1980a). The chromium content of the products was determined from the absorbance at 424 nm for bidentate CrATP (ϵ = 24 M^{-1} cm^{-1}) and 428 nm for bidentate CrADP (ϵ = 19 M^{-1} cm^{-1}) (Cleland & Mildvan, 1979), and the nucleotide content was determined from the absorbance at 259 nm ($\epsilon = 15.4 \times 10^3$ M^{-1} cm^{-1}). In addition, the Cr^{3+} concentration was determined after hydrolysis by the EDTA method (Marczenko, 1976). Cr^{3+} forms a violet-colored complex with EDTA in slightly acidic medium with a maximum absorbance at 540 nm (ϵ = 140 M^{-1} cm^{-1}). Hydrolysis was accomplished by adjusting the Cr–nucleotide solutions to pH 9 with NH_4OH and heating at 100 °C for 10 min. The pH was adjusted to 4 before the addition of EDTA. The concentrations determined by the different methods were in good agreement with a Cr^{3+} to nucleotide ratio of 1.

The bidentate complexes were purified by reverse-phase HPLC on analytical and preparative Altex Ultrasphere ODS C_{18} columns with 10 mM methanesulfonic acid, pH 2.5, for CrATP or 10 mM ethanesulfonic acid, pH 2.5, for CrADP as the isocratic buffer and ion-pairing agent (Gruys & Schuster, 1982). The flow rate was maintained at 1 mL/min. As described by Gruys and Schuster (1982), four major peaks were eluted after a smaller peak of contaminating material when CrATP was used. Peaks 2 and 3 were rechromatographed for better separation. Fractions in the middle of each peak were pooled, and the purity was determined from the elution profile of a sample reinjected in the column. Each purified isomer was contaminated 5–10% by another isomer. The pooled fractions containing each isomer were concentrated to about 1 mM by rotary flash evaporation and stored at −20 °C.

Kinetic Studies. Initial velocities using ATP, ATPαS, and ATPβS as substrates in the presence of Mg^{2+} or Cd^{2+} were determined by the assay method described previously (Rabinowitz & Pricer, 1962). 10-CHO-H_4folate produced is converted to 5,10-methenyl-H_4folate under acidic conditions. The concentration of this product is determined from the absorbance at 350 nm (ϵ = 24 900 M^{-1} cm^{-1}). The reaction mixture contained 2 mM 6(*RS*)-H_4folate, 20 mM sodium ascorbate, 40 mM sodium formate, 50 mM KCl, and 10 mM triethanolamine hydrochloride, pH 8.0. The metal to nucleotide ratio was maintained at 2:1. Because of the slower reaction rates obtained with the analogues, the enzyme concentration was higher than used in experiments with ATP (400-fold for ATPαS and 2000-fold for ATPβS), and in some cases, the incubation time was 30 min instead of 10 min. In the initial velocity measurements for the yeast enzyme 100 mM ammonium formate was used instead of 40 mM sodium formate. Lack of inhibition of the enzymes by Cd^{2+} was shown by varying the Cd^{2+} concentration from 1 to 6 mM in the presence of 2 mM ATP. No decrease in velocity was obtained as the Cd^{2+} concentration was increased. In addition, kinetic constants for MgATP and CdATP obtained by using an ATP to metal ratio of 2:1 for the bacterial and yeast enzymes were the same as with a 2:1 ratio of metal to nucleotide. The concentrations of the metal–nucleotide or metal–thionucleotide complexes were calculated by using stability constants reported by Pecoraro et al. (1984): 5.0×10^4 M^{-1} for MgATP, 2.95×10^4 M^{-1} for MgATPαS, 1.10×10^4 M^{-1} for MgATPβS, 2.29×10^4 M^{-1} for CdATP, 8.33×10^4 M^{-1} for CdATPαS, and 2.75×10^5 M^{-1} for CdATPβS.

Inhibition by the Cr–nucleotide analogues was studied at pH 6 to minimize the pH-dependent epimerization of the Δ and Λ isomers (Dunaway-Mariano & Cleland, 1980b) and the hydrolysis of the analogues to free Cr^{3+} and nucleotide. Studies showed that at this pH value the production of 10-CHO-H_4folate was linear with time over a 5-min period. The reaction components were the same as described above except that the buffer was 0.1 M *N*-morpholinoethanesulfonate, the Mg^{2+} to ATP ratio was maintained at 5:1, and the enzyme concentration was approximately 500-fold higher than that normally used at pH 8.0. Under the conditions of the assay at pH 6 (5 min) we could not detect any epimerization of the analogues as determined by HPLC analysis of the reaction mixture.

Kinetic constants were determined from reciprocal plots using a linear least-squares fit. Values of the correlation coefficients ranged from 0.96 to 0.99.

Results

Phosphorothioate Analogues as Substrates. All substrates used showed normal Michaelis–Menten kinetics. A summary of the kinetic constants obtained with the bacterial enzyme

Table I: Kinetic Constants with the Bacterial Enzyme

substrate	screw sense	K_m (μM)	V (mM min^{-1} mg^{-1})	V/K_m (min^{-1} mg^{-1})
MgATP		206	303	1.47×10^3
CdATP		58	139	2.40×10^3
(S_P)-MgATPβS	Δ	526	0.33	0.627
(S_P)-CdATPβS	Λ	2615	0.13	0.0497
(R_P)-MgATPβS	Λ	2245	0.055	0.0244
(R_P)-CdATPβS	Δ	2335	0.35	0.15
(S_P)-MgATPαS	Δ	2080	19.9	9.57
(S_P)-CdATPαS	Λ	1680	12.4	7.38
(R_P)-MgATPαS	Λ	1050	10.1	9.62
(R_P)-CdATPαS	Δ	2280	15.8	6.94

Table II: Kinetic Constants with the Yeast Enzyme

substrate	screw sense	K_m (μM)	V (mM min^{-1} mg^{-1})	V/K_m (min^{-1} mg^{-1})
MgATP		111	141	1.27×10^3
CdATP		51	49	0.96×10^3
(S_P)-MgATPβS	Δ	859	0.11	0.128
(S_P)-CdATPβS	Λ	1412	0.046	0.0325
(R_P)-MgATPβS	Λ	1279	0.053	0.0414
(R_P)-CdATPβS	Δ	637	0.32	0.502
(S_P)-MgATPαS	Δ	674	2.76	4.09
(S_P)-CdATPαS	Λ	1710	4.44	2.60
(R_P)-MgATPαS	Λ	1440	4.61	3.20
(R_P)-CdATPαS	Δ	1330	4.85	3.65

Table III: Ratio of V/K_m Values for Phosphorothioate Analogues

	$(V/K_m)_{Mg}/(V/K_m)_{Cd}$	
nucleotide	bacterial enzyme	yeast enzyme
(S_P)-ATPβS	12.6	3.94
(R_P)-ATPβS	0.16	0.082
(S_P)-ATPαS	1.30	1.57
(R_P)-ATPαS	1.39	0.88

is presented in Table I. It is apparent that Cd^{2+} can effectively replace Mg^{2+} in the reaction with ATP as the nucleotide substrate. When a sulfur atom is substituted on the α-phosphorus, the activity as judged by the first-order rate constant V/K_m is affected to a large extent; a sizable decrease in V is accompanied by an increase in K_m. Placement of the sulfur on the β-phosphorus had an even larger effect on both V and V/K_m. ATPγS did not exhibit detectable activity as a substrate but was an effective competitive inhibitor. The results obtained with the yeast enzyme are presented in Table II and show a similar effect of sulfur substitution. Table III summarizes ratios of V/K_m values for the S_P and R_P isomers obtained for both enzymes.

From the results in Tables I–III, several facts become evident. Substitution of S for O in the nonbridging positions on the α- or β-phosphorus atom increases the K_m values substantially (over 10-fold in most cases), although major differences between the analogues do not exist. From an examination of V values it is apparent that, with Mg^{2+} as the divalent cation, the S_P (Δ) isomer of ATPβS is preferred by both enzymes. When Cd^{2+} was present, the R_P (Δ) isomer was the most active. However, as emphasized by Pecoraro et al. (1985), the correct kinetic constant to compare is V/K_m. The same results are obtained when this constant is compared. A change in stereoselectivity upon changing the cation would be reflected in the $(V/K_m)_{Mg}/(V/K_m)_{Cd}$ values. These should be >1 for one isomer and <1 for the other (Eckstein, 1985). The data clearly show this for the ATPβS diastereomers (Table III). In addition to demonstrating a reversal for preference from the S_P to the R_P isomer when Cd^{2+} was substituted for Mg^{2+}, the data demonstrate a preference for the Δ screw sense isomer by both enzymes. When ATPαS was used as the substrate, the value of the kinetic constants suggested no preference for the S_P or R_P isomer regardless of whether Mg^{2+} or Cd^{2+} was the activating cation (Tables I–III).

Table IV: Inhibition Constants of ATPβS Isomers and ATPγS Using the Bacterial Enzyme

substrate	inhibitor	K_i (μM)
MgATP	(S_P)-ATPβS	1117
MgATP	(R_P)-ATPβS	572
CdATP	(S_P)-ATPβS	293
CdATP	(R_P)-ATPβS	136
MgATP	ATPγS	193

Table V: Inhibition Constants of CrATP and CrADP Isomers

		K_i (μM)	
analogue	screw sense	bacterial enzyme	yeast enzyme
CrATP isomers[a]			
HPLC peak 1	Δ	144	818
HPLC peak 2	Λ	54	52
HPLC peak 3	Λ	72	42
HPLC peak 4	Δ	138	507
CrADP isomers			
HPLC peak 1	Δ	91	ND[b]
HPLC peak 2	Λ	53	ND[b]

[a] The HPLC peaks refer to the elution position during purification of the isomers as described under Experimental Procedures. [b] ND = not determined.

ATPβS Analogues as Competitive Inhibitors. Because reaction rates were much slower with the ATPβS isomers, they could be tested as inhibitors in assays using an enzyme concentration that produced no detectable product from the analogues. These results are presented in Table IV. Both were found to be competitive inhibitors of MgATP and CdATP. The K_i values of the S_P and R_P isomers were similar, suggesting that both bind with near equal affinity. If there is a difference, the R_P isomer may bind with an affinity about twice that of the S_P isomer. Thus, although the Λ screw sense isomer is much less active as a substrate, it appears to bind as well as or better than the Δ screw sense isomer.

Inhibition Studies with Cr^{3+} Analogues. Substrate activity for the CrATP analogues could not be detected under the assay conditions used. On the other hand, these compounds were effective competitive inhibitors. Table V summarizes the inhibition constants obtained with each of the Cr^{3+} analogues by using the bacterial and yeast enzymes. When purified β,γ-bidentate CrATP was used, the left-handed (Λ) screw sense isomers (HPLC peaks 2 and 3) inhibited both enzymes more effectively than the two Δ isomers. The difference appears to be more pronounced with the yeast enzyme. The greater effectiveness of the Λ isomer also is indicated by the inhibition studies with CrADP (Table V). The results of these experiments with the Cr–nucleotide analogues are consistent with those from the ATPβS inhibition studies, that the isomer that is the less preferred substrate (Λ) acts as the stronger inhibitor. Thus, binding affinities and reactivity of the screw sense isomers have different steric requirements.

Discussion

The results with the ATPβS isomers as substrates clearly show that formyltetrahydrofolate synthetase from *C. cylindrosporum* and the same activity in the C_1-tetrahydrofolate synthase from yeast are stereoselective for the Δ screw sense (right-handed) isomer of the metal–nucleotide complex. Other synthetases are about equally divided in their preference for the Δ or Λ isomers. Preference for the Δ isomer of metal–

ATPβS as a substrate has been observed for Met-tRNA synthetase (Smith & Cohn, 1982), Trp-tRNA synthetase (Piel et al., 1983), and carbamoyl phosphate synthetase (Pillai et al., 1980) while the opposite isomer, Λ, is preferred by glutamine synthetase (Pillai et al., 1980) and Phe-tRNA synthetase (Connolly et al., 1980). In contrast to the preference for the Δ isomer for catalysis by formyltetrahydrofolate synthetase, inhibition studies with the ATPβS, CrATP, and CrADP isomers indicate that the Λ screw sense isomer binds with an equal or stronger affinity than the Δ isomer. In the case of liver fructokinase it has also been concluded that the inactive isomer binds equally as well as the active isomer (Pecoraro et al., 1985). Perhaps this phenomenon could be explained by assuming that the AMP portion of the nucleotide substrate fits into a specific site, placing the γ-phosphate of the Δ isomer at the appropriate distance from the catalytic center. If the Λ isomer were to bind with the AMP portion in the same site, the γ-phosphate would be somewhat removed from the catalytic center. Since both the Δ and Λ isomers of the β,γ-bidentate complex would be present in a MgATP solution, it must be considered that the enzyme operates in the presence of a competitive inhibitor of the metal–nucleotide substrate.

The reversal in stereoselectivity (from S_P to R_P) observed with ATPβS when Mg^{2+} was replaced by Cd^{2+} and the lack of stereoselectivity when ATPαS was the substrate with either Mg^{2+} or Cd^{2+} indicate that the divalent cation is coordinated to the β-phosphate but not the α-phosphate group. Other enzymes that show this pattern include acetate kinase, adenylate kinase, hexokinase (Cohn, 1982), and fructokinase (Pecoraro et al., 1985). Lack of stereoselectivity as we found with ATPαS can have several explanations in addition to the lack of metal binding (Eckstein, 1985). For example, structural constraints imposed by the enzyme to force both Mg^{2+} and Cd^{2+} to bind to the same ligand (Cohn, 1982), the higher proportion of the tridentate species ($\sim$75%) in CdATPαS compared to that in MgATPαS ($\sim$27%) (Pecoraro et al., 1984), and the lower affinity of Cd^{2+} for the sulfur atom in ATPαS compared to that in ATPβS (Pecoraro et al., 1984) may be other possible explanations. However, our conclusions are supported by EPR studies of enzyme–Mn^{2+}–nucleotide complexes using [α-^{17}O]ADP, [α-^{17}O]ATP, [β-^{17}O]ADP, and [β-^{17}O]ATP. These results demonstrate Mn^{2+} coordination to the β-phosphate but not the α-phosphate (Smithers, Himes, and Reed, unpublished results). The results suggest, but do not prove, that the reactive metal–nucleotide substrate is a β,γ-bidentate complex. A β-monodendate complex cannot be ruled out from these studies.

Under the assay conditions used we found the CrATP analogues to be inactive. Cleland (1982) has proposed that substrate activity of the CrATP analogues is observed only when the enzyme does not have to insert one of its side chains into the inner coordination sphere of the metal. It is questionable, however, whether any meaning can be attached to the lack of activity with formyltetrahydrofolate synthetase. First, conditions were not such that we could have detected a single turnover. Second, the reaction probably proceeds via the formation of an enzyme-bound formyl phosphate (Kofron, Smithers, Reed, and Himes, unpublished results), and if either of the analogues were active, the reaction would probably stop with the formation of a CrADP–formyl phosphate complex in which the Cr^{3+} would be complexed to the β-phosphate of ADP and the phosphate in formyl phosphate.

One purpose of these studies was to compare the catalytic and binding site requirements for the nucleotide in the procaryotic and eucaryotic enzymes because of the major differences in their structures. The procaryotic protein is a tetramer of identical subunits (M_r 60 000) with four active sites (Harmony & Himes, 1973). The eucaryotic enzyme (M_r 220 000) is a dimer, and the individual polypeptides contain three different enzyme activities that catalyze successive steps in one-carbon metabolism (Paukert et al., 1976). Interestingly, limited proteolysis of the enzyme produces a 76 000-dalton protein that still contains the formyltetrahydrofolate synthetase activity. This part of the protein may be similar to the 60 000-dalton subunit of the procaryotic enzyme. The nucleotide structural requirements are similar for both enzymes, but there are some differences. The Δ isomer of ATPβS was preferred by both, changing from Mg^{2+} to Cd^{2+} resulted in a reversal in preference for the S_P to the R_P isomer when ATPβS but not ATPαS was the substrate, and the Λ isomer of CrATP was the more potent inhibitor. Differences include the fact that the preference for (S_P)-MgATPβS over (R_P)-MgATPβS was by a factor of 26 for the bacterial enzyme but only a factor of 3 for the yeast enzyme. In the case of CdATPβS the situation was reversed. The yeast enzyme preferred the R_P isomer by a factor of 16, but the bacterial enzyme's preference was by a factor of 3. Differences were also noted in the CrATP inhibition studies. With the bacterial enzyme the K_i of the Λ isomer was about 50% that of the Δ isomer. In the case of the yeast enzyme the K_i value for the Λ isomer was 5–10% of the value for the Δ isomer. These differences may reflect the existence of different types of interactions between functional groups in the two enzymes and the nonbridging, noncoordinated atom on the β-phosphate.

Registry No. MgATP, 1476-84-2; CdATP, 72052-13-2; (Δ)-CrATP, 71630-18-7; (Λ)-CrATP, 71748-27-1; (Δ)-CrADP, 73000-97-2; (Λ)-CrADP, 73037-58-8; (Δ)-MgATPβS, 99416-10-1; (Λ)-MgATPβS, 99416-09-8; (*S*)-ATPβS·Mg, 72052-07-4; (*R*)-ATPβS·Mg, 72052-08-5; MgATPαS, 99829-11-5; (*S*)-ATPαS·Mg, 72052-16-5; (*R*)-ATPαS·Mg, 72052-17-6; (Δ)-CdATPβS, 99413-65-7; (Λ)-CdATPβS, 99528-34-4; (*S*)-ATPβS·Cd, 72052-14-3; (*R*)-ATPβS·Cd, 72052-15-4; CdATPαS, 99829-12-6; (*S*)-ATPαS·Cd, 72052-18-7; (*R*)-ATPαS·Cd, 72052-19-8; ATPγS, 35094-46-3; formyltetrahydrofolate synthetase, 9023-66-9.

REFERENCES

Burgers, P. M. J., & Eckstein, F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4798–4800.

Burgers, P. M. J., Sathyanarayana, B. K., Saenger, W., & Eckstein, F. (1979) *Eur. J. Biochem. 100*, 585–591.

Cleland, W. W. (1982) *Methods Enzymol. 87*, 159–179.

Cleland, W. W., & Mildvan, A. S. (1979) *Adv. Inorg. Biochem. 1*, 163–192.

Cohn, M. J. (1982) *Acc. Chem. Res. 15*, 326–332.

Connolly, B. A., Von der Haar, F., & Eckstein, F. (1980) *J. Biol. Chem. 255*, 11301–11307.

Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279–3286.

Davison, B. L., Leighton, T., & Rabinowitz, J. C. (1979) *J. Biol. Chem. 254*, 9220–9226.

Dunaway-Mariano, D., & Cleland, W. W. (1980a) *Biochemistry 19*, 1496–1505.

Dunaway-Mariano, D., & Cleland, W. W. (1980b) *Biochemistry 19*, 1506–1515.

Eckstein, F. (1983) *Angew. Chem., Int. Ed. Engl. 22*, 423–439.

Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367–402.

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Gruys, K. J., & Schuster, S. M. (1982) *Anal. Biochem. 125*, 66–73.

Harmony, J. A. K., & Himes, R. H. (1973) *CRC Crit. Rev. Biochem. 1*, 501–535.
Jaffe, E. K., & Cohn, M. J. (1978a) *Biochemistry 17*, 652–657.
Jaffe, E. K., & Cohn, M. J. (1978b) *J. Biol. Chem. 253*, 4823–4825.
Jaffe, E. K., & Cohn, M. J. (1979) *J. Biol. Chem. 254*, 10839–10845.
Marczenko, Z. (1976) in *Spectrophotometric Determination of Elements*, p 219, Wiley, Chichester, England.
Paukert, J. L., Straus, L. D., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 5104–5111.
Paukert, J. L., Williams, G. R., & Rabinowitz, J. C. (1977) *Biochem. Biophys. Res. Commun. 77*, 147–154.
Pecoraro, V. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262–5271.
Pecoraro, V. L., Rendina, A. R., & Cleland, W. W. (1985) *Biochemistry 24*, 1619–1622.
Piel, N., Freist, W., & Cramer, F. (1983) *Bioorg. Chem. 12*, 18–33.
Pillai, R. P., Raushel, F. M., & Villafranca, J. J. (1980) *Arch. Biochem. Biophys. 199*, 7–15.
Rabinowitz, J. C., & Pricer, W. E., Jr. (1967) *J. Biol. Chem. 237*, 2898–2902.
Samuel, C. E., D'Ari, L., & Rabinowitz, S. C. (1970) *J. Biol. Chem. 245*, 5115–5121.
Smith, L. T., & Cohn, M. (1982) *Biochemistry 21*, 1530–1534.
Staben, C. (1985) Ph.D. Thesis, University of California, Berkeley, CA.
Whitehead, T. R., Lacayo, N., & Rabinowitz, J. C. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1076.

Inhibition of Angiotensin Converting Enzyme by Aldehyde and Ketone Substrate Analogues†

Damian Grobelny‡ and Richard E. Galardy*
Department of Biochemistry and Sanders-Brown Research Center on Aging, University of Kentucky, Lexington, Kentucky 40536
Received June 20, 1985

ABSTRACT: Three classes of carbonyl-containing substrate analogues and partial substrate analogues have been tested for their ability to inhibit angiotensin converting enzyme. (4-Oxobutanoyl)-L-proline is proposed to occupy the S_1' and S_2' subsites on the enzyme, thus locating its aldehyde carbonyl group at the position of the active site zinc atom. This aldehyde is 70% hydrated in aqueous solution and could mimic a tetrahedral intermediate occurring during enzyme-catalyzed substrate hydrolysis, but its K_i is only 760 μM. Carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninal is proposed to occupy the S_1 through S_4 subsites on the other side of the zinc atom. Its weak K_i of 60 μM is nearly equipotent to its parent peptide terminating in phenylalanine. However, ketoace, (5*RS*)-(5-benzamido-4-oxo-6-phenylhexanoyl)-L-proline [Almquist, R. G., Chao, W. R., Ellis, M. E., & Johnson, H. L. (1980) *J. Med. Chem. 23*, 1392–1398], one of the third class of inhibitors proposed to occupy subsites S_1 through S_2' on both sides of the zinc atom, has a K_i of 0.0006 μM under our assay conditions, orders of magnitude more potent than its parent peptide. The carbonyl carbon of ketoace is less than 3% hydrated in aqueous solution as determined by carbon-13 nuclear magnetic resonance spectroscopy. If the hydrate is the species bound to converting enzyme, its K_i must be less than 18 pM. Ketoace is a slow-binding inhibitor of converting enzyme, but its overall K_i is dependent on its concentration and therefore prevents calculation of kinetic constants for slow binding. The K_i of (5-benzamido-4-hydroxy-6-phenylhexanoyl)-L-proline, the alcohol analogue of ketoace, is 3 μM. Lengthening ketoace to give (5*RS*)-[5-(carbobenzoxyprolinamido)-4-oxo-6-phenylhexanoyl]-L-proline presumably allows occupation of the additional subsite S_2. However, this lengthened inhibitor has a K_i 10 000-fold weaker than that of ketoace.

Angiotensin converting enzyme is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that catalyzes the hydrolysis of the carboxy-terminal dipeptide histidylleucine from the decapeptide angiotensin I to produce the pressor octapeptide angiotensin II. Several potent inhibitors of the enzyme have been shown to be orally active antihypertensive agents in animals and man (Cushman & Ondetti, 1980; Sweet et al., 1981).

Aldehydes and ketones have been proposed to be transition-state analogues for proteases due to their ability to add either an enzyme-bound nucleophile or a water molecule to form a tetrahedral adduct that mimics a tetrahedral intermediate known to occur in amide hydrolysis [Lewis & Wolfenden (1977a,b) and references cited therein]. Strong inhibition by aldehyde and ketone substrate analogues has been reported for several zinc metalloproteases: angiotensin converting enzyme (Almquist et al., 1980, 1982; Meyer et al., 1981; Gordon et al., 1984; Natarajan et al., 1984), leucine aminopeptidase (Andersson et al., 1982), and carboxypeptidase A [Galardy & Kortylewicz (1984) and references cited therein]. α-Fluoro ketones are also powerful inhibitors of converting enzyme and carboxypeptidase A (Gelb et al., 1985). Evidence for formation of a tetrahedral enzyme–inhibitor

† This study was supported by NIH Grant HL 27368.
‡ Present address: Institute of Organic and Polymer Technology, Technical University of Wroclaw, 50-370 Wroclaw, Poland.

0006-2960/86/0425-1072$01.50/0