A Study of the Substrate and Inhibitor Specificities of AMP Aminohydrolase, 5′-Nucleotidase, and Adenylate Kinase with Adenosine Carboxylates of Variable Chain Length

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A series of AMP analogs in which a terminal carboxylate residue, linked to C4′ of the ribose moiety of adenosine by zero, one, or two methylene groups (**1, 2, 3**) or by the unsaturated ethylidene link (**4**) replaces the phosphate anion, is tested for activity as substrates or effectors of three enzymes known to interact with AMP with a different degree of specificity. **2**–**4** are substrates of AMP aminohydrolase, **3** and **4** are competitive inhibitors of adenylate kinase, and all acids produce competitive inhibition of the least specific enzyme, 5′-nucleotidase. These activities can be correlated with the intramolecular flexibility of anionic substituent and adenine base which in turn is expressed in typical shifts of the proton magnetic resonance signal of purine H-8. The uronic acid **1**, having a rigid molecular conformation, is inactive towards two AMP-dependent enzymes and little active with the third, indicating that this type of compound is not suitable as a nucleotide antagonist whereas nucleoside carboxylates of type **2** and **3** have a higher potential as effectors of nucleotide metabolism.

Introduction

A nucleotide molecule is composed of three parts with rather different chemical properties, *viz.* the substituted purine or pyrimidine base, the hydrophilic ribose or 2′-deoxyribose residue, and the exocyclic phosphate group. Enzymes engaged in mononucleotide metabolism frequently exhibit more or less stringent specificity for only *two* of the substructures, involving the interaction of phosphate anions with cationic enzyme residues (usually arginines) [1] on one hand, and more diverse contributions of hydrogen bonds, polar, or hydrophobic interactions between protein groups and the bases or the sugar on the other hand. Typical examples are AMP deaminase or many nucleotide kinases which have base but no sugar specificity [2, 3], and ribonucleotide reductases which are ribotide-specific and entirely base-unspecific [4]. Enzymes of high specificity for all three moieties of a nucleotide substrate are much less frequent. Such structure specificities have been investigated with the aid of numerous base- and sugar-modified derivatives of the natural nucleotides. However, these molecules may adopt different conformations about the N-glycosidic bond (*anti* or *syn*) and the exocyclic bonds (*gauche, gauche* or *trans, gauche*) [5]. Although the *anti* and *gauche, gauche* conformations predominate in solution [6], certain conformation changes in enzyme-bound nucleotides are conceivable and have, in fact, been observed [7]. Therefore, in the group of enzymes with dual specificity described above it is not only necessary to establish the chemical nature of specificity-determining substrate groups, but knowledge of their relative intramolecular arrangement is also required for an understanding of enzyme catalysis. This aspect has found little attention in the study of nucleotide-converting enzymes, presumably because model compounds of variable molecular dimensions are not generally available in the nucleotide series.

We have previously reported that adenosine 5′-phosphate (AMP) and a synthetic analog, 5′-deoxyadenosine 5′-acetic acid (**3**) have closely comparable structures and enzymatic activity [8], indicating that a carboxylate anion can efficiently mimic a nucleotide's phosphate group. AMP is a particularly interesting and biochemically important mononucleotide: Besides its role as a nucleic acid precursor it is thought to participate in regulation of the cellular energy charge [9], and it is an allosteric effector of enzymes like glycogen phosphorylase b [10] which are unrelated to nucleotide and nucleic acid biosynthesis. The availability of several adenosine 5′-carboxylates of variable chain length (**1**–**4**) has prompt-

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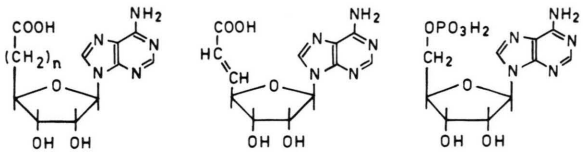
ed us to analyze in detail the influence of intramolecular anion-to-adenine interaction, and of the distance between the two half-molecules, upon the activity of different AMP-utilizing enzymes.



1: $n = 0$, adenosine 5′-uronic acid.
2: $n = 1$, 5′-deoxyadenosine 5′-carboxylic acid.
3: $n = 2$, 5′-deoxyadenosine 5′-acetic acid.
4: 5′-carboxymethylene-5′-deoxy-5′-dehydroadenosine. (= didehydro-**3**).

Materials and Methods

The adenosine carboxylic acids **1, 2, 3,** and **4** were synthesized by published procedures [11–13] and were purified by crystallization or by anion exchange column chromatography on Dowex 1 × 2 (formate form). Rabbit muscle AMP aminohydrolase (grade IV) and snake venom (Crotalus atrox) 5′-nucleotidase (grade V) were supplied by Sigma Chemicals, München. Other enzymes, AMP, ATP, and NADH were obtained from Boehringer GmbH, Mannheim.

Enzyme kinetics were recorded at 25.0 °C using a Zeiss PMQ 3 spectrophotometer and quartz cuvettes of 1.0, 5.0, or 10.0 mm light path. The data were treated graphically by the methods of Lineweaver-Burk, Dixon-Webb, or Cornish-Bowden [14] to obtain V, K_m, and K_i values.

AMP aminohydrolase activity was determined in 0.05 M MES-Tris buffer (pH 6.0–6.5) or 0.05 M HEPES-Tris buffer (pH 7.0–7.5), containing 0.2 M KCl, by following the absorbance change at 265 nm. Substrate concentrations were 0.05 to 1 mM and enzyme concentrations 0.03 to 1 μg/ml.

Assays of 5′-nucleotidase were performed in 0.1 M Tris-HCl buffer (pH 6.5) using adenosine deaminase (from calf intestine, 0.2 μg/ml) as auxiliary enzyme. Substrate concentrations were 0.025 to 0.25 mM, and enzyme solutions (which undergo significant activity losses within hours after preparation) were adjusted to yield $\Delta A_{265} = 0.01\ min^{-1}$ in the presence of 0.05 mM AMP.

Assays of adenylate kinase (from rabbit or pig muscle) contained 0.1 M Tris-HCl buffer (pH 7.6), 0.1 M KCl, 1 mM $MgSO_4$, 0.4 mM phosphoenol pyruvate, 0.4 mM NADH, and 40 μg/ml of a pyruvate kinase/lactate dehydrogenase (from rabbit muscle) mixture; AMP concentrations were 0.05–20 mM. The reaction was started by addition of 0.1 μg adenylate kinase and was followed by the absorbance change at 340 nm.

Results

We chose to compare three widely distributed enzymes which cover different types of interaction with their common substrate, AMP. Muscle AMP aminohydrolase (AMP deaminase, EC 3.5.4.6) catalyzes an addition-elimination process at the adenine ring and requires a monoanionic group for substrate binding [2]. Conversely, muscle adenylate kinase (myokinase, EC 2.7.4.3) has an adenine-specific AMP binding site [15] and catalyzes phosphorylation of the 5′-phosphate. Snake venom 5′-nucleotidase (EC 3.1.3.5) specifically hydrolyzes the 5′-phosphate ester bond and shows a preference, but no absolute specificity for adenine as a base [16]. Although otherwise the three proteins differ widely in molecular size, subunit composition, and regulatory properties their AMP sites appear to be similar in nature: They all act on ribonucleoside, 2′-deoxyribonucleoside, and other sugar-modified nucleoside 5′-phosphates, but not on 3′-nucleotides or on ribose-5-phosphate indicating that the relative position of phosphate and adenine groups is critical for enzyme activity.

AMP deaminase is a tetrameric enzyme of very complex kinetic behaviour [17–19] which requires potassium ions for activity. Three of the adenosine carboxylates (**2, 3,** and **4**) can replace AMP as substrate (Table I). Acid **3,** which is isosteric with AMP, differs from the natural substrate only by a twofold larger apparent K_m value and fivefold slower reaction at pH 6.5. Acids **2** and **4** react much more slowly and have up to tenfold higher K_m values, whereas adenosine 5′-uronate (**1**) is totally inactive at all pH values and concentrations tested. The slow reaction rate of **2** is of obvious advantage for studying the pH dependence of AMP deaminase: The low enzyme concentrations required for deamination of fast substrates, like AMP or **3,** lead to inactivation of the enzyme [17] and hence to inaccuracies in the determination of V and K_m below pH 6.5, but adenosine carboxylate **2** requires high

Table I. Relative reaction rates and apparent K_m values of AMP and AMP analogs with rabbit muscle AMP aminohydrolase.

Substrate	pH 6.0		pH 6.5		pH 7.0		pH 7.5	
	V_{rel}	K_m [mM]	V_{rel}	K_m [mM]	V_{rel}	K_m [mM]	V_{rel}	K_m [mM]
AMP	(450	3)	100	0.45				
1	inactive		inactive		inactive		inactive	
2	0.2	1.2	0.6	4.5	2.5	5.5	3	10
3	(20	1)	21	0.8				
4	1.2	1.3	3.5	1.9				

Kinetic data were determined at 25.0 °C in the presence of 0.2 M KCl. The reaction rate of AMP at pH 6.5 (= 100%) is V=0.20 nkat. Data for the fast reacting substrates at pH 6 are not very accurate for reasons mentioned in the text but the order of magnitude of the figures in parentheses could be reproduced with certainty.

Table II. Inhibition of adenylate kinases by AMP analogs at 25.0 °C, pH 7.6, in the presence of 1 mM Mg^{2+}.

Compound	Rabbit muscle enzyme	Pig muscle enzyme
1	inactive	inactive
2	inactive	inactive
3	K_i = 1.7 mM	K_i = 2.1 mM
4	K_i = 4.6 mM	K_i = 5.8 mM
AMP	K_m = 0.3 mM	K_m = 0.3 mM

Table III. AMP hydrolysis and inhibition of snake venom 5′-nucleotidase by AMP analogs at 25.0 °C.

Compound	pH 6.5	pH 8.5
1	K_i = 0.13 mM	K_i = 0.40 mM
2	K_i = 0.05 mM	K_i = 0.13 mM
3	K_i = 0.10 mM	K_i = 0.22 mM
AMP	K_m = 0.014 mM	K_m = 0.033 mM

enough enzyme concentrations in the pH range from pH 6 to 7.5 to show normal hyperbolic kinetics under conditions of the spectrophotometric assay, eliminating the need for stopped flow methods. An interesting observation is that the rate of deamination of 2 and 4 increases while their affinity to the enzyme is lowered at higher pH values, but that this trend is noted for AMP when going to lower pH.

The interaction of adenosine carboxylates with muscle adenylate kinase was studied using the rabbit and the porcine enzyme which differ only little in amino acid composition. Accordingly, the data summarized in Table II are very similar for both proteins. We found no evidence for substrate activity of any of the carboxylates in the presence of ATP plus enzyme using the spectrophotometric assay; if an adenosine carboxylic-phosphoric acid anhydride were formed eben more slowly it would certainly escape detection and isolation due to its hydrolysis. However interaction of a carboxylate with the enzymes' AMP sites is possible because acids **3** and **4** inhibit the phosphorylation of AMP. Again, 5′-deoxyadenosine 5′-acetic acid (**3**) is the more effective compound with an inhibition constant about 6 to 7 times the Michaelis constant of AMP. Fig. 1 indicates that the inhibition is clearly of the competitive type; each method of plotting the data yields the same result.

5′-Nucleotidase is least specific towards the adenosine carboxylates. Substrate activity of the

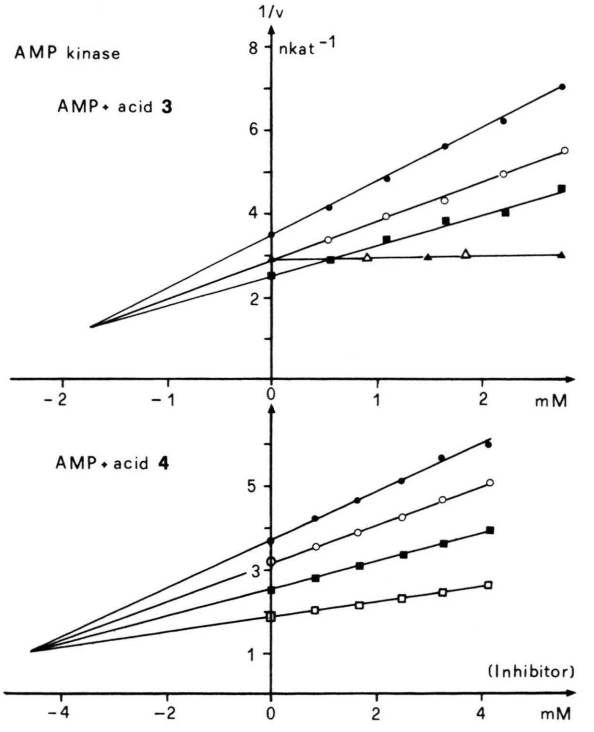


Fig. 1. Dixon plots showing competitive inhibition of rabbit muscle adenylate kinase-catalyzed AMP phosphorylation in the presence of 5′-deoxyadenosine 5′-acetic acid (**3**) (above) and the unsaturated acid **4** (below). AMP concentrations were 45 μM (●), 58 μM (○), 75 μM (■), or 115 μM (□). No inhibition is seen in presence of the acids **1** or **2** (▲, △).

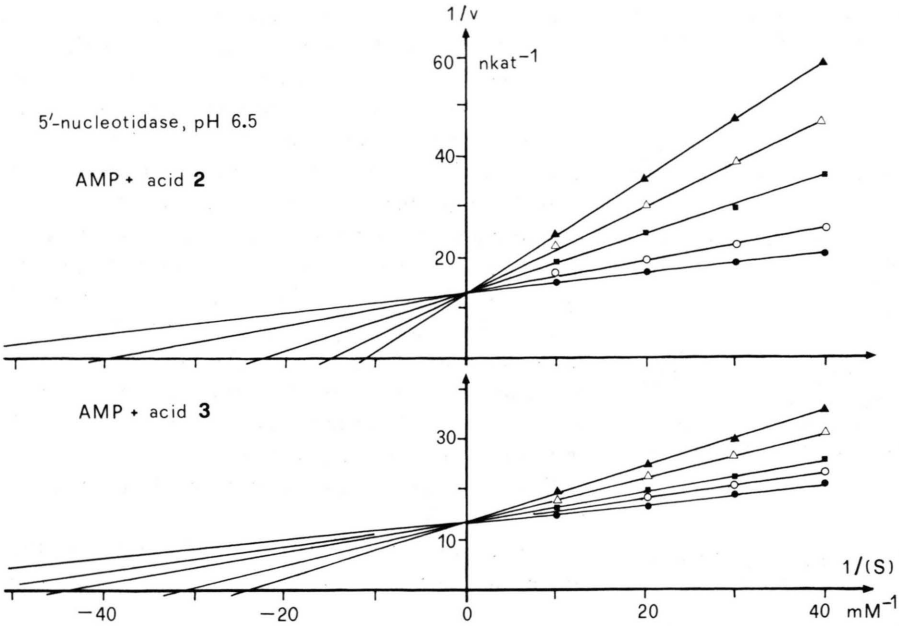


Fig. 2. Lineweaver-Burk plots for 5′-nucleotidase-catalyzed hydrolysis in the presence of inhibitors (pH 6.5, 25 °C). Above: AMP (●); AMP plus 50 μM (○), 150 μM (■), 250 μM (△), or 350 μM (▲) 5′-deoxyadenosine 5′-carboxylic acid **2**. Below: AMP plus 5′-deoxyadenosine 5′-acetic acid (**3**) (same symbols as above). Adenosine 5′-uronic (**1**) produces the same type of competitive inhibition (not shown); **4** was not tested.

Table IV. Chemical shift difference $\Delta\delta$ of H-8 as a measure of anion-to-adenine interaction and enzymatic activities of AMP and AMP analogs.

Compound	Exocyclic substituent	$\Delta\delta$ (H 8) ppm	Substrate or inhibitor activity *vs.*		
			AMP deaminase	AMP kinase	5′-nucleotidase
1	$-COO^-$	0.43	inactive	inactive	weak inhibitor
2	$-CH_2COO^-$	0.12	poor substrate	inactive	good inhibitor
3	$-CH_2CH_2COO^-$	0.16	good substrate	inhibitor	inhibitor
4	$-CH=CHCOO^-$	0.13	poor substrate	weak inhibitor	
AMP	$-CH_2OPO_3H^-$	0.27	substrate		substrate
	$-CH_2OPO_3^{--}$	0.39		substrate	

nucleotide analogs having non-hydrolyzable 5′-bonds cannot be expected but the homologous acids **1**, **2** and **3** are inhibitors of AMP hydrolysis both at low and high pH (Table III). In this case 5′-deoxyadenosine 5′-carboxylate (**2**) is most effective ($K_i \approx 3\ K_m$) and the uronic acid **1**, although not inactive like in the two other enzyme systems, is the least potent inhibitor. All three analogs produce competitive inhibition of 5′-nucleotidase (Fig. 2). In contrast to the situation observed with AMP deaminase, the data in Table III indicate that AMP hydrolysis and inhibitor action have the same pH-dependence.

Discussion

Little information is as yet available about the amino acid residues and architecture of AMP sites within the three enzyme molecules. Only one of them, muscle adenylate kinase, has been studied by X-ray crystallography [20]. In this situation and in view of the obvious specificity similarities for adenine-containing, 5′-anionic substrates and inhibitors a comparison of the relative activities of AMP and adenosine carboxylates can help to a better understanding of enzyme – substrate interactions. Previously used model compounds include 5′-substituted

AMP and 6′-substituted homoadenosine 6′-phosphonic acid derivatives [21 – 23] but the presence of two epimers, which have different enzymatic activities, is a complicating factor in the analysis of these systems.

One point of interest is the negative charge of a nucleotide or analog. Due to the phosphate ionization constant, $pK_{a,2} = 6.05$, AMP exists as a mixture of the mono- and dianionic species at physiologic pH while the carboxylic acids are always monoanions (**1**: $pK_a = 4.0$; **2 – 4**: $pK_a = 4.7$). In case of AMP deaminase it has been concluded from the substrate activity of AMP amidate and other derivatives [2] that the enzyme acts on monoanions, and the good activity of **3** immediately confirms that specificity. Acid **3** is, in fact, a better substrate than the phosphonic acid analog of AMP ($V_{rel} = 20\%$ $K_m = 1.7$ mM at pH 6.5) [21] which has essentially the same steric dimensions. Enzymatic activity of the carboxylic acids is nevertheless affected by a pH increase (*cf.* Table I), indicating pH-dependent changes in the AMP site rather than in substrate ionization. It is clear that charges within the catalytic site will be only one determinant of AMP deaminase activity besides, for example, the pH dependence of K^+ activation [18] or the existence of an activating nucleotide site of low (and not yet very well defined) specificity [19]. However, the interesting parallelism of increased apparent K_m values (lower affinity) and increased velocity observed for the fast reacting natural substrate (AMP) as well as for the slowly reacting carboxylates (**2, 4**), despite opposite pH dependencies, can be hardly fortuitous but should reflect the nature and cooperation of amino acid residues in the catalytic site.

5′-Nucleotidase also appears to have a preference for monoanions. All K_m and K_i values for AMP and competitive inhibitors are lower at pH 6.5 (where AMP has an approximately equal proportion of the singly and doubly charged species) than at pH 8.5 (Table III); if a dianion were required for binding to the catalytic site, the K_m of AMP would be expected to decrease at the higher pH and K_i of the carboxylates should deviate more strongly from K_m. The increase in K_m and K_i values at pH 8.5 may rather indicate beginning deprotonation of an amino acid residue at the active site. Accordingly, the carboxylates are again better models of AMP than 6′-deoxyphomoadenosine 6′-phosphonates: Whereas **1, 2,** and **3** are competitive inhibitors of AMP hydrolysis, the phosphonates inhibit 5′-nucleotidase in a non-competitive or even more complex fashion and are less efficient [21, 22].

Only two of the adenosine carboxylates are competitive inhibitors of adenylate kinase at the pH optimum of 7.6, where 95% of AMP molecules are dianionic (Table II). It is reasonable to assume that in the Mg^{2+}-activated enzymes one negative charge of a bound nucleotide is specifically neutralized by a metal ion while the phosphate group is being phosphorylated. As a carboxylate cannot engage in two interactions at a time its affinity towards the kinase must be decreased, in accord with the significantly larger difference between apparent K_m and K_i values (as compared with nucleotidase). In this system homoadenosine 6′-phosphonates can be both substrates or inhibitors [21, 22] but substrate activity is low (0.04 – 2% of AMP) despite the presence of a doubly ionizable phosphonate group.

Taken together, the degree of specificity observed in the reactions of carboxylate and phosphonate analogs of AMP with the three proteins is connected with the functions of these enzymes in a sensible way: All model compounds tested so far interact with the least specific, degrading enzyme, 5′-nucleotidase; several, but not all AMP analogs are accepted by AMP aminohydrolase which is thought to function in the control of adenine nucleotide concentrations in muscle; even more stringent structural requirements are shown by adenylate kinase, an important enzyme of cellular energy metabolism. In contrast, the size of the protein molecules does not appear to determine the specialization of AMP sites.

Another correlation can be recognized between the molecular conformations and enzyme activities of AMP and its analogs. The gross spatial arrangement of C5′ anion and adenine base (*gauche, gauche/anti*), which is stabilized by ion-dipole attractive forces [24], is the same in all compounds but enzyme reactions are known to be sensitive to more subtle conformation changes. Based upon the proton magnetic resonance spectra of numerous adenine nucleosides and nucleotides in aqueous solution we have shown that the chemical shift difference $\Delta\delta$ of the purine proton H 8,

$$\Delta\delta = \delta\,(\text{nucleoside}) - \delta\,(\text{adenine}),$$

is a reliable measure of intramolecular purine-to-C5′ interaction [6]. The data summarized in Table IV demonstrate that this parameter is indeed useful for

an interpretation of enzyme activities: Medium $\Delta\delta$ values which reflect a certain conformational flexibility are usually associated with substrate or inhibitor properties, but the exceptionally strong intramolecular (hydrogen bond) interaction $-COO^- \cdot$ H8-adenine- in adenosine uronate (**1**) makes that compound totally rigid, inactive in two enzyme systems, and least active in the third. The order of $\Delta\delta$, **2** ≈ **4** < **3** < AMP, is directly parallel to the poor (or missing) activity of **2** and **4** and the higher activities of **3** and AMP in the two more specific enzymes, AMP deaminase and AMP kinase. The unsaturated acid **4** must be less active than **3** (having the same chain length) because the lack of rotation about its double bond makes the molecule less flexible to assume an optimum, AMP-like orientation within an active site. In 5′-nucleotidase that site appears adapted to shorter intramolecular dimensions than in the other enzymes but a requirement for flexibility is still evident in the different activities of **2** and **1**. All these observations suggest that the exocyclic anion of a nucleotide interacts with enzymes in an "induced fit" process, in analogy to induced fit phenomena in the interaction of substituted purine bases with nucleotide-dependent enzymes [4].

In conclusion, we predict that nucleoside carboxylates would be able to replace nucleotides in a variety of other enzyme systems provided they possess the proper molecular dimensions. One obvious reason for such exchangeability is that phosphates and carboxylates may engage in the same type of electrostatic plus hydrogen bond interactions with active site arginine residues [1]. The practical aspect is that carboxylate analogs could serve as potentially useful non-hydrolyzable antagonists of nucleotide metabolism *in vivo*. Adenosine and thymidine 5′-uronic acids, which are easily prepared by oxidation of the nucleosides' terminal CH_2OH group, have been tested for biological properties [25, 26] but have not shown any promising activity. This is not surprising in view of our present *in vitro* results where the short-chain uronates are inactive or little active. As several routes for the synthesis of "elongated" carboxylic acids [12, 13] are now available the experimental basis for a search of nucleotide antagonists is greatly improved.

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