INTERACTION OF SULFUR-CONTAINING ANALOGS OF ATP WITH ADENYLATE CYCLASE

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1. Introduction

Analogs of ATP bearing a sulfur atom on either the γ- or the α-phosphate group (fig. 1) have recently been synthesized and tested for biological activity in various enzyme systems. Notably, adenosine 5′-O-(1-thiotriphosphate) (ATPαS) was found to be resistant against pyrophosphatases [unpublished] and adenosine 5′-O-(3-thiotriphosphate) (ATPγS) against alkaline phosphatase [1]. Presently, we have investigated the possible substrate or inhibitor functions of these compounds with adenylate cyclase, an enzyme usually contaminated by substrate consuming phosphorylytic activities. Although we found that ATPγS serves as a substrate for adenylate cyclase from Ehrlich ascites cells, the compound was also degraded by other phosphorylytic enzymes present in the preparation. ATPαS (or degradation products thereof) was found to inhibit the adenylate cyclase, and the isomeric mixture of ATPαS showed competitive inhibition with an apparent K_i of 0.035 mM.

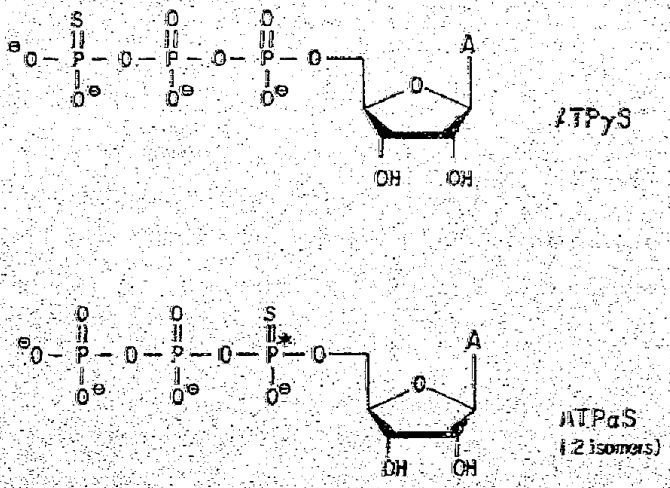

Fig. 1. Chemical structures of adenosine 5′-(3-thiotriphosphate) = ATPγS, and adenosine 5′-(1-thiotriphosphate) = ATPαS.

2. Materials and methods

Adenylate cyclase from Ehrlich ascites cells was prepared as a washed 600 g particulate fraction as described earlier [2] and stored in small aliquots under liquid nitrogen. Assays contained in a total volume of 0.05 ml the following: 40 mM Tris-(hydroxyaminomethane)–HCl (pH 8), 5 mM $MgCl_2$, 10 mM aminophylline, 0.1–0.2 mg/ml enzyme and, if added, 10 mM sodium fluoride. If ATP (labelled or unlabelled) was the substrate, 10 mM sodium creatine phosphate and 0.1 mg/ml creatine kinase were also included. For direct determination of enzyme activity 0.1 mM [α-^{32}P]ATP (600 000 cpm)

was added, and cyclic AMP production was measured as described earlier [3]. If ATPγS or unlabelled ATP was the substrate, the concentration was 1 mM and work-up was as follows. Reactions were stopped by placing tubes into a boiling water bath for 3 min. After addition of 0.2 ml H_2O and mixing, tubes were centrifuged in the cold at 2500 *g* for 5 min. The supernatant solution was stored frozen until ready for assay of cyclic AMP content which was measured in 5–20 μl aliquots by the binding protein method of Gilman [4] using a cyclic AMP binding protein prepared from beef heart muscle.

The ATPαS [5] was synthesized and the isomers separated as described elsewhere [6]. ATPγS and [^{3}H]ATPγS were prepared as published [1,7]. Purity checks and degradation measurements of [^{3}H]ATPγS were carried out by thin layer ion exchange chromatography using polymine thin layer plates (PEI, Macherey and Nagel) and 0.75 M potassium phosphate, pH 3.5 for development (R_f values: ATPγS – 0.08; ATP – 0.19). Biochemicals were purchased from Sigma Chemical Co., St. Louis, or Boehringer-Mannheim, New York, and α-[^{32}P]ATP from International Chemical and Nuclear Company, Irvine, California.

3. Results

3.1. *Experiments with ATPγS*

Addition of 1 mM ATPγS to standard assays of adenylate cyclase resulted in virtually immediate and effective reduction of the rate of formation of radioactive cyclic AMP by about 80%. This effect was dose dependent, both with basal and fluoride-stimulated activity (fig. 2).

With cold ATP or ATPγS as substrates in adenylate cyclase assays, cyclic AMP formation, as well as its stimulation by adrenaline and fluoride, could be demonstrated (table 1). Adenosine and ATPαS (see below) which both inhibit adenylate cyclase also inhibited cyclic AMP formation from either ATP or ATPγS as substrates (table 2). Chromatographic analysis of unlabelled ATPγS revealed no detectable contamination with ATP or ADP. Thus, it is strongly indicated that cyclic AMP is formed directly from ATPγS, and not from ATP as a contaminant.

With [^{3}H]ATPγS as substrate, it was found that

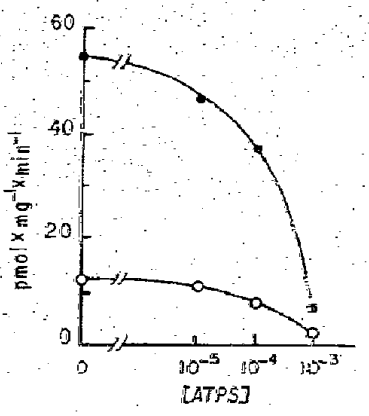

Fig. 2. Apparent inhibition of Ehrlich cell adenylate cyclase by ATPγS in the presence (●—●—●) and absence (○—○—○) of 10 mM NaF. [α-^{32}P] ATP was 0.1 mM.

Table 1
Effect of stimulants on adenylate cyclase from Ehrlich ascites cells assayed with ATP and ATPSγ as substrates

Assay condition	pmole · mg^{-1} · min^{-1}*	
	ATP	ATPSγ
Basal activity	36 ± 7	19 ± 3
10 mM NaF	230 ± 16	81 ± 9
0.1 mM Adrenaline	129 ± 13	64 ± 14

* Means and standard errors for duplicate cyclic AMP determinations of triplicate cyclase assays are listed.

Table 2
Inhibitor effects on adenylate cyclase from Ehrlich ascites cells assayed with ATP or ATPSγ

Substrate	Inhibitor*	pmole · mg^{-1} · min^{-1}**
ATP	None	215 ± 17
	ATPαS	35 ± 7
	Adenosine	67 ± 10
ATPSγ	None	73 ± 8
	ATPαS	34 ± 3
	Adenosine	33 ± 2

* Concentrations of inhibitors and substrates were all 1 mM, and 10 mM NaF was present in all assays.
** Values were corrected for blanks (no enzyme). Means and standard errors for duplicate cyclic AMP determinations of duplicate cyclase incubations are listed.

the compound was degraded by phosphorylytic enzymes present in Ehrlich cell adenylate cyclase. The products included ADP, AMP and nucleosides or hypoxanthine, and 50% of ATPγS (1 mM) were hydrolyzed within 10 min, assayed under the same conditions as for cyclase measurements.

3.2. *Experiments with ATPαS*

When the isomer mixture of ATPαS was included in adenylate cyclase assays, strong and immediate inhibition of cyclic AMP formation similar to that observed with ATPγS was observed. However, no cyclic AMP formation was apparent in incubations with enzyme and ATPαS as measured by the protein binding assay. The product with ATPαS as substrate would be the isomeric thiophosphate analogs of cyclic AMP which are strong inhibitors of cyclic AMP binding and potent activators of cardiac protein kinase (unpublished). The stereoisomers of ATPαS (absolute conformations unknown) were found to be nearly equipotent in inhibiting adenylate cyclase (90–95% at 0.5 mM). The inhibition observed with the isomer mixture of ATPαS appeared to be competitive, yielding a K_i value of 0.035 ± 0.006 mM (K_m = 0.053 ± 0.009 mM), as analysed by a weighted fit to the rate data as outlined by Cleland [8]. However, inhibition was also noted to occur with adenosine-5′-O-(1-thiodiphosphate) (ADPαS) and, to a lesser degree, with adenosine-5′-O-thiophosphate (AMPS) (fig. 3). It is conceivable that kinetic data reported here relate to ADPαS or to both, ADPαS and ATPαS.

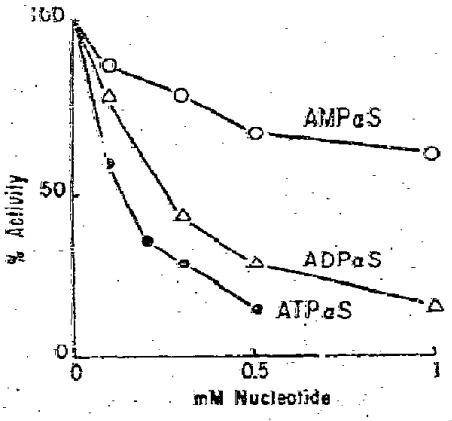

Fig. 3. Inhibition of Ehrlich cell adenylate cyclase by ATPαS (●–●–●), ADPαS (△–△–△) and AMPS (○–○–○). [α-^{32}P] ATP was 0.1 mM.

4. Discussion

The presence of a terminal phosphothioate group in the ATP molecule apparently does not impair its substrate function with adenylate cyclase from Ehrlich cells. Preliminary studies with enzymes from other tissues indicated that ATPγS may serve generally as a substrate for adenylate cyclase. The relatively rapid degradation of ATPγS by contaminating ATPases does not allow the use of this nucleotide for routine purposes in adenylate cyclase assays; however, with a highly purified enzyme this possibility could still be considered. In the present study no effort was made to characterize the type and origin of the specific enzyme(s) degrading ATPγS. It was observed that fluoride does not inhibit the rate of ATPγS degradation to the same extent as usually seen with ATP, suggesting that different enzymes could be involved. The adenylylimidodiphosphate [9] which is resistant to degradation by ATPases, thus is still a preferable alternate cyclase substrate, although we found it to be less effective than either ATP or ATPγS in preliminary studies involving different adenylate cyclases.

The inhibitory effect of ATPαS on adenylate cyclase is of interest since to date only few inhibitors of the enzyme have been found, including compounds such as adenosine, which acts noncompetitively [10], and its analogs [11,12]. The adenosine α,β-methylene triphosphate has been reported to inhibit liver adenylate cyclase competitively [13]. Present findings that ATPαS, or ADPαS, act as rather potent competitive inhibitors raises the hope that further chemical modification of ATP may lead to the development of strong, and perhaps enzymatically resistant, inhibitors of adenylate cyclase. These may be of use in in vitro studies of the enzyme or may be of value as pharmacological agents in intact tissues. The fact that the diastereoisomers of ATPαS (or their corresponding thiodiphosphates) show similar degrees of inhibitory potency indicates that there are no narrow structural requirements at the triphosphate group in respect to binding to adenylate cyclase.

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