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Use of a Vinyl Phosphonate Analog of ATP as a Rotationally Constrained Probe of the C5′—O5′ Torsion Angle in ATP Complexed to Methionine Adenosyl Transferase

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An analog of adenosine 5′-triphosphate (ATP) was synthesized in which the C4′—C5′—O—P^{α} system is replaced by a *trans* C4′—CH=CH—P^{α} system. In the form of 1 : 1 complexes with Mg, this analog and its counterpart with a C4′—CH_2—CH_2—P^{α} system were linear competitive inhibitors, with respect to MgATP, of the MAT-II (normal tissue) and MAT-T (hepatoma tissue) forms of rat ATP : L-methionine-*S*-adenosyltransferase (MAT); $K_m(\mathrm{ATP})/K_i$ values ranged from 0.4 to 2.4. 2′-Deoxy-ATP was a weak substrate, $K_m(\mathrm{ATP})/K_m = 0.035$, of MAT-II and a weak competitive inhibitor, $K_m(\mathrm{ATP})/K_i = 0.07$, of MAT-T. These findings, together with interactions of the MAT forms with other substrates and inhibitors, indicate that binding of ATP to these transferases is accompanied by little rotation about the C5′—O5′ bond, and that C4′ and P^{α} are in a *trans*-type relationship in enzyme-bound ATP.

INTRODUCTION

Adenosine 5′-triphosphate (ATP, **1a**) is a substrate of many enzymes, but, as summarized below, little information is available regarding many aspects of the conformation of enzyme-bound ATP. Numerous conformations of ATP are theoretically possible. The most pronounced conformational differences in ATP arise from variations in modes of puckering of its furanose ring and in the torsional angles about the N9—C1′, C4′—C5′, C5′—O5′, O5′—P^{α}, and α,β-phosphoanhydride bonds. The β,γ-phosphoanhydride bonds in enzyme-bound ATP usually comprise part of a six-membered ring that results from β,γ coordination of ATP with Mg to form a 1 : 1 complex in which P^{β} has become chiral. Evidence for the absolute configuration at P^{β} of MgATP bound to various ATP-utilizing kinases has been obtained using Cr(III) and Co(III) complexes of ATP (*1*) or β-thio-ATP diastereomers (*2, 3*). NMR studies indicate that the C1′—N9 torsional angles of MgATP complexed to pyruvate kinase (*4*), adenylate kinase (*5, 6*), or protein kinase (*7*) and of MgdATP complexed to DNA polymerase (*8*) are such as to position H8 over the ribofuranose ring as depicted in Fig. 1. Consistent with this, 8,5′-cyclo-ADP, in which the C1′—N9 bond is fixed in a similar conformation, is a substrate of pyruvate kinase (*9*). Evidence for modes of furanoid puckering in MgATP bound to protein kinase (*7*) or adenylate kinase (*6*) has been reported

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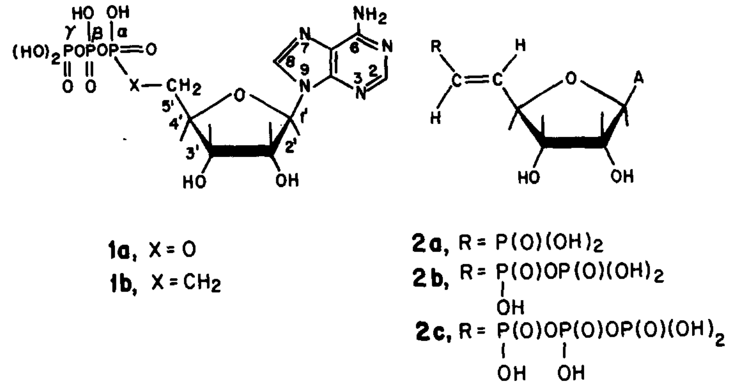

FIG. 1. Structures of ATP (**1a**) and its phosphonate isostere **1b**, and the *trans* vinyl phosphonate analogs of AMP (**2a**), ADP (**2b**), and ATP (**2c**). A = adenin-9-yl.

from NMR studies. The C4′—C5′ conformation of MgATP bound to pyruvate kinase appears to be predominantly *gauche-trans* from NMR studies (*10*) and also from substrate activity exhibited by the 5′ epimer of 8,5′-cyclo-ADP that possesses a *gauche-trans* type of 4′-5′ conformation (*9*).

The vinyl phosphonate analogs **2a** and **2b** of AMP and ADP, respectively, in which C4′ and P^{α} are *trans* to each other, have substrate properties that support a *trans* disposition of C4′ and P^{α} about the C5′—O5′ bond of AMP complexed to AMP kinase and of MgADP complexed to pyruvate kinase (*11*). We describe here a chemical synthesis of the vinyl phosphonate analog **2c** of ATP and its use as a probe of the conformation about the C5′—O5′ bond of MgATP bound to an enzyme that cleaves this bond. To this end we examined kinetically the inhibitor properties of **2c** toward two known forms of rat ATP : L-methionine *S*-adenosyltransferase (EC 2.5.1.6). This enzyme catalyzes attack of the sulfur of methionine on C5′ of ATP to form *S*-adenosylmethionine, the principal biological methyl donor (*12*). The alkyl phosphonate **1b** (*13*), by reason of its structural relationship to both **1a** and **2c**, was also examined as an inhibitor.

MATERIALS AND METHODS

General

N,*N*-Dimethylformamide (DMF) was distilled from CaH_2 and stored over molecular sieves. Paper chromatography was carried out by the ascending technique on Whatman No. 1 paper in isobutyric acid–concd. NH_4OH–water (66 : 1 : 33). Electrophoresis was carried out on Whatman No. 1 paper at pH 3.6 (0.05 M citrate). The uv spectra were obtained with a Cary Model 15 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tennessee. HPLC was performed with a Waters Model 204 chromatograph equipped with a Model M-6000A dual solvent delivery system and a Model 660 programmer. Analyses employed a Waters RCM-100 unit containing a μ-Bondapak C_{18} column eluted at 1.2 ml/min with 0.1 M KH_2PO_4–0.025 M Bu_4NHSO_4 (pH 5) with a linear gradient of 10–40% MeOH over 10 min.

9-[5′,6′-Dideoxy-6′-(hydroxypyrophosphoroxy)phosphinyl-β-D-ribo-hex-5′-trans-enofuranosyl]adenine (**2c**). The AMP analog **2a** (*11*) (0.4 mmol) was converted to the corresponding 5′-phosphoroimidazolidate and this was reacted with tri-n-butylammonium pyrophosphate according to the Hoard–Ott procedures (*14*). After filtration of the mixture and evaporation of the DMF used as solvent, the residue was dissolved in EtOH–H_2O (1 : 1, 100 ml) and the solution was brought to pH 11 with Et_3N to remove 2′,3′ cyclic carbonate residues (*15*). After 4 h at 23°C, volatiles were removed *in vacuo* and EtOH was evaporated from the residue to remove residual Et_3N. The crude product (0.4 mmol) was chromatographed at 5°C on a DEAE-cellulose column (4 × 20 cm). Elution with a linear gradient of 0–0.35 M $Et_3NH \cdot HCO_3$ (4 liters), vacuum evaporation of appropriate fractions, and several evaporations of EtOH from the residue gave the triethylammonium salt of **2c**. This was dissolved in sufficient MeOH so that addition of 1 M NaI (7.5 equiv) in MeOH gave no precipitate. The MeOH solution was evaporated to a minimum volume and diluted with acetone (10 vol). The precipitate was washed with acetone and dried at 25°C over P_2O_5 to give the sodium salt of **2c** as a white powder (68% yield) that showed a single uv-absorbing component with paper chromatography (R_f 0.22), electrophoresis (mobility relative to ATP, 0.97 : 1.00), and HPLC (R_t 15.5 min; ATP, 15 min); uv max at pH 6.8, 260 nm (ε = 14,900).

Anal. Calcd for $C_{11}H_{12}N_5O_{12}P_3Na_4 \cdot 2H_2O$: C, 21.06; H, 2.61; N, 11.16; P, 14.81. Found: C, 21.06; H, 3.00; N, 10.86; P, 15.10.

Enzyme kinetic studies. Dextran (industrial grade, molecular weight 15,000–20,000) and 2′-deoxy-ATP were obtained from Sigma Chemical Co. Dialysis tubing (Spectrapor, molecular weight cutoff 12,000) and a collodion bag concentration–dialyzer apparatus (molecular weight cutoff 25,000) were obtained from A. H. Thomas Company.

A cytosol extract of rat Novikoff hepatoma cells (2 ml; packed by centrifugation) was prepared as described (*16*) and was chromatographed on a column of DEAE-cellulose as described (*17*), after which the preparation was concentrated to ca. 1 ml in a dialysis bag immersed in dextran powder. The preparation was dialyzed overnight against 0.05 M Tris hydrochloride (pH 7.8)–50 mM KCl–5 mM $MgCl_2$–5 mM $HS(CH_2)_2OH$, and then concentrated (with simultaneous dialysis against the same mixture) with a Selectron collodion bag apparatus to ca. 0.6 ml. This stock solution of MAT-T was stored at −20°C. Portions were diluted 10-fold in the above buffer to provide a working solution. Under the conditions given below, K_m^{Met} was 15 μM [reported (*16*), 21 μM], and K_m^{ATP} was 0.14 mM [reported (*16*), 0.14 mM].

A preparation of MAT-II was obtained by extraction from rat kidney (*18*) and partial purification with DEAE-cellulose (*17*) by described methods and was then simultaneously dialyzed and concentrated as described above for MAT-T. A working solution was obtained by 10-fold dilution in the above buffer. K_m^{Met} was 5 μM [reported (*17*), 3.6 μM].

Assay mixtures (0.1 ml) contained 150 mM KCl–15 mM $MgCl_2$–5 mM dithiothreitol–50 mM Tris hydrochloride, pH 8.2. L-[*methyl*-^{14}C]Methionine (New England Nuclear Co., 54 Ci/mol) and MgATP were included at the levels specified below. Reactions were started by addition of 10 μl of working enzyme solution containing 4.7×10^{-6} units of MAT-T or 10.9×10^{-6} units of MAT-II (1 unit forms 1 μmol of *S*-adenosylmethionine per minute). Reactions were carried out at 37°C for 30 min (MAT-T) or 10 min (MAT-II) and terminated by cooling in an ice bath and addition of 10 μl of 4 N $HClO_4$-10 mM L-methionine. Each suspension was centrifuged and 50 μl of supernatant was applied to a 2.3-cm disk of phosphocellulose paper. Disks were washed as described (*19*), and then immersed in a toluene solution of phosphors and counted in a Packard liquid scintillation spectrometer (Model 2425). Blanks were provided by incubations carried out in the absence of ATP. Reaction velocities were linear for at least 30 min and were proportional to the amount of enzyme added at the levels of enzyme activity employed.

Substrate constants were determined from double-reciprocal plots of velocity vs substrate concentration; all plots were linear. K_m values for methionine (54 Ci/mol) were determined at 2 mM ATP. Substrate constants for ATP and substrate and inhibition constants for ATP analogs were determined in the presence of 60 μM [^{14}C]methionine (18 Ci/mol). This methionine level was noninhibitory. When dATP was studied as a substrate, a 10-fold higher level of MAT per assay was used than in the remaining studies.

Inhibition studies were made with six to eight levels of MgATP in the range 0.05–0.5 mM for each of two inhibitor levels that were in the range $1\text{–}5 \times K_i$ and for control mixtures lacking inhibitor. Inhibitors were tested as their 1 : 1 complexes formed by admixture of stock solutions with equimolar amounts of $MgCl_2$. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs slopes of double-reciprocal plots of velocity vs substrate level. All the latter plots were linear. All double-reciprocal plots in inhibition studies intersected on the $1/V$ axis as illustrated in Fig. 2.

The 2′-deoxy-ATP studied as a substrate and inhibitor of MAT-II and MAT-T was analyzed by HPLC on a C_{18} column eluted with aqueous 0.5 M $Et_3NH \cdot HCO_3$ and a linear gradient of 0–2% MeOH. It contained 0.04–0.08% of a single uv-absorbing contaminant that possessed the same retention time as ATP.

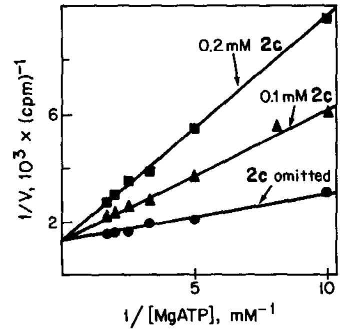

FIG. 2. Inhibition of MAT-II by **2c**.

TABLE 1

SUBSTRATE AND INHIBITOR CONSTANTS OF ATP ANALOGS WITH RAT KIDNEY (MAT-II) AND NOVIKOFF HEPATOMA (MAT-T) FORMS OF METHIONINE ADENOSYLTRANSFERASE

Enzyme	Compound	V_{max} (rel%)	$K_m{}^a$ (mM)	K_i^b (mM)
MAT-II	**1a** (ATP)	100	0.14	
	2c			0.058
	1b			0.075
	2′-dATP	6.5	4.0	
MAT-T	**1a** (ATP)	100	0.14	
	2c			0.33
	1b			0.31
	2′-dATP			2.0

[a] Concentration for half-maximal velocity.
[b] Inhibition constant.

RESULTS AND DISCUSSION

The studies employed a form of methionine adenosyltransferase (designated here as MAT-II) that is predominant in most normal rat tissues (*18, 20*), and a second form (MAT-T) that occurs in several transplantable rat tumors (*16, 21*). Enzyme-catalyzed reactions were studied at the pH and the levels of the obligatory cations K^+ and Mg^{2+} known to promote optimal catalytic efficiency (*16, 19, 22*). The level of methionine was near saturating and noninhibitory. ATP and its analogs were added as 1 : 1 Mg complexes in order to minimize variations in the level of uncomplexed Mg^{2+}.

With both forms of MAT, the ATP analogs **1b** and **2c** produced linear competitive inhibition with respect to MgATP, as exemplified in Fig. 2. The two analogs were effective inhibitors (Table 1), as evidenced by $K_m(\text{ATP})/K_i$ values ranging from 0.42 (**2c** with MAT-T) to 2.4 (**2c** with MAT-II). The dissociation constants of MgATP with these enzymes are unreported; however, the values may actually be higher than the K_m values of MgATP insofar as 8-methylthio-ATP has a K_m of 0.20 mM and a K_i of 0.53 mM with MAT-T (*23*) under the conditions of the present studies. This tends to reinforce the view that the affinities of **1b** and **2c** for the MgATP sites are comparable to that of MgATP itself. The conformationally constrained vinyl phosphonate **2c** appears to bind to the MgATP sites at least as well as the relatively flexible analog **1b**, showing that rotation about the C5′—O5′ bond of ATP is not necessary for efficient binding of ATP to these enzymes.

Our studies indicated that 2′-deoxy-ATP is a weak substrate of MAT-II with a K_m value that is approximately 30 times higher than the K_m of ATP (Table 1). It was not possible to establish unequivocally substrate activity of 2′-deoxy-ATP with MAT-T, because the observed activity was relatively weak and could have arisen from traces of ATP that appeared to be present in the 2′-deoxy-ATP as evidenced by HPLC analysis. However 2′-deoxy-ATP, as its 1 : 1 Mg complex, was a weak linear competitive inhibitor of MAT-T with respect to MgATP (Table 1), indicating that the 2′ hydroxyl of ATP plays an important role in the reversible binding of ATP to MAT-T as well as to MAT-II. ATP and 2′-deoxy-ATP, like the more extensively studied nucleotides AMP and 2′-deoxy-AMP (*24*), presumably exhibit only minor conformational differences from each other in aqueous solution, and hence the 2′ hydroxyl more likely functions in ATP binding by directly interacting with the enzyme rather than by favorably influencing the conformation of unbound ATP. The tripolyphosphate portion of ATP also appears to participate in reversible binding of ATP to methionine adenosyltransferases, insofar as inorganic tripolyphosphate is a powerful competitive inhibitor with respect to ATP (*12*). Furthermore, MAT-catalyzed reactions involve stereospecific attack of methionine sulfur on C5′ with cleavage of the C5′—O5′ bond (*12*), and are presumably preceded by enzymatic activation of the α-phosphoryl group. The foregoing catalytic properties and substrate and inhibitor specificities of these enzymes suggests that precise positioning of the C4′—C5′—O5′—P^{α} system within the active site is necessary for efficient catalysis, and that this could well require simultaneous interactions of the enzymes with the 2′ hydroxyl and the triphosphate moiety of ATP. On such a view, the present findings favor a *trans*-type disposition of C4′ and P^{α} about the C5′—O5′ bond of MAT-bound ATP. The inhibition constants of **2c** indicate that the degree of *trans* character could be as much as 100% of maximal. A *trans* disposition about C5′—O5′ has been indicated for 85% of free (non-enzyme-bound) AMP at pH 8 from NMR studies (*25–27*), and a similar C5′—O5′ conformation is thus possible in free ATP. A *trans* C5′—O5′ conformation in crystals of hydrated disodium ATP has been indicated from X-ray diffraction studies (*28*). In addition, X-ray crystallography gave evidence for *trans* C5′—O5′ conformations in the range θ = 150–167° (*cis* is 0°) in nicotinamide adenine dinucleotide bound to lactate dehydrogenase (*29*), malate dehydrogenase (*30*), and glyceraldehyde-3-phosphate dehydrogenase (*31*), and in ADP ribose bound to alcohol dehydrogenase (*32*).

The inhibitor properties of the vinyl phosphonate **2c** with methionine adenosyl transferases suggest its potential usefulness as a probe of the conformation around the C5′—O5′ bond in ATP bound to other enzymes.

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