Modified nucleotides as substrates and inhibitors of adenylate kinase from different sources

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Abstract The substrate and inhibitory properties of modified nucleotides with respect to adenylate kinase from rabbit muscles, human placenta and *Escherichia coli* were studied. A number of 5′-hydrogenphosphonates and 5′-fluorophosphates of modified nucleotides were shown to inhibit the phosphorylation reaction catalyzed by these enzymes. A clear difference between phosphonates of 3′-deoxyribonucleotides and the corresponding ribo- and 2′,3′-dideoxyribonucleotides was found. 3′-Azido-2′,3′-dideoxythymidine and its phosphorus derivatives did not inhibit the adenylate kinase reaction.

Key words: Adenylate kinase; Modified nucleotide; Phosphorylation; Substrate specificity

1. Introduction

High anti-HIV activity of 5′-hydrogenphosphonates [1–7] and 5′-fluorophosphates [8–10] of modified nucleotides in virus-infected cell cultures has recently been reported. Their metabolism, however, has been investigated inadequately, and the data are contradictory. For instance, 5′-hydrogenphosphonates of modified thymidine are stable in the presence of phosphatases [11] whereas in human blood serum they are dephosphorylated, although 10–100 times slower than the corresponding 5′-phosphates [12]. Furthermore, it was recently reported that 3′-azido-3′-deoxythymidine (AZT) inhibited the reaction [2ADP→ATP+AMP] catalyzed by adenylate kinase from rabbit muscle and did not affect the reverse reaction [16]. This result seems unusual as kinases are known to be specific towards the nature of the nucleic base in substrate molecule.

We present here data on the phosphorylation of a series of nucleoside 5′-hydrogenphosphonates and 5′-fluorophosphates (Ia–c)–(IVa–c) (Scheme 1) as well as AZT and its derivatives by adenylate kinase from different sources.

2. Materials and methods

Adenylate kinase from rabbit muscles was purchased from Sigma; enzyme from *E. coli* and from human placenta was isolated according to Rikhter et al. [13], with slight modifications. Yeast hexokinase was obtained from Fluka. The synthesis of compounds Ib,c [14], IIb,c [6,9], IIIa–c [6,9], IVa,c [15], IVb [1] was carried out as described. Other nucleotides were from Sigma.

Kinetic assay for the reaction [ATP+AMP→2ADP] was carried out at 37°C in an incubation mixture (20 μl) containing 25 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 0.05 mM [γ-^{32}P]ATP (30 Ci/mmol), 2×10^{-4} U of adenylate kinase and 0.5 mM AMP (or the tested nucleotide). For evaluation of inhibitory properties, variable amounts of modified nucleotides were added.

Measurement of the reverse reaction [2ADP→ATP+AMP] rate was performed in an incubation mixture (20 μl) containing 25 mM Tris-HCl (pH 7.7 at 37°C), 5 mM $MgCl_2$, 0.05 mM [β-^{32}P]ADP (30 Ci/mmol), 2 mM glucose, 2×10^{-4} U of adenylate kinase, 10^{-4} U of hexokinase and, where necessary, the appropriate amount of the inhibitor.

Samples (1 μl) were taken from the incubation mixture at 10-min intervals and analyzed by TLC on polyethyleneimine-cellulose plates in 0.5 M KH_2PO_4. The TLC plates were dried and radioautographed. Each spot was cut according to the radioautograph and the radioactivity was counted in a scintillation counter by means of the Cherenkov effect.

3. Results and discussion

Substrate and inhibitory properties of modified nucleotides I–IV were evaluated in the reactions catalyzed by adenylate kinase from rabbit muscles, *E. coli* and human placenta. The latter two enzymes were only partly purified to a specific activity of 0.2 U/mg [13], but were free of phosphatase, nonspecific ATPase and other NMP-kinase impurities.

Only phosphates Ia–IIIa were phosphorylated in the presence of all three kinases. The phosphorylation rates for IIa and IIIa vs. that for AMP (Ia) are shown in Table 1. None of the phosphonates (Ib–IIIb) and fluorophosphates (Ic–IIIc) was phosphorylated, but most exhibited inhibitory activity when added to the reaction mixture. Fig. 1 demonstrates the reaction rates catalyzed by adenylate kinase from human placenta at various concentrations of the inhibitors. The inhibitor to AMP concentration ratios at which the reaction rate is decreased by 50% are listed in Table 2.

Since the inhibitory effect was seen only if the inhibitors were added in a large excess with respect to AMP and, correspondingly, in still larger excess with respect to ATP, it was not improbable that the inhibition could be caused by an impurity that is undetectable by the usual analytic methods. To exclude this possibility, we carried out experiments using [U-^{14}C]AMP. The reaction mixture contained 0.05 mM [U-^{14}C]AMP with a specific activity of 585 Ci/mol and ATP was in excess with respect to both AMP and the substances tested. The results were in a good agreement with those obtained by the standard procedure (data not shown).

Fig. 2 shows the results of examination of the inhibitory properties of AZT (IVa). The reaction proceeded from ADP to AMP+ATP in the presence of hexokinase to make it irreversible. No inhibitory effect of AZT could be seen. The same results were obtained with the two other tested adenylate kinases (data not shown).

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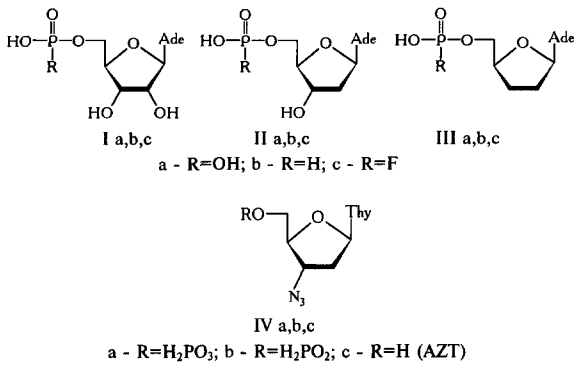

Scheme 1.

The data obtained demonstrated that only adenylates with the unmodified phosphate group (Ia–IIIa) were phosphorylated by all adenylate kinases. AMP and 2′,3′-dideoxyribo-adenosine 5′-phosphate (IIIa) were found to be better substrates than dAMP (Table 1). A similar relationship was demonstrated for the inhibitory properties of ribo-, 2′-deoxyribo- and 2′,3′-dideoxyribohydrogenphosphonates (Ib–IIIb) and fluorophosphates (Ic–IIIc).

2′-Deoxyriboadenylates (IIb,c) did not inhibit the reaction under study up to a 100-fold molar excess with respect to AMP (Table 2).

Thus, the modified 3′-deoxyriboadenylates (IIa–c) clearly differ as substrates and inhibitors from the ribo (Ia–c) and 2′,3′-dideoxyribo (IIIa–c) analogs. The effect might be due to different interactions with the enzyme of 'symmetric' (ribo- and 2′,3′-dideoxyribo-) and 'asymmetric' (3′-deoxyribo-) glycones.

The results obtained suggest that the structure of the phosphate residue in adenylates is crucial for the phosphorylation catalyzed by adenylate kinases. These data agree with those for nucleoside 5′-fluoromethylphosphonates, which could neither be phosphorylated by human cellular nucleotidyl kinases, nor compete with natural 5′-nucleotides [17]. Since compounds IIIb–c are not phosphorylated in the presence of human adenylate kinase, there must be another metabolic pathway for developing their anti-HIV activity.

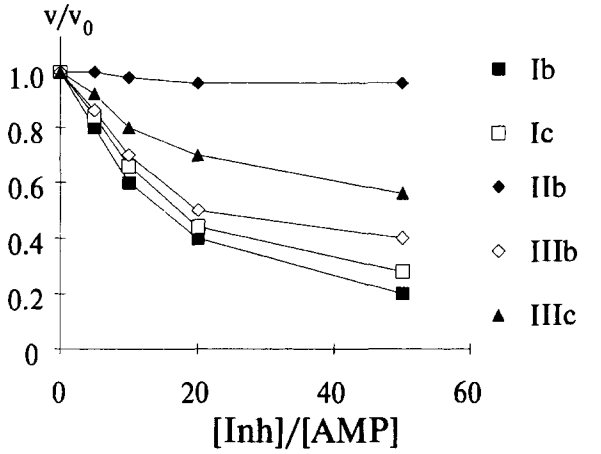

Fig. 1. Dependence of AMP phosphorylation rate (v) catalyzed by human placental adenylate kinase on inhibitor (Inh) concentration; v_0, reaction rate in the absence of inhibitor.

Our results on inhibition of adenylate kinases by AZT (IVa) or its derivatives (IVa,b) disagree with the data of Barile et al. [16]. All the studied enzymes were not inhibited by compounds IVa–c up to a 100-fold excess with respect to AMP or ADP concentration, without regard to the reaction direction.

Thus, the tested adenylate kinases exhibit high specificity towards the structure of the phosphorus-containing fragment in the substrate molecule. This property can be of use for the design of new antiviral agents targeted at DNA synthesis catalyzed by viral DNA polymerases, particularly reverse transcriptases. The latter are known to display reduced specificity towards dNTP modified at the phosphorus atom and can

Table 1
Ratio of nucleoside phosphate phosphorylation rate to that for AMP

Substrate	Adenylate kinase		
	Rabbit muscle	*E. coli*	Human placenta
IIa	0.75	0.4	0.53
IIIa	0.95	0.9	0.95

Table 2
Inhibition of AMP phosphorylation by nucleoside derivatives

Compound	$[Inh]_{50}$/[AMP]		
	Rabbit muscle	*E. coli*	Human placenta
Ib	20	10	15
IIb	–	–	–
IIIb	25	20	20
Ic	20	> 50	15
IIc	–	–	–
IIIc	> 50	> 50	> 50
IVa–c	–	–	–

$[Inh]_{50}$/[AMP] is the ratio of inhibitor and AMP concentrations at which 50% inhibition was observed; '–' indicates that inhibition was not found up to a 100-fold molar excess of the compound tested with respect to AMP.

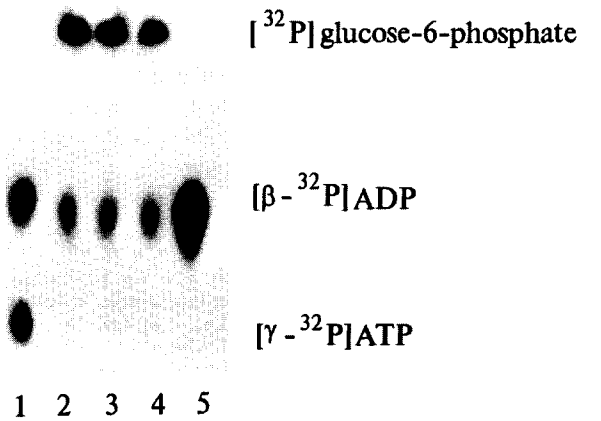

Fig. 2. Phosphorylation in the presence of IVa (AZT) catalyzed by adenylate kinase from rabbit muscles. For details of the reaction conditions, see Section 2; 1, without hexokinase and AZT; 2, without AZT (reference); 3, 4 mM AZT; 4, 10 mM AZT; 5, [β-^{32}P]ADP.

convert compounds bearing phosphonate residues instead of phosphate ones in the triphosphate moiety [18–23].

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