Interactions of the Epimeric 5′-*C*-Methyl and 5′-*C*-Carbamyl Derivatives of Adenosine Monophosphate with Adenosine Monophosphate Utilizing Enzymes†

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ABSTRACT: The two 5′ (allo and talo) epimers of 5′-*C*-carbamyl-2′,3′-*O*-isopropylideneadenosine have been synthesized and phosphorylated, in addition to the corresponding 5′-*C*-methyl epimers, to furnish the four title compounds. The respective V_{max} values of *allo*- and *talo*-5′-methyl-AMP relative to AMP were 0.4 and 38% for rabbit AMP-aminohydrolase, 0.57 and 0.22% for *Crotalus* 5′-nucleotidase, 0.04 and 0.9% for pig AMP-kinase, and 0.006 and 1.05% for rabbit AMP-kinase. The V_{max} values of *allo*- and *talo*-5′-carbamyl-AMP relative to AMP were 0.001 and 0.01% for rabbit AMP-kinase. Only *talo*-5′-methyl-ADP was a substrate of rabbit pyruvate kinase. These and previous findings with calf adenosine aminohydrolase reveal a general preference for utilization of *talo*-5′-methyladenosine derivatives as substrates. Previous postulates of the conformation of enzyme-bound adenosine and AMP suggest that the enzyme-bound 5′-methyl-*talo* compounds would be relatively compact in shape and hence be preferentially accommodated within enzyme sites. *allo*- and *talo*-5′-methyl-AMP were competitive inhibitors of AMP-aminohydrolase (K_i = 0.10 and 0.27 mM, respectively); with pig AMP-kinase the allo epimer was a noncompetitive inhibitor (K_i = 0.22 mM) whereas the talo epimer was competitive (K_i = 0.30 mM). The 5′-carbamyl-AMP epimers were noncompetitive inhibitors of AMP-aminohydrolase. The data indicate that the complexes of AMP with AMP-aminohydrolase and with AMP-kinase can accommodate a 5′-*C*-methyl group in either the allo or talo configuration, although bulk tolerance of the AMP-kinase complex towards a 5′-*C*-methyl group in the allo configuration is very limited.

Previous studies (Hampton *et al.*, 1973a,b) investigated the effect on enzymic interactions of adenosine 5′-phosphate (AMP) of introducing additional bulk in AMP in the region of the oxygen atom (O-5′) which bridges the phosphate and ribose segments. In the analogs synthesized for that purpose the $CH_2OPO_3H_2$ system of AMP was replaced by $CH_2CH(CN)PO_3H_2$ and $CH_2CH(OH)PO_3H_2$, respectively, and it was found that the complexes of AMP with AMP-kinase and with AMP-aminohydrolase contain sufficient space near O-5′ to accommodate either an hydroxyl group or a cyano group. In the present report these studies have been extended by an investigation of the effect of added bulk at the adjoining area of AMP centered about C-5′. For this purpose the two 5′ (talo and allo) epimers of 5′-*C*-methyl-AMP[1] (Ia and IIa, respectively) were obtained by chemical phosphorylation of the previously described 2′,3′-*O*-isopropylidene derivatives of the corresponding 5′-*C*-methyladenosines (Howgate and Hampton, 1972); in addition, the hitherto unknown epimeric 5′-*C*-carbamyl derivatives of 2′,3′-*O*-isopropylideneadenosine were synthesized and converted to the talo and allo epimers of 5′-*C*-carbamyl-AMP (Ib and IIb). This report describes substrate and inhibitor properties of the four compounds with AMP-aminohydrolase of rabbit muscle, 5′-nucleotidase of snake venom, and AMP-kinases of rabbit and pig muscle.

Materials and Methods

Ultraviolet spectra were obtained in buffered aqueous solutions with a Cary Model 15 spectrophotometer and nuclear magnetic resonance (nmr) spectra with a Varian XL-100-15 instrument. A Bendix automatic polarimeter 1169 was used to obtain specific rotations. Paper chromatography and cellulose thin-layer chromatography (Eastman 6065 sheets) were carried out in: (A) ethanol–1 M ammonium acetate (7:3); (B), 2-propanol–concentrated ammonia–water (7:1:2); (C) *tert*-amyl alcohol–formic acid–water (3:2:1); (D) *tert*-butyl alcohol–methyl ethyl ketone–water–concentrated ammonia (4:3:2:1). Silical gel thin-layer chromatography (tlc) was carried out with (E) chloroform–methanol (7:3). Paper electrophoresis was carried out in (1) 0.05 M triethylammonium bicarbonate (pH 7.5) and (2) 0.05 M acetate buffer (pH 4.5).

allo-5′-Methyl-AMP (IIa). The procedure was based on that of Yoshikawa *et al.* (1967, 1969). Phosphorus oxychloride (0.088 ml, 0.94 mmol) and dry trimethyl phosphate (0.06 ml) were added to 9-(6′-deoxy-2′,3′-*O*-isopropylidene-β-D-allofuranosyl)adenine (20 mg, 0.06 mmol) (Howgate and Hampton, 1972) previously dried *in vacuo* at 78.5°. The mixture was stirred under anhydrous conditions at 5°; after 3 hr the nucleoside dissolved. Tlc on cellulose (solvent A) indicated that the reaction was complete after 42 hr. Volatiles were removed at 0.1 mm, 25°. The yellow syrup was cooled to −5° and ice-water (0.5 ml) was rapidly added followed by cold (−5°) saturated LiOH solution to pH 1.5. The solution was heated with stirring at 70° (bath temperature) for 30 min in a closed flask. Tlc (solvent A) indicated that deacetonation was complete with apparently no formation of adenine. The solution

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[1] Abbreviations used are: *talo*-5′-methyl-AMP, 9-(6′-deoxy-5′-*O*-phosphoryl-α-L-talofuranosyl)adenine; *allo*-5′-methyl-AMP, 9(6′-deoxy-5′-*O*-phosphoryl-β-D-allofuranosyl)adenine; *talo*-5′-carbamyl-AMP 9-(5′-*O*-phosphoryl-α-L-talofuranuronamide)adenine; *allo*-5′-carbamyl-AMP, 9-(5′-*O*-phosphoryl-β-D-allofuranuronamide)adenine.

TABLE 1: Paper Chromatography and Electrophoresis.

Compound	R_F Values System A	B	C	Electrophoretic Mobility (cm) pH 7.5	pH 4.5
AMP	0.11	0.11	0.31	31.2	18.5
allo-5′-Methyl-AMP	0.16	0.13	0.40	29.9	17.4
talo-5′-Methyl-AMP	0.19	0.15	0.42	30.0	18.0
allo-5′-Carbamyl-AMP	0.07	0.07		27.1	
talo-5′-Carbamyl-AMP	0.04	0.05		26.8	

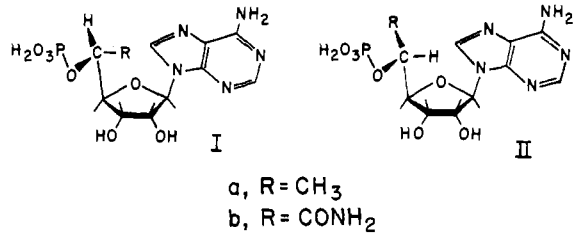

FIGURE 1: Structures of the AMP derivatives studied: (Ia) *talo*-5′-methyl-AMP; (Ib) *talo*-5′-carbamyl-AMP; (IIa) *allo*-5′-methyl-AMP; (IIb) *allo*-5′-carbamyl-AMP. Systematic names for these compounds are listed in footnote 1.

was brought to pH 8.0 with LiOH solution and chromatographed at 5° on a column (2.5 cm, diameter 2.2 cm) of Dowex 1 (Cl^-) (400 mesh) with a linear gradient of 800 ml of 0.005 N HCl into 800 ml of 0.001 N HCl. The material obtained by lyophilization of appropriate fractions was chromatographed on Whatman 3MM paper in solvent B using two developments to give the product as a white powder (17 mg). 9-(6′-Deoxy-5′-*O*-phosphoryl-β-D-allofuranosyl)adenine (dried at 78.5° (0.1 mm)) had λ_{max} 257 nm (ϵ 15.1 × 10^3 calculated for a dihydrate) at pH 2.0, λ_{max} 259 nm (ϵ 15.4 × 10^3) at pH 11.0; $[\alpha]_D^{24.5}$ −21 ± 1° (*c* 0.64, water). The nucleotide migrated as a single spot on paper electrophoresis and chromatography (Table I). The yield (determined spectroscopically) was 72%. It was quantitatively converted into the known 9-(6′-deoxy-β-D-allofuranosyl)adenine (Howgate and Hampton, 1972) by venom 5′-nucleotidase, as shown by paper chromatography and electrophoresis.

talo-5′-Methyl-AMP (Ia). 9-(6′-Deoxy-2′,3′-*O*-isopropylidene-α-L-talofuranosyl)adenine (Howgate and Hampton, 1972) was phosphorylated as described for its allo epimer. The nucleotide Ia (yield, 76%) showed the same ultraviolet spectral properties as IIa and had $[\alpha]_D^{24.3}$ −28 ± 0.5° (*c* 1.34, water). It migrated as a single spot on paper electrophoresis and chromatography (Table I). The nmr spectrum was determined on a 0.17 M solution in D_2O (pD 10) of Ia which had been lyophilized three times from D_2O: (100 MHz, Me_4Si external) δ 9.15 (s, 1, H-8), 8.67 (s, 1, H-2), 6.59 (d, 1, H-1′, *J* = 6 Hz), 5.2 (H-2′, partially obscured by HDO), 5.05 (m, 1, H-3′), 4.88 (t, 1, H-4′), 4.67 (m, 1, H-5′), 1.82 (d, 3, *J* = 6.0 Hz, methyl). Assignments of chemical shifts are based on the spectra of 9-(6′-deoxy-2′,3′-*O*-isopropylidene-α-talofuranosyl)adenine (Howgate and Hampton, 1972) and of AMP (Feldman and Agarwal, 1968).

9-(2′,3′-O-Isopropylidene-β-D-allofuranuronamide)adenine. The 5′-aldehyde of isopropylideneadenosine (300 mg) (Pfitzner and Moffatt, 1963; Gleason and Hogenkamp, 1971) was suspended in acetone cyanohydrin (3 ml) and warmed on a steam bath until the aldehyde dissolved (5 min). The solution was kept at room temperature for 30 min, then treated with 30% H_2O_2 (1 ml) and 10 N NaOH (to pH 10). After 30 min, the solution was neutralized with acetic acid and evaporated. The residue was chromatographed on a silica gel plate (20 cm × 20 cm × 2 mm) with chloroform–methanol (4:1). Three major zones were present with R_F values 0.7, 0.5, and 0.4. The zone with R_F 0.7 was unreacted 2′,3′-*O*-isopropylideneadenosine (20 mg) from the 5′-aldehyde preparation. The zone with R_F 0.5 was eluted with 10% methanolic chloroform, and the resulting solid crystallized from water–methanol to give small needles (63 mg): mp 235–238° dec; $[\alpha]_D^{20}$ −110° (1:1 ethanol–H_2O, *c* 1.0); uv λ_{max} (pH 2) 256 nm (ϵ 15,000) (pH ≥ 7), 259 nm (ϵ 15,300); nmr (100 MHz, Me_2SO-d_6, Me_4Si external) δ 8.73 (s, 1, H-8), 8.56 (s, 1, H-2), 7.78 (broad, 4, $-NH_2$ and $-CONH_2$), 7.02 (d, 1, *J* = 5.0 Hz, –OH), 6.53 (d, 1, *J* = 4.0 Hz, H-1′), 5.57 (d of d, 1, *J* = 4.0 and 6.5 Hz, H-2′), 5.34 (d of d, 1, *J* = 2.1 and 6.5 Hz, H-3′), 4.89 (d of d, 1, *J* = 2.1 and 3.1 Hz, H-4′), 4.55 (d of d, 1, *J* = 3.1 and 5.0 Hz, H-5′), 1.92 (s, 3), and 1.68 (s, 3, isopropylidene).

Anal. Calcd for $C_{14}H_{18}N_6O_5 \cdot 0.25H_2O$: C, 47.41; H, 5.25; N, 23.65. Found (material dried at 100°, 0.1 mm): C, 47.42; H, 5.09; N, 23.14.

9-(2′,3′-O-Isopropylidene-α-L-talofuranuronamide)adenine. The zone with R_F 0.4 from the preceding chromatogram was eluted with 10% methanolic chloroform and the resulting solid crystallized from methanol to give fluffy needles (91 mg): mp 275–278° dec; $[\alpha]_D^{20}$ −55° (1:1 ethanol–water, *c* 1.0); uv λ_{max} (pH 2) 256 nm (ϵ 15,100) (pH > 7), 258 nm (ϵ 15,600); nmr (100 MHz, Me_2SO-d_6) δ 8.77 (s, 1, H-8), 8.56 (s, 1, H-2), 7.68 (s, 2, exchange–NH_2), 7.61 and 7.55 (2 peaks, 2, exchange –CO–NH_2), 6.99 (d, 1, *J* = 6.5, exchange, –OH), 6.46 (d, 1, *J* = 4.0 Hz, H-1′), 5.47 (d of d, 1, *J* = 5.7 and 3.9 Hz, H-2′), 5.39 (d of d, 1, *J* = 5.7 and 2.0 Hz, H-3′), 5.01 (d of d, 1, *J* = 2.0 and 2.2 Hz, H-4′), 4.52 (d of d, 1, *J* = 2.2 and 6.5 Hz, H-5′), 1.91 (s, 3), and 1.66 (s, 3, isopropylidene).

Anal. Calcd for $C_{14}H_{18}N_6O_5$: C, 48.00; H, 5.18; N, 23.99. Found (dried at 100°, 0.1 mm): C, 48.19; H, 5.06, N, 23.92.

The absolute configuration of the foregoing two nucleosides was assigned by comparison of their specific rotations with those of the known analogs in which a methyl group replaces the 5′-carbamyl group. In the 5′-methyl-substituted nucleosides (Howgate and Hampton, 1972) the allo epimer, $[\alpha]_D$ −90°, is 51° more levorotatory than the talo epimer; in the 5′-carbamyl nucleosides the allo epimer is 55° more levorotatory than the talo epimer.

talo- and allo-5′-Carbamyl-AMP (Ib and IIb). 9-(2′,3′-*O*-Isopropylidene-α-L-talofuranuronamide)adenine (5 mg) was treated in pyridine (1 ml) at 22° for 24 hr with *o*-phenylene phosphorochloridate (3 molar excess), a reagent of general utility for the synthesis of nucleotides (Khwaja and Reese, 1966, 1971; Khwaja *et al.*, 1970). Water (0.3 ml) was added and after 16 hr at 5° the *o*-hydroxyphenyl ester of the 2′,3′-*O*-isopropylidene derivative of Ib was isolated by paper electrophoresis at pH 7.5 and heated for 1.25 hr at 80° in aqueous HCl (pH 2). The residue from lyophilization was treated for 30 min with 0.4 ml of a 1% solution of bromine in 0.1 M aqueous barium acetate[2] after which the barium salt of Ib (30% yield) was precipitated with ethanol and converted with an ion-

[2] The *o*-hydroxyphenyl group could also be removed, but in poor yield, by treatment with large amounts of *Crotalus* venom phosphodiesterase at pH 8.8, or with 2 M NaOH to which eight volumes of 30% H_2O_2 was added in portions during 5 days, 25°.

TABLE II: Kinetic Parameters of the Epimeric 5′-*C*-Methyl and 5′-*C*-Carbamyl Derivatives of AMP with AMP-aminohydrolase and 5′-Nucleotidase.

Compound	V_{max} (Rel)	K_m (mM)	K_i (mM)
AMP-aminohydrolase			
AMP	100	0.56	
allo-5′-Methyl-AMP	0.4	0.24	0.099[b]
talo-5′-Methyl-AMP	38	1.35	0.275[b]
allo-5′-Carbamyl-AMP	<0.001[a]		0.215[c]
talo-5′-Carbamyl-AMP	0.006	0.02	0.050[c]
5′-Nucleotidase			
AMP	100	0.065	
allo-5′-Methyl-AMP	0.57	0.370	0.041[c]
talo-5′-Methyl-AMP	0.22	0.454	0.193[c]
allo-5′-Carbamyl-AMP	<0.1[a]		

[a] No detectable substrate activity. The upper limit of V_{max} shown was calculated on the assumption that the K_m value was the same as that of AMP. [b] Competitive inhibition. [c] Mixed competitive–noncompetitive inhibition.

exchange resin to the potassium salt for use in the enzymatic studies. The material was homogeneous as judged by paper chromatography and electrophoresis (Table I) and possessed the same ultraviolet absorption maxima as Ia.

The use of dioxane–triethylamine, dioxane–2,6-lutidine, or acetonitrile–2,6-lutidine as reaction solvent with a 6 molar excess of *o*-phenylene phosphorochloridate gave very low yields of Ib. The phosphorylation method of Yoshikawa *et al.* (1967) produced no Ib.

9-(2′,3′-*O*-Isopropylidene-β-D-allofuranuronamide)adenine was phosphorylated by the same reagent in dioxane (1 ml) containing triethylamine (0.15 ml). The subsequent basic treatment sufficed to remove the *o*-hydroxyphenyl group in this case. The isopropylidene derivative of IIb was purified by paper electrophoresis prior to acidic treatment and isolation of unblocked IIb (70% yield).

Enzyme Kinetic Studies. All assays were done at 20°. Initial velocities were measured with a Cary Model 15 spectrophotometer and in all cases were linear and proportional to the level of primary enzyme and independent of the level of secondary enzymes used in coupled assays. Each substrate study employed four or more concentrations of substrate and each inhibition study employed, in addition, two or more levels of inhibitor. Substrate constants were obtained from Burk–Lineweaver plots, all of which were linear, and inhibition constants from replots of inhibitor concentration *vs.* slope which were also linear in all cases.

AMP-aminohydrolase (rabbit muscle) was a grade 4 preparation from Sigma. The reaction was carried out in 0.01 M potassium citrate (0.01 M KCl), pH 6.5 (final volume, 1 ml), and was followed by the decrease in absorbance at 265 nm. For comparison of AMP and the *talo*-5′-methyl-AMP substrate properties, and for study of inhibition of AMP deamination by the *allo*-5′-methyl-AMP, 0.076 μg of enzyme was added for each determination; 0.019 μg was used to study inhibition by the *talo*-5′-methyl-AMP and 0.38 μg for the substrate activity of the *allo*-5′-methyl-AMP. Compounds Ib and IIb were studied with 50 μg of enzyme. The enzyme was diluted into 1 M KCl prior to use.

5′-Nucleotidase (Sigma, grade II, from *Crotalus adamanteus*) was assayed by following the decrease in absorbance at 265 nm in a coupled assay with adenosine deaminase (Sigma, Type I from calf intestinal mucosa) in 1 ml of 0.1 M Tris-Cl buffer (pH 8.5). Studies of the inhibition by the epimeric 5′-methyl-AMP derivatives employed 0.9 μg of nucleotidase and 0.01 μg of adenosine deaminase per determination, and studies of their substrate activities employed 0.17 mg of nucleotidase and 0.1 mg of adenosine deaminase.

AMP-kinase (pig muscle) was from Boehringer. Pyruvate kinase (rabbit muscle, Type II) and lactate dehydrogenase (rabbit muscle, Type I) were from Sigma Chemical Co. For studies of inhibition of transformation of AMP the system contained lactate dehydrogenase (25 μg), pyruvate kinase (4.8 μg), and AMP-kinase (0.1 μg) 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), and NADH (0.38 mM). Substrate studies with the epimeric 5′-methyl-AMP nucleotides were performed under the same conditions except that 40 μg of pyruvate kinase was used and that 4 μg of AMP-kinase was employed with the talo epimer and 30 μg with the allo epimer.

AMP kinase of rabbit muscle and the pyruvate kinase (rabbit muscle) used in this assay were from Boehringer. Assays with AMP as substrate used 10 μg of the pyruvate kinase and 0.2 μg of the AMP-kinase, the remaining conditions being identical with those employed with the pig AMP-kinase. With *allo*-5′-methyl-AMP as substrate, assays contained increased amounts of pyruvate kinase (50 μg) and AMP-kinase (40 μg) and with the talo epimer (Ia) the conditions were identical except that 10 μg of AMP kinase was used. Studies with Ib used the same conditions as for Ia; with IIb, 150 μg of AMP-kinase was used.

Results

Adenylate Aminohydrolase. The rate of deamination of both 5′-*C*-methyl-AMP derivatives was a hyperbolic function of substrate concentration. The maximal velocity of the talo epimer was 95 times that of the allo epimer (Table II). Both compounds were linear competitive inhibitors and the *allo* epimer was approximately three times more effective. Substrate activity of *allo*-5′-carbamyl-AMP IIb could not be detected, *talo*-5′-carbamyl-AMP Ib exhibited 96% of the expected decrease in absorbance at 265 nm, and the product had the same ultraviolet spectral properties as inosine (maximum at 249 nm at pH 6.5). Compounds Ib and IIb displayed linear competitive–noncompetitive inhibition of the phosphorylation of AMP. With AMP as substrate, the values of V_{max} (1100 μmol/min per mg of protein) and of K_m (0.42–0.70 mM) were similar to reported values (Smiley *et al.*, 1967; Murray and Atkinson, 1968).

5′-Nucleotidase. The rate of dephosphorylation of the two 5′-*C*-methyl epimers was a hyperbolic function of nucleotide concentration. The V_{max} of AMP was 1.57 μmol/min per mg of protein; enzymatic hydrolysis of the two epimers was much slower (Table II). Both epimers were linear inhibitors of AMP dephosphorylation and showed mixed-type inhibition kinetics. Inhibition by the allo epimer was somewhat more competitive and it was bound to the enzyme four times more strongly. The products of dephosphorylation and deamination were characterized as 9-(6′-deoxy-β-D-allofuranosyl)hypoxanthine and 9-(6′-deoxy-α-L-talofuranosyl)hypoxanthine by their chromatographic and spectral properties. A mixture of 310 μg of either the *talo*- or *allo*-5′-methyl-AMP, 21 units of nucleotidase, and 220 units of adenosine deaminase in 8 ml of Tris-Cl buffer (pH 8.5) was stored at 20° for 2 days. The

TABLE III: Chromatography of Products of the Action of 5′-Nucleotidase and Adenosine Aminohydrolase on the 5′-*C*-Methyl-AMP Epimers.

Compound	R_F Solvent D	R_F Solvent E
9-(6′-Deoxy-β-D-allofuranosyl)-adenine	0.74	0.55
9-(6′-Deoxy-α-L-talofuranosyl)-adenine	0.79	0.54
9-(6′-Deoxy-β-D-allofuranosyl)-hypoxanthine[a]	0.47	0.37
9-(6′-Deoxy-α-L-talofuranosyl)-hypoxanthine[a]	0.52	0.35
Product from *allo*-5′-methyl-AMP	0.48	0.37
Product from *talo*-5′-methyl-AMP	0.51	0.35

[a] Obtained by the action of adenosine aminohydrolase on the corresponding adenine nucleoside.

TABLE IV: Kinetic Parameters of the 5′-Methyl- and 5′-Carbamyl-AMP Epimers with Pig and Rabbit AMP-kinases.

Compound	V_{max} (Rel %)	K_m (mM)	K_i (mM)
Pig AMP-kinase			
AMP	100	0.20	
allo-5′-Methyl-AMP	0.04	0.46	0.225[a]
talo-5′-Methyl-AMP	0.9	0.53	0.305[b]
Rabbit AMP-kinase			
AMP	100	0.42	
allo-5′-Methyl-AMP	0.006	0.40	
talo-5′-Methyl-AMP	1.05	0.46	
allo-5′-Carbamyl-AMP	0.0012	0.022	
talo-5′-Carbamyl-AMP	0.010	0.014	

[a] Noncompetitive inhibition. [b] Competitive inhibition.

enzymes were heat denatured and removed after concentration of the solutions to small volume and addition of isopropyl alcohol (20 ml) to each mixture. Chromatography on cellulose (solvent D) and silica gel (solvent E) showed only one uv-absorbing component was present in each and these corresponded in R_F to 9-(6′-deoxy-β-D-allofuranosyl)- and 9-(6′-deoxy-α-L-talofuranosyl)hypoxanthines, respectively (Table III). The compounds isolated from the action of 5′-nucleotidase and adenosine aminohydrolase on the epimeric 5′-methyl-AMP derivatives had ultraviolet spectra at pH 11.0, 7.5, and 2.0 identical with those of inosine.

AMP-kinases. V_{max} for AMP with the pig muscle enzyme was found to be 93 μmol/min per mg of protein and with the rabbit muscle enzyme 115 μmol/min per mg of protein. Table IV shows that the talo epimers Ia and Ib are considerably better substrates than the allo epimers IIa and IIb. The initial velocity values of IIa were doubled prior to making the double-reciprocal plots because the ADP analog formed from IIa was found not to be a substrate of pyruvate kinase. This was demonstrated in experiments which employed enhanced levels of lactate dehydrogenase (750 μg), pyruvate kinase (750 μg), and rabbit AMP-kinase (200 μg) in the assay medium, when AMP gave 98% of the calculated decrease in absorbance at 340 nm, *talo*-5′-methyl-AMP gave 103%, and *allo*-5′-methyl-AMP gave 52%. Formation of NAD was completed within 50 min by the allo epimer (23 μm initially) and within 3.5 hr by the talo epimer (45 μm).

The allo epimer IIa gave linear noncompetitive inhibition whereas the talo epimer Ia gave linear competitive kinetics.

Discussion

Substrate Properties. The talo epimer of 5′-*C*-methyl-AMP (Ia) was 175-fold more effective than its allo isomer (IIa) as a substrate of rabbit myokinase and 95-fold more effective as a substrate of AMP-aminohydrolase. The substrate effectiveness with adenosine aminohydrolase of the talo and allo epimers of 5′-*C*-methyladenosine (V_{max} = 28 and 0.4%, respectively, that of adenosine; Hampton *et al.*, 1972a) closely resemble that of their 5′-phosphate derivatives Ia and IIa with AMP aminohydrolase. A fourth enzyme exhibiting the same substrate stereospecificity is rabbit pyruvate kinase for which only the *talo*-5′-methyl-ADP had detectable substrate activity and its V_{max} would be at least 125 times greater than the hypothetical V_{max} of *allo*-5′-methyl-ADP calculated on the assumption that the two epimers had identical Michaelis constants. The pronounced substrate properties of 8,5′-cyclo-AMP with 5′-nucleotidase, AMP-aminohydrolase, and AMP-kinase (Hampton *et al.*, 1972b) indicate that the 1′,9 and 4′,5′ torsion angles of enzyme-bound AMP are probably such that H-8 is positioned near C-5′ and O-5′ is positioned between H-3′ and H-4′ (depicted in Figure 1 for the AMP derivatives of the present study). The conformation of adenosine when bound to adenosine aminohydrolase has been concluded (Hampton *et al.*, 1972a) to be similar to that of AMP; further, the 5′ epimer of 8,5′-cyclo-ADP produced by AMP-kinase is a substrate of rabbit pyruvate kinase (Hampton *et al.*, 1972b) to indicate that in this instance the conformation of enzyme-bound ADP also is probably similar to that of enzyme-bound AMP. Enzyme-bound *talo*-5′-methyl-AMP can be expected to assume a relatively compact conformation (Ia) similar to that of AMP itself in which the methyl group is situated near H-8. In contrast, the conformation of enzyme-bound *allo*-5′-methyladenosine and its 5′-mono- and diphosphates (*e.g.*, IIa) is considerably less compact because the methyl group is now oriented in a direction between O-4′ and H-4′, and the relatively weak activity of the allo compounds as substrates of adenosine aminohydrolase, AMP-aminohydrolase, AMP-kinase, and pyruvate kinase could arise because the complexes of the normal substrates with these enzymes possess limited bulk tolerance in the region of the 5′-hydrogen which is substituted by a methyl group in the allo derivative.

The talo configuration was also preferentially utilized as a substrate of AMP-kinase and AMP-aminohydrolase in the case of the 5′-carbamyl-AMP derivatives. Substitution of the carbamyl group for a methyl group markedly reduced substrate effectiveness with both enzymes, and while this might to some extent be associated with increased bulk of the 5′ substituent, interpretation of the results in terms of probable conformations of enzyme-bound substrates is made difficult by the many possibilities which exist for facile hydrogen bonding of the carbonyl and amino portions of the carbamyl group to O-4′, O-5′, and the phosphoryl group in these 5′-carbamyl-AMP derivatives.

The 5′-methyl-AMP epimers were much better substrates for AMP-aminohydrolase than for 5′-nucleotidase or the two AMP-kinases. The α-cyano- and α-hydroxyphosphonate

analogs of AMP (Hampton *et al.*, 1973a,b) likewise are better substrates for AMP-aminohydrolase than for AMP-kinase and these and the present findings presumably reflect a tendency for reaction rates to diminish in proportion to the closeness of the substituent groups in the AMP analogs to the site of the catalyzed reaction.

Inhibition Studies. talo-5′-Methyl-AMP was a linear competitive inhibitor of pig muscle AMP-kinase (Table IV), and in view of the substrate activity of this compound the K_i value (305 μM) is probably a measure of dissociation at the AMP site. The dissociation constant of the enzyme–AMP complex of pig AMP-kinase, as in the case of the corresponding rabbit enzyme (Noda, 1962), is probably similar in magnitude to the Michaelis constant (200 μM), and the present studies thus indidate that sufficient space is available within the kinase–AMP complex to accommodate a 5′-*C*-methyl group in the talo configuration. When the methyl group is in the allo configuration, inhibition of the kinase reaction becomes noncompetitive.

The dissociation constant of AMP with AMP-aminohydrolase has not been reported. However, the pronounced substrate activity (Table II) of the *talo*-5′-methyl-AMP suffices to show that the added methyl group is well tolerated within the aminohydrolase–AMP complex. The *allo*-5′-methyl-AMP is likewise a substrate and a linear competitive inhibitor and the K_i value indicates an affinity for the AMP site slightly higher than that of the talo epimer.

Previous work (Hampton *et al.*, 1973a,b) with analogs of AMP in which the $CH_2OPO_3H_2$ system was replaced by CH_2-$CH(R)PO_3H_2$ showed that the complexes of AMP with AMP-kinase and AMP-aminohydrolase are able to accommodate a cyano or hydroxyl group in the O-5′ region. These analogs were comprised in both cases of a mixture of two 6′-epimeric forms, and hence no conclusions can yet be drawn regarding details of the spatial relationship of the two adjoining areas of bulk tolerance at O-5′ and C-5′, respectively, of AMP in its complexes with AMP-kinase and AMP-aminohydrolase.

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