BIOORGANIC CHEMISTRY 5, 31–35 (1976)

Evidence for the Conformation About the C(5′)–O(5′) Bond of AMP Complexed to AMP Kinase: Substrate Properties of a Vinyl Phosphonate Analog of AMP

ALEXANDER HAMPTON[1], FRANCIS KAPPLER, AND FLORIAN PERINI

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received September 15, 1975

A vinyl phosphonate analog of adenosine 5′-phosphate (AMP) was synthesized in which the CH_2–O–P system of AMP is replaced by CH=CH—P. The V_{max} values of this analog relative to AMP were 0.7% with rabbit muscle AMP aminohydrolase, 13.4% with rabbit muscle AMP kinase, and 6.6% with pig muscle AMP kinase. The vinyl analog of ADP produced by the kinases was a substrate of rabbit muscle pyruvate kinase. These results, together with substrate specificity properties at the AMP sites of the enzymes indicate that the C(4′)–C(5′)–O(5′)–P system of AMP is of *trans* character during conversion of AMP to ADP by pig or rabbit AMP kinase.

AMP (**I**, Fig. 1) is theoretically capable of existing as numerous conformers, the most important of which are defined by the torsion angles about the C(1′)–N(9), C(4′)–C(5′), and C(5′)–O(5′) bonds and by modes of puckering of the ribofuranose ring. The substrate properties of cyclo derivatives of AMP (*1*, *2*) indicate that when AMP is bound

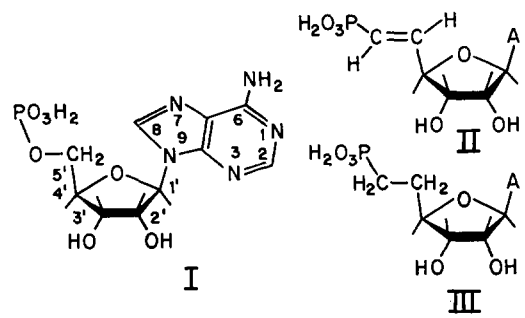

FIG. 1. Structures of AMP (**I**), its *trans* vinyl phosphonate analog (**II**), and its saturated phosphonate isostere (**III**). The 4′,5′ torsion angle was selected arbitrarily. *A*, adenine.

to the catalytic site of 5′-nucleotidase, AMP aminohydrolase, or AMP kinase the C(1′)–N(9) torsion angle is such as to position H(8) in the vicinity of C(4′) as depicted in Fig. 1. No information appears to be available as yet on the ribofuranose conformation or the C(4′)–C(5′) torsion angle of enzyme-bound AMP. The present report approaches the question of the C(5′)–O(5′) torsion angle of enzyme-bound AMP through a study

[1] Author to whom correspondence should be addressed.

of the substrate properties with AMP aminohydrolase (EC 3.5.4.6) and AMP kinase (EC 2.7.4.3) of a hitherto undescribed vinyl phosphonate analog of AMP (compound **II**) in which C(4′) and the phosphorus are fixed in a *trans* relationship to each other. Compound **II** was synthesized by removal of blocking groups from the known diphenyl ester of its 2′,3′-*O*-isopropylidene derivative. Substrate properties with the same enzyme preparations of the saturated phosphonate isostere **III** of AMP (*3*, *4*) were determined for purposes of comparison because this compound is structurally intermediate between AMP and the vinyl phosphonate **II**.

MATERIALS AND METHODS

Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville Tennessee.

Disodium Salt of 9-(5′,6′-Dideoxy-6′-phosphono-β-D-ribo-hexofuranosyl)adenine (**III**)

The acid form of **III** (*3*, *4*) (35 mg, 0.1 mmole) was freed from a minor uv-absorbing contaminant by downward chromatography for 3 days in solvent *A* (Table 1) and then dissolved in water and applied to a column of DEAE-cellulose (HCO_3^-) (2 cm × 10 cm). Compound **III** was eluted with 0.4 *M* Et_3NHCO_3. Volatiles were removed in vacuo, and

TABLE 1

PAPER CHROMATOGRAPHY AND ELECTROPHORESIS

Compound	R_f Values		Electrophoretic mobility	
	A[a]	*B*[b]	pH 7.6[c]	pH 4.5[d]
I	0.07	0.23	1.00	1.00
II	0.06	0.31	0.96	0.85
III	0.06	0.28	0.86	0.88

[a] *n*-PrOH–NH_4OH–H_2O (7 : 1 : 2).
[b] *n*-BuOH–HOAc–H_2O (5 : 2 : 3).
[c] 0.05 *M* Et_3NHCO_3.
[d] 0.05 *M* NaOAc.

the residue was coevaporated with ethanol, then dissolved in methanol (2 ml) and 1 *M* NaI in acetone (0.25 ml) was added followed by acetone (50 ml). The precipitate was washed with acetone (3 × 20 ml) and dried at 22°/0.01 mm to give the sodium salt (40 mg) as a white powder that was homogeneous on paper chromatograms and electrophoretograms (Table 1); uv max, pH 3 257 nm (ε = 15,200), pH 12 259 nm (ε = 15,500); nmr (220 MHz, D_2O) δ 8.66 (*s*, 1, H-8), 8.55 (*s*, 1, H-2), 6.37 (*d*, 1, $J_{1',2'}$ = 5 Hz, H-1′), 5.13 (*m*, 1, H-2′), 4.62 (*t*, 1, $J_{2',3'}$ = 6 Hz, H-3′), 4.51 (*m*, 1, H-4′), 2.32 (*m*, 2, H-5′, H-5″ or H-6′, H-6″), 2.04 (*m*, 2, H-5′, H-5″ or H-6′, H-6″).

Anal. Calcd for $C_{11}H_{14}N_5O_6PNa_2 \cdot 2.5H_2O \cdot CH_3OH$: C, 30.91; H, 4.97; N, 15.02; P, 6.64. Found: C, 30.87; H, 4.77; N, 15.32; P, 6.62.

9-(5',6'-Dideoxy-6'-phosphono-β-D-ribo-hex-5'-trans-enofuranosyl)adenine (II)

9-(5',6' - Dideoxy-6' - diphenylphosphono-2',3' - *O*-isopropylidene-β-D-ribo-hex-5' - enofuranosyl)adenine (0.20 g, 0.37 mmole) (*3*) was dissolved in aqueous 90% trifluoroacetic acid (10 ml) and kept at 22° for 15 min. Volatiles were removed *in vacuo*; 9 *M* NH_4OH (10 ml) was added and the excess evaporated; 2 *N* NaOH (10 ml) was then added, followed by enough tetrahydrofuran to give a solution. This was kept at 22° for 30 min and concentrated *in vacuo* to ca. 5 ml. The solution was made 0.1 *M* in Tris–HCl and 0.2 *M* in magnesium acetate and brought to pH 8.8 with HCl. Snake venom phosphodiesterase (10 mg; Sigma Chemical Co., type IV) from *Crotalus atrox* was added. Incubation was carried out at 37°C for 24 hr. The enzyme was heat denatured and removed by centrifugation. The centrifugate was adjusted to pH 3.5 and stirred at 22°C for 1 hr with Darco-G60 (3*g*). The charcoal was filtered and washed with water and extracted with aqueous ethanol (1 : 1) containing 1% NH_4OH. The filtrate was concentrated and chromatographed on five sheets (20 × 54 cm) of Whatman No. 1 paper for 3 days (descending method) in solvent *A*. Compound **II** was eluted with water and adsorbed on a DEAE–HCO_3^- cellulose column (20 × 5 cm). Elution with 0.4 *M* Et_3NHCO_3 gave, after evaporation and four coevaporations with ethanol, the triethylammonium salt of **II**. This was dissolved in methanol (3 ml) and 1 *M* NaI in acetone (1.5 ml) was added followed by acetone (75 ml). The precipitate was washed with acetone (3 × 10 ml) and dried at 22°C/0.01 mm over P_2O_5 to give the sodium salt of **II** as a white powder (142 mg; 83%) that was homogeneous in the systems of Table 1; uv max, pH 2 257 nm ($\varepsilon = 14{,}900$), pH 12 259 nm ($\varepsilon = 15{,}200$); nmr (220 MHz, D_2O) δ 8.65 (*s*, 1, H-8), 8.50 (*s*, 1, H-2), 6.85 (*ddd*, 1, $J_{4',5'} = 5$ Hz, $J_{5',6'} = 17$ Hz, $J_{P,5'} = 22$ Hz, H-5'), 6.45 (*d*, 1, $J_{1',2'} = 6$ Hz, H-1'), 6.40 (*t*, 1, $J_{5',6'} = 17$ Hz, $J_{P,6'} = 16$ Hz, H-6'), 5.13 (*t*, 1, $J_{2',3'} = 6$ Hz, H-3'), 4.71 (*t*, 1, $J_{4',5'} = 5$ Hz, H-4'). (H-2' was partially obscured by the HDO peak; a CH_3OH signal was not seen because CH_3OH was removed during evaporation of D_2O from the sample prior to obtaining the spectrum.)

Anal. Calcd for $C_{11}H_{12}N_5O_6PNa_2 \cdot 2.5H_2O \cdot CH_3OH$: C, 31.04; H, 4.54; N, 15.08; P, 6.67. Found: C, 30.96; H, 4.05; N, 15.44; P, 6.74.

Enzyme Kinetic Studies

Reactions were followed at 20°C in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 ml. In all systems, the initial velocity with AMP analogs as substrate was linear and proportional to the level of primary enzyme and independent of the level of secondary enzymes used in coupled assays. Substrate constants were determined from Lineweaver–Burk plots, all of which were linear; for each study four or five levels of substrate were used.

AMP aminohydrolase (Sigma, grade IV, from rabbit muscle) activity was measured by the decrease in absorbance ($\Delta\varepsilon = 8400$) at 265 nm in a system containing of 0.01 *M* citrate buffer (pH 6.5), 25 m*M* KCl, 0.25 and 1.25 μg (for AMP and its analogs, respectively) of enzyme, and either AMP (40–118 μ*M*), **II** (43–152 μ*M*), or **III** (31–88 μ*M*).

In a reaction with **II** that was allowed to proceed to completion the decrease in absorbance was 97% of the theoretical amount and the uv spectrum was the same as that of IMP.

Pig and rabbit muscle adenylate kinases were purchased from Boehringer and studied in 1 ml of 0.1 *M* Tris–HCl (pH 7.6) containing $MgSO_4$ (0.92 m*M*), KCl (0.11 *M*), phosphoenolpyruvate (0.31 m*M*), NADH (0.38 m*M*), ATP (0.28 m*M*), pyruvate kinase (Boehringer; 10 μg), lactate dehydrogenase (Sigma Chem. Co.; 10 μg), adenylate kinase (0.052 μg for AMP and 1.14 μg for the AMP analogs), and either AMP (122 μM–1.25 m*M*), **II** (132–622 μM), or **III** (133 μM–1.33 m*M*). A stock solution that contained all the components except AMP and the AMP analogs was made daily and stored at 22°C for 1 hr and then at 0°C; dilution of 100-μl portions to 1 ml with 0.1 *M* Tris–HCl gave the above assay levels. The reaction was started by addition of AMP or its analogs and the decrease in absorbance at 340 nm was measured. When the stock solution was prepared in this manner, no decrease in absorbance occurred prior to addition of the nucleotide. The total decrease in absorbance with **II** as substrate was 97% of the maximum, showing that the vinyl analog of ADP produced is a substrate of the pyruvate kinase.

RESULTS AND DISCUSSION

Substrate specificity studies with rabbit AMP aminohydrolase (*2, 5*) show that during the catalytic process important interactions occur between the enzyme and at least three portions of AMP, namely, the adenine ring, the phosphate moiety, and the 3′-OH. The V_{max} value of the vinyl phosphonate **II** (Table 2) is comparable to the values (0.22 and 1.0) of 3′-deoxy AMP (*2*) and adenosine (*5*), respectively, whereas the V_{max} value

TABLE 2

SUBSTRATE CONSTANTS OF II AND III

Enzyme	Compound	V_{max} (rel%)	K_m (m*M*)
AMP aminohydrolase	AMP	100[a]	0.83
	II	0.7	0.53
	III	21.8	0.39
AMP kinase (rabbit muscle)	AMP	100[b]	0.50
	II	13.4	2.7
	III	6.1	3.6
AMP kinase (pig muscle)	AMP	100[c]	0.25
	II	6.6	2.0
	III	3.9	3.3

[a] V_{max} for AMP was 397 μmole/min/mg protein.
[b] V_{max} was 210 μmole/min/mg protein.
[c] V_{max} was 180 μmole/min/mg protein.

of the more flexible alkyl phosphonate **III** is 30-fold higher than that of **II**. This suggests that the additional rigidity of **II** due to its vinyl group may prevent the normal interactions between its phosphoryl and/or 3′-OH group and the enzyme. Since the V_{max} of

II is less than that of adenosine, the phosphoryl group of **II** may not interact at all with the enzyme, and for this particular enzyme no conclusions, therefore, can be drawn regarding the conformation of the C(4′)–C(5′)–O(5′)–P system of enzyme-bound AMP.

For AMP kinases it follows from the nature of the catalysed reaction that the phosphoryl group of enzyme-bound AMP interacts with the enzyme and that a specific orientation of the O(5′)–P bond within the active site is required to bring about activation of the phosphoryl group and its subsequent stereospecific attack on the γ phosphate of enzyme-bound ATP. A second factor necessary for the action of these kinases is interaction of part of the adenine ring (possibly the basic N(1)–C(6)–N^6 amidine system) with a specific enzymic site, because IMP and GMP are not substrates (*6*, *7*). The moderately high V_{max} values (Table 2) of **II** indicate that both these factors are operative for this analog. Inspection of a molecular model of AMP shows that when the C(5′)–O(5′) torsion angle is varied it is not possible, by rotation of other bonds, to maintain constant the position and orientation of the O(5′)–P bond in relation to the adenine ring. The substrate activity of **II** thus favors the view that the C(5′)–O(5′) torsion angle of AMP kinase-bound AMP is similar to that of **II**, and therefore, that C(4′) and P are in a *trans*-type relationship. That the relatively flexible AMP analog **III** is a poorer substrate than **II** (Table 2) is possibly due to the weaker acidity of its phosphoryl group.

A *trans* disposition about C(5′)–O(5′), as indicated here for AMP kinase-bound AMP, has also been concluded to be present in 85 % of free AMP at pH 8 on the basis of nuclear magnetic resonance studies and conformational energy calculations (*8–10*). The conformation about the C(1′)–N(9) bond of enzyme-bound AMP has likewise been concluded to be similar to the C(1′)–N(9) conformation of free AMP (*1*, *2*).

ACKNOWLEDGMENTS

This work was supported by USPHS Research Grant CA-11196 from the National Cancer Institute, an award from the Pennsylvania Science and Engineering Fund, and by grants to the Institute for Cancer Research (USPHS Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania). The 220-MHz spectra were taken at the University of Pennsylvania at the Middle Atlantic Regional NMR Facility supported by NIH Grant RR-542.

REFERENCES

1. A. Hampton, P. J. Harper, and T. Sasaki, *Biochemistry* **11**, 4965 (1972).
2. A. Hampton and T. Sasaki, *Biochemistry*, **12**, 2188 (1973).
3. G. H. Jones and J. G. Moffatt, *J. Amer. Chem. Soc.*, **90**, 5337 (1968).
4. G. H. Jones, E. K. Hamamura, and J. G. Moffatt, *Tetrahedron Lett.*, 5731–5734 (1968).
5. C. L. Zielke and C. H. Suelter, *J. Biol. Chem.*, **246**, 1313 (1971).
6. L. Noda, *Enzymes*, **8**, 279 (1973).
7. N. C. Price, G. H. Reed, and M. Cohn, *Biochemistry*, **12**, 3322 (1973).
8. R. H. Sarma, R. J. Mynott, D. J. Wood, and F. E. Hruska, *J. Amer. Chem. Soc.*, **95**, 6457 (1973).
9. R. H. Sarma, C-H. Lu, F. E. Evans, N. Yathindra, and M. Sundaralingam, *J. Amer. Chem. Soc.*, **96**, 7337 (1974).
10. F. E. Evans and R. H. Sarma, *J. Biol. Chem.*, **249**, 4754 (1974).