Substrate Properties of 8,2′- and 8,3′-O-Cyclo Derivatives of Adenosine 5′-Monophosphate with Adenosine 5′-Monophosphate Utilizing Enzymes†

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ABSTRACT: The 5′-phosphates of 3′-deoxyadenosine, 9-β-D-arabinofuranosyladenine, 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine, and 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine were synthesized by phosphorylation of the corresponding nucleosides and examined together with the 5′-phosphates of 2′-deoxyadenosine and 9-β-D-xylofuranosyladenine as substrates of rabbit adenosine 5′-phosphate (AMP) aminohydrolase, snake venom 5′-nucleotidase, and rabbit AMP kinase. The respective V_{max} values of the 8,2′- and 8,3′-cyclonucleotides relative to AMP were 0.85 and 0.55% with 5′-nucleotidase, 0.008 and less than 0.001% (AMP aminohydrolase), and 0.057 and 0.045% (AMP kinase). These findings, together with the substrate properties of the remaining compounds and additional substrate specificity characteristics of the enzymes, accord with a previous conclusion that the adenine–ribose torsion angle of enzyme-bound AMP is such that H-8 is oriented in the vicinity of C-4′.

Previous studies (Hampton *et al.*, 1972) showed that 8,5′-cycloadenosine 5′-phosphate substitutes efficiently for adenosine 5′-phosphate (AMP) (Figure 1) as a substrate of 5′-nucleotidase, AMP aminohydrolase, and AMP kinase, and it was concluded that enzyme-bound AMP probably possesses an anti-type adenine–ribose torsion angle in which H-8 is positioned above C-4′ (Figure 1). In the present report the question of the adenine–ribose torsion angle of enzyme-bound AMP has been further examined through a study of the substrate properties of 8,2′-*O*-cyclo-AMP (Ib)[1] and 8,3′-*O*-cyclo-AMP (IIb) with the above three enzymes. These nucleotides were obtained by chemical phosphorylation of the previously described cyclonucleosides Ia and IIa (Ikehara, 1969). Substrate properties with the same enzymes of 2′-dAMP, 3′-dAMP (IIIb), ara-AMP (IVb), and xylo-AMP (Vb) were also examined, since these nucleotides possess structural features intermediate between those of AMP and 8,2′- and 8,3′-*O*-cyclo-AMP.

Experimental Section

Materials and General Methods. 5′-Nucleotidase (grade II, *Crotalus adamanteus* venom), AMP aminohydrolase (grade IV, rabbit muscle), lactic dehydrogenase (type I, rabbit muscle), adenosine aminohydrolase (type I, calf intestinal mucosa), phosphoenolpyruvate, 3′-deoxyadenosine (IIIa) (grade III), and 2′-dAMP were purchased from Sigma. Rabbit and pig muscle adenylate kinases were obtained from Boehringer. 9-β-D-Arabinofuranosyladenine and NADH were from Pabst Laboratories. The 8,2′- and 8,3′-cycloadenosines (Ia and IIa) were provided by Dr. M. Ikehara and xylo-AMP was provided by Dr. H. P. C. Hogenkamp.

Ascending chromatography was performed on Whatman No. 1 paper (preparative runs on acid-washed Whatman No. 3MM paper) in solvent systems A (2-propanol–concentrated NH_4OH–water (7:1:2)) and B (1-butanol–acetic acid–water (5:2:3)). Paper electrophoresis was performed with Whatman No. 1 paper in 0.05 M triethylammonium bicarbonate buffer (pH 7.5) at 57 V/cm (40 min). Phosphate ester components of the chromatograms and electrophoretograms were detected by a molybdate spray followed by ultraviolet irradiation (Hanes and Isherwood, 1949; Bandurski and Axelrod, 1951). For chromatographic and electrophoretic data see Table I.

The nucleoside 5′-monophosphates Ib–IVb were obtained by direct phosphorylation of the unprotected nucleosides Ia–IVa with $POCl_3$ in trimethyl phosphate; the reaction conditions were based on procedures by which Yoshikawa *et al.* (1967, 1969) selectively phosphorylated unprotected ribonucleosides at the 5′ position. Ib–IVb were purified by downward paper chromatography in system A and obtained as amorphous powders after lyophilization of the aqueous eluates; the ultraviolet spectral properties were identical with those of the corresponding nucleosides Ia–IVa, and upon chromatography in solvents A or B or paper electrophoresis, Ib–IVb showed only a single component, and this was ultraviolet absorbing and phosphorus containing. The yields, which were in general good, and the reaction conditions are summarized in Table II. Selective phosphorylation at the 5′-hydroxyl groups of Ia–IVa was confirmed by the complete hydrolysis of Ib–IVb with snake venom 5′-nucleotidase in 0.1 M Tris-Cl buffer (pH 8.5) at 37°. These results show that the Yoshikawa procedure for selective 5′-phosphorylation of a

† From The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111. *Received February 23, 1973.* This work was supported by U. S. Public Health Service Grant CA-11196 from the National Cancer Institute, American Cancer Society Grant T-490, an award from the Pennsylvania Science and Engineering Fund, and by grants to the Institute for Cancer Research (U. S. Public Health Service Grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania).

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[1] Abbreviations used are: 2′-dAMP, 2′-deoxyadenosine 5′-phosphate; 3′-dAMP, 3′-deoxyadenosine 5′-phosphate; 8,2′-*O*-cyclo-AMP, 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine 5′-phosphate; 8,3′-*O*-cyclo-AMP, 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine 5′-phosphate; ara-AMP, 9-β-D-arabinofuranosyladenine 5′-phosphate; xylo-AMP, 9-β-D-xylofuranosyladenine 5′-phosphate; P-enolpyruvate, phosphoenolpyruvate.

TABLE I: Paper Chromatography and Electrophoresis.

Compd	R_F in Solvent A	R_F in Solvent B	M_{AMP}[a] pH 7.5
Ia	0.41	0.54	
IIa	0.39	0.53	
IIIa	0.43	0.51	
IVa	0.39	0.50	
AMP	0.08	0.26	1.00
Ib	0.06	0.32	1.08
IIb	0.05	0.31	0.97
IIIb	0.08	0.38	0.98
IVb	0.08	0.34	0.94
Vb	0.08	0.35	0.94

[a] Electrophoretic mobility relative to AMP.

TABLE II: Conditions Used for Phosphorylation of Nucleosides.[a]

Nucleoside No.	mg	$(MeO)_3PO$ (μl)	$POCl_3$ (μl)	H_2O (μl)	Time (hr)	Yield of 5′-Nucleotide[b] (%)
Ia	27	250	20		10	62
IIa	7.2	50	5		10	65
IIIa	10	50	10		8	32
IVa	107	2.8 (ml)	110	7	8	69

[a] All reactions were carried out at 5°. [b] Determined spectrophotometrically subsequent to paper chromatography.

nucleoside can be successful in the absence of a 2′,3′-*cis*-diol system.[2]

Enzyme assays were carried out by measuring the rate of change of absorbance at a suitable wavelength in a Cary Model 15 spectrometer using 1-cm cells containing a final volume of 1 ml. In all systems the initial velocity with AMP as substrate was linear and proportional to the concentration of primary enzyme and independent of the concentration of secondary enzymes used in coupled assays. Kinetic parameters were obtained from Lineweaver–Burk plots in which no less than four different substrate concentrations were employed.

5′-Nucleotidase was assayed by following the decrease in absorbance at 265 nm in a coupled assay with adenosine deaminase in 1 ml of 0.1 M Tris-Cl buffer, pH 8.5. The decrease in optical density was measured at 265 nm where for AMP $\Delta\epsilon$ for the conversion is 6600. The amount of 5′-nucleotidase employed was 2.25 μg for AMP, 18 μg for 2′- and 3′-dAMP, and 67.5 μg for 8,2′-*O*-cyclo-, ara-, and xylo-AMP. Adenosine deaminase concentration varied from 50 to 750 μg/ml of assay solution according to the substrate activity of the adenosine derivative under study.

[2] The pyrophosphoryl chloride–*m*-cresol method of Imai *et al.* (1969) has been successfully employed in the same fashion for the synthesis of other AMP analogs lacking a 2′,3′-*cis*-diol system (Follman and Hogenkamp, 1971).

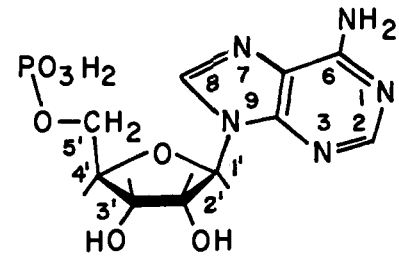

FIGURE 1: AMP depicted in an anti conformation.

The rate of dephosphorylation of 8,3′-*O*-cyclo-AMP by 5′-nucleotidase was followed by chromatography because 8,3′-*O*-cycloadenosine was not a substrate of adenosine deaminase. Solutions (50–250 μM) of 8,3′-*O*-cyclo-AMP in 100 μl of 0.1 M Tris-Cl buffer, pH 8.5, containing 15 μg of 5′-nucleotidase were kept for 5 min at 20° after which 25 μl of Cl_3CCOOH was added. Chromatography was then carried out in solvent A and IIa was eluted with water and determined spectrophotometrically.

The AMP aminohydrolase reactions were carried out in 0.01 M citrate buffer (pH 6.5) containing 25 mM KCl. Measurement and calculation of $\Delta\epsilon$ were the same as with 5′-nucleotidase. The amount of enzyme employed was 0.1 μg for AMP, 5 μg for 3′-dAMP and ara-AMP, and 50 μg for xylo-, 8,2′-*O*-cyclo-, and 8,3′-*O*-cyclo-AMP.

AMP kinase was assayed in 1 ml of 0.1 M Tris-Cl (pH 7.6) containing KCl (0.1 M), $MgSO_4$ (1 mM), ATP (0.28 mM), *P*-enolpyruvate (0.87 mM), NADH (0.38 mM), and pyruvate kinase and lactic dehydrogenase as noted below. The OD change at 340 nm was initiated by the addition of the compound studied. The amount of pig muscle AMP kinase used was: 0.43 μg for AMP, 0.86 μg for 2′- and 3′-dAMP, and 86 μg for 8,2′- and 8,3′-*O*-cyclo-AMP. The rabbit muscle AMP kinase employed was 0.77 μg for AMP, 2.46 μg for 2′- and 3′-dAMP, and 40 μg for 8,2′- and 8,3′-*O*-cyclo-AMP. The concentration of lactic dehydrogenase and pyruvate kinase varied from 50 to 172 μg/ml of assay solution according to the substrate activity of the compound studied.

Results

All Burk–Lineweaver plots were linear; the Michaelis constants and maximal velocity values obtained therefrom are listed in Tables III–V.

Chromatography in system A showed that when 50 μg of 8,2′-*O*-cyclo-AMP, ara-AMP, or 2′- or 3′-dAMP were in-

TABLE III: AMP Analogs as Substrates of 5′-Nucleotidase of *Crotalus adamanteus* Venom.

Compound	K_m (μM)	Rel V_{max}
AMP	27	100.0[a]
8,2′-*O*-Cyclo-AMP	96	0.85
2′-dAMP	238	15.8
Ara-AMP	192	0.61
8,3′-*O*-Cyclo-AMP	333	0.55
3′-dAMP	143	10.7
Xylo-AMP	550	1.37

[a] V_{max} for AMP was 1.43 μmol/min per mg of protein.

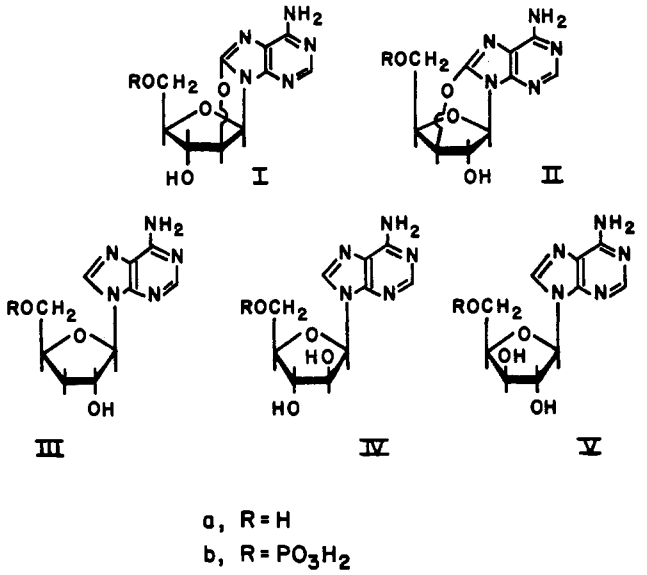

FIGURE 2: Structures of AMP analogs studied: Ib, 8,2′-*O*-cyclo-AMP; IIb, 8,3′-*O*-cyclo-AMP; IIIb, 3′-dAMP; IVb, ara-AMP; Vb, xylo-AMP. Systematic names for these compounds are listed in ref 1.

cubated at 37° for 3 hr with 900 μg of the nucleotidase and 750 μg of adenosine deaminase in 100 μl of Tris-Cl buffer, pH 8.5, they were replaced by materials of R_F 0.31, 0.31, 0.33, and 0.33, respectively, all of which at pH 7.0 showed an absorption maximum at 250 nm.

Discussion

The cyclonucleotides Ib and IIb can be regarded as derived from AMP by three successive structural modifications: (1) replacement of the 2′- or 3′-OH by H to give 2′- and 3′-dAMP, (2) introduction of a 2′- or 3′-OH in an inverted position to give ara-AMP and xylo-AMP, respectively, and (3) formation of 8,2′-O and 8,3′-O bonds.

With one exception noted below, replacement of a ribose OH of AMP by H led to a five- to tenfold diminution in maximal velocity with the four enzymes studied. The influence on V_{max} of the 2′ and 3′ hydroxyls of AMP could arise not only from direct interaction with enzymic groups but also indirectly *via* an influence on the conformation of AMP; for example, O-3′ could affect the orientation of O-5′ by exerting repulsive forces on it, while the 2′-OH may form a hydrogen bond with N-3 as suggested by certain proton magnetic resonance (pmr) evidence (Schweizer *et al.*, 1968). With AMP aminohydrolase, replacement of the 3′-OH of AMP by H causes an exceptionally large (500-fold) reduction in V_{max}, suggesting that in this case the 3′-OH promotes catalysis mainly by direct interaction with the enzyme.

Introduction of sugar hydroxyls into 2′- and 3′-dAMP to give ara- and xylo-AMP additionally diminished V_{max} by a factor of 7–400. If ara- and xylo-AMP are bound to the enzymes with the 8,5′-type glycosidic torsion angle postulated for AMP (Hampton *et al.*, 1972) then 8,3′-*O*-cyclo-AMP might be expected to be a less effective substrate than xylo-AMP and 8,2′-cyclo-AMP to be less effective than ara-AMP. A pronounced effect of this type was in fact observed in the case of AMP aminohydrolase where 8,3′ cyclization of xylo-AMP produced a 20-fold or greater reduction in V_{max} and 8,2′ cyclization of ara-AMP produced a 250-fold reduction. The effect was less pronounced with AMP kinase where 8,2′ cyclization caused a 20-fold reduction in V_{max}, whereas 8,3′ cyclization did not alter V_{max}. In the case of 5′-nucleotidase, 8,3′ cyclization reduced substrate activity only twofold and 8,2′ cyclization had no effect. The differing responses of the three enzymes to cyclization of ara- and xylo-AMP correlate with variations in the specificity of their interaction with the adenine ring of AMP. This specificity is most pronounced for AMP aminohydrolase which acts upon none other than adenine-derived nucleotides (Zielke and Suelter, 1971, and references therein), is less marked for AMP kinase which utilizes CMP in addition to AMP (Noda, 1962), and is low for the venom 5′-nucleotidase which dephosphorylates ribose 5-phosphate at 2% the rate of AMP (Heppel and Hilmoe, 1951). Since ara- and xylo-AMP were actually poorer substrates than ribose 5-phosphate for the 5′-nucleotidase, it is possible that their adenine rings may interact weakly if at all with this enzyme, thus accounting for the observation that 8,2′ or 8,3′ cyclization is virtually without effect on substrate activity.

The present data indicate that the rate of AMP aminohydrolase reactions will be highly dependent upon accurate positioning on the enzyme of the 3′-OH as well as of the reaction center at C-6, and deviations from the optimal ribose–adenine torsion angle of the enzyme-bound substrate can hence be expected to readily impair catalysis. With AMP

TABLE IV: AMP Analogs as Substrates of AMP Aminohydrolase.

Compound	K_m (mM)	Rel V_{max}
AMP	0.5	100.0[a]
8,2′-*O*-Cyclo-AMP	0.1	0.0083
2′-dAMP	2.3	18.5
Ara-AMP	0.3	2.6
8,3′-*O*-Cyclo-AMP		*b*
3′-dAMP	0.2	0.22
Xylo-AMP	0.5	0.0026

[a] V_{max} for AMP was 1210 μmol/min per mg of protein. [b] No detectable substrate activity.

TABLE V: AMP Analogs as Substrates of Pig and Rabbit AMP Kinases.

Compound	AMP Kinase (Pig Muscle) K_m (mM)	AMP Kinase (Pig Muscle) Rel V_{max}	AMP Kinase (Rabbit Muscle) K_m (mM)	AMP Kinase (Rabbit Muscle) Rel V_{max}
AMP	0.19	100.0[a]	0.50	100.0[b]
8,2′-*O*-Cyclo-AMP	0.07	0.0031	0.31	0.057
2′-dAMP	1.67	32.0	0.28	20.9
Ara-AMP			1.43	1.3
8,3′-*O*-Cyclo-AMP	0.04	0.0027	0.16	0.045
3′-dAMP	2.00	40.0	1.00	20.3
Xylo-AMP			1.71	0.052

[a] V_{max} for AMP was 88 μmol/min per mg of protein. [b] V_{max} for AMP was 121 μmol/min per mg of protein.

kinase, on the other hand, the interactions between the enzyme and the adenine ring or the ribose hydroxyls are less exacting than with AMP aminohydrolase and a given alteration in the ribose–adenine torsion angle should therefore be better tolerated; such an alteration would also tend to be better tolerated by AMP kinase because the concomitant rotation of the 4′,5′ bond which can occur would tend to protect the phosphate reaction center from unfavorable effects on catalysis. It is concluded that the substrate properties of 8,2′- and 8,3′-*O*-cyclo-AMP with AMP aminohydrolase and AMP kinase are consistent with the previous postulate that in enzyme-bound AMP H-8 is located in the area above C-4′ and, further, these properties provide evidence that rotation of the ribose–adenine bond so as to position H-8 first over H-3′, then over H-2′, progressively reduces substrate activity.

Acknowledgments

The authors thank Dr. Morio Ikehara for providing 8,2′-anhydro-8-hydroxy-9-β-D-arabinofuranosyladenine and 8,3′-anhydro-8-hydroxy-9-β-D-xylofuranosyladenine, and Dr. H. P. C. Hogenkamp for providing 9-β-D-xylofuranosyladenine 5′-phosphate.

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Specific Binding of Ribonucleic Acid by Antiadenosine Antibodies†

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ABSTRACT: Antibodies to the four major RNA bases were elicited by immunization with nucleoside–protein conjugates. Reactivity of RNA with antinucleoside antibody was investigated by the double-antibody technique which measures the primary interaction between antibody and antigen. In order to demonstrate a reaction between antinucleoside antibody and RNA, serum ribonuclease activity had to be eliminated, and at the levels of RNase found in the antisera this could be done with Na_2SO_4. In the presence of 0.2 M Na_2SO_4, antiadenosine (anti-A) reacted with all RNA preparations tested, except tRNA. No reaction with RNA could be detected with antibody to guanosine, cytidine, or uridine under identical conditions although these were at least as reactive with DNA as was anti-A. The specificity of RNA–anti-A interaction was characterized further by inhibition experiments. Only adenosine and adenine-containing nucleosides, nucleotides, or polynucleotides inhibited the binding of tritiated *Escherichia coli* RNA. The inhibitory activity of poly(A) was lost when it was complexed with poly(U) in a hydrogen-bonded duplex.

Immunochemical techniques have been of great value in studies on the structure and function of proteins and polysaccharides. Recently, nucleic acid reactive antibodies have been obtained, both experimentally and from sera of patients with systemic lupus erythematosus, thereby making possible similar immunochemical studies of nucleic acids.

One method for obtaining antibodies that react with nucleic acids involves the use of hapten–protein conjugates (Butler *et al.*, 1962; Erlanger and Beiser, 1964; Sela *et al.*, 1964; Halloran and Parker, 1966; Karol and Tanenbaum, 1967). A general procedure, based on periodate oxidation of the free vicinal hydroxyl groups, was developed by Erlanger and Beiser (1964) for coupling any ribonucleoside or ribonucleotide to a protein carrier. Specific antibodies were obtained for the five common bases present in RNA and DNA. The specificity of these antisera, determined by precipitation, complement fixation, and hapten inhibition, was directed primarily against the homologous purine or pyrimidine base. The antisera cross-reacted with denatured DNA from all species tested, but did not react with native DNA. All attempts to demonstrate direct reaction of the antisera with

† From the Department of Microbiology, Columbia University, New York, N. Y. 10032. *Received December 19, 1972.* Taken from the dissertation submitted by B. J. Rosenberg in partial fulfillment of the requirements for the Ph.D. degree at Columbia University, New York, N. Y. Supported by Grant AI-06860 from the National Institutes of Health.

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