INTERACTION OF SULFUR-CONTAINING ATP ANALOGS WITH RABBIT MUSCLE PHOSPHOFRUCTOKINASE

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1. Introduction

Analogs of ATP bearing a sulfur atom on either α, β or γ phosphate groups were tested as substrates or competitive inhibitors for various phosphotransferases [1–5]. Since both ATPαS and ATPβS exist in two diastereomeric forms, arbitrarily named A and B, use of these analogs offers information on the stereospecificity, as well as the stereochemistry, of the phosphoryl group transfer reactions. The regulatory enzyme phosphofructokinase (ATP; D-fructose-6-P-1-phosphotransferase, EC 2.7.1.11) is an excellent model system for such investigations, since adenine nucleotides are used by this enzyme both as substrates and as allosteric effectors.

2. Materials and methods

2.1. *Chemicals*

Isomers of ATPαS and ATPβS were synthesized as in [5,6]. o^1ITP was prepared by oxidative deamination of adenosine-1-oxide 5′-triphosphate [7,8]. Other natural or modified nucleotides, enzymes and substrates were supplied by Boehringer Mannheim.

Abbreviations ATPαS, adenosine 5′-*O*-(1-thiotriphosphate), ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate), AMPS, adenosine 5′-*O*-phosphorothionate; o^1ITP, inosine-1-oxide 5′-triphosphate, F6P, fructose-6-phosphate, sATP, 6-mercaptopurine ribonucleoside 5′-triphosphate; sAMP, 6-mercaptopurine ribonucleoside 5′-monophosphate

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2.2. *Assay of phosphofructokinase activity*

The reaction medium at 24°C contained per 1 ml solution: 40 mM imidazole buffer (pH 7), 50 mM KCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 0.05 mM NADH, 1 mM dithiothreitol, 0.1 mg bovine serum albumin, 0.8 U triosephosphate isomerase, 0.8 U α-glycerolphosphate dehydrogenase, 1.5 U aldolase, and different concentrations of F6P and nucleotides. The reaction was started with purified rabbit muscle phosphofructokinase and the $A_{366\ nm}$ decrease was monitored with an Eppendorf 1101 M photometer, equipped with a W+W Type 4410 recorder (full-scale deflection, 0.25 *A* units). Auxiliary enzymes as well as phosphofructokinases were desalted prior to the assays by dialysis against 0.1 M imidazole buffer (pH 7), 0.2 mM EDTA and 1 mM dithiothreitol.

3. Results

3.1. *ATP analogs as substrates or competitive inhibitors of phosphofructokinase*

As shown in table 1 both diastereoisomers of ATPαS are substrates for rabbit muscle enzyme, exhibiting kinetic parameters closely related to the natural nucleotide. On the other hand, both A and B isomers of ATPβS are very weak substrates for phosphofructokinase, their efficiency expressed as k^*/k ratio is only 0.34% and, respectively, 0.14%, that of ATP. As in the case of ATPαS, we have not found significant differences in the behavior of the stereoisomers of ATPβS. As expected, ATPγS is not a substrate for rabbit muscle enzyme, however it is a competitive inhibitor.

Table 1
Kinetic constants of rabbit muscle phosphofructokinase for sulfur-containing ATP analogs

Nucleotide	K_m or K_i (μM)	V_m	100 k^*/k
ATP	15 ± 2	1 000	100
ATPαS(A)	15 ± 2	0 779	77 9
ATPαS(B)	18 ± 2	0.429	35 7
ATPβS(A)	48 ± 6	0.011	0.34
ATPβS(B)	64 ± 5	0 006	0.14
ATPγS	60 ± 5	–	–

The assays were carried out in imidazole buffer (pH 7) with 0 4 mM F6P. V_m are relative to the normal substrate (40 6 μmol/min/mg protein), k and k^* represent the relative V_m/K_m ratios for ATP and its analogs. The K_i value for ATPγS was determined from plots of $1/v$ against 1/ATP at various concentrations of analog. The results are mean value of 4 separate experiments

Table 2
Allosteric inhibition of rabbit muscle phosphofructokinase by ATP, ATPαS(A) and its reactivation by cAMP and AMPS

Nucleotide (mM)				V (μmol/min/mg of protein)
ATP	ATPαS(A)	cAMP	AMPS	
0.12	–	–	–	28 3
1.00	–	–	–	2 1
1 00	–	0 20	–	18 4
1.00	–	1.00	–	23.5
1.00	–	–	0 20	5 2
1 00	–	–	1 00	14.6
–	0.12	–	–	20 7
–	1 00	–	–	2 9
–	1 00	0 20	–	13 2
–	1 00	1.00	–	17.7

Assays were carried out in imidazole buffer (pH 7) with 0 04 mM F6P, 6 mM $MgCl_2$ and nucleotides as indicated

3.2. *Allosteric effects of sulfur-containing ATP and AMP analogs*

Likewise ATP, ATPαS, A and B are capable of producing an inhibition of phosphofructokinase by binding to the regulatory site. The I_{50} values (the corresponding concentrations for 50% inhibition under identical experimental conditions, i.e., imidazole buffer, (pH 7), 0.04 mM F6P and 6 mM $MgCl_2$) are 0.4 mM for ATP, 0.48 mM for ATPαS(A) and 0.34 mM for ATPαS(B). At 1 mM triphosphates the phosphofructokinase activity was inhibited by 94% (ATP), 86% (ATPαS(A)) and 92% (ATPαS(B)), respectively. The allosteric inhibition by ATP or ATPαS was counteracted by AMP or cAMP (table 2). The allosteric effects of ATPβS are either absent (in the case of the B isomer), or much weaker (I_{50} for ATPβS(A) is 1.8 mM). In order to distinguish an allosteric effect of ATPγS from that of competitive inhibition, we used o^1ITP, which is a phosphate donor similar to ATP but lacking the allosteric effects, at pH 7 and 8, in the presence of various ATPβS concentrations but with constant o^1ITP/ATPγS ratio [9]. From these experiments, not shown here, emerged that ATPγS lacked the ability of allosteric inhibition.

3.3. *Irreversible inhibition of phosphofructokinase by incubation with ATPαS(B) and AMPS*

AMPS like AMP or cAMP is able to stimulate the ATP-inhibited phosphofructokinase, but in a lesser extent than the natural nucleotides (table 2). However, by incubating phosphofructokinase with AMPS a progressive inhibition of the enzyme is observed (fig.1). Similar behavior was shown by ATPαS(B), the other analogs being less effective or ineffective. ATP or other triphosphates, but not AMP or cAMP, were able to protect the enzyme against the inactivation by AMPS or ATPαS(B). These results are interpreted as being the consequence of the interaction of the above mentioned analogs with SH groups from the active site of phosphofructokinase, since dithiothreitol prevents the enzyme inhibition.

4. Discussion

With a few exceptions, ATP analogs with modifed purine bases are good substrates for rabbit muscle phosphofructokinase, this enzyme showing a rather broad specificity for nucleotides as phosphoryl group donors [9–11]. The interaction of nucleotides with the allosteric site of phosphofructokinase is more complex than that with the substrate site, and requires both an intact adenine moiety and an intact terminal phosphate group for full activity [9]. Our results with ATP analogs containing sulfur in the phosphate chain support this point of view. However, some points are to be raised:

(i) All sulfur-containing ATP analogs bind similarly

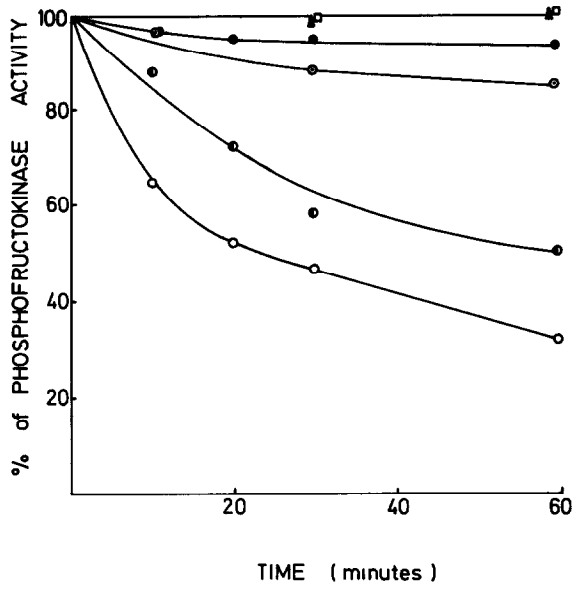

Fig.1 Inactivation of rabbit muscle phosphofructokinase by AMPS and ATPαS(B) The reaction mixture contained at 4°C and 0 5 ml final volume, 50 mM Tris–HCl (pH 8), 0.2 mM EDTA, 0.1 mg phosphofructokinase and 0.5 mM nucleotide as specified. At different time intervals samples were taken out (5 μl) for measurement of phosphofructokinase activity as in section 2, at optimal concentrations of substrates, i.e., 0.3 mM ATP and 1 mM F6P. (□) Control, with ATP; (▲) ATPγS, (○) AMPS, (○) ATPαS(A), (○) ATPαS(B), (●) AMPS + ATP.

to the active site of phosphofructokinase, regardless of which phosphate group bears the sulfur atom;

(ii) The catalytic and allosteric properties of ATP analogs decrease progressively from ATPαS to ATPγS. In other words, the presence of the sulfur atom in the close proximity of the phosphate bonds, increases the stability of these bonds towards cleavage, without affecting to any considerable extent the binding of the analogs to the phosphofructokinase. In this way one can explain why ATPγS, which is not a substrate for hexokinase or other phosphotransferases [2] is a substrate for *E. coli* DNA-dependent RNA polymerase [6], methionyl-tRNA-synthetase [1], or adenylate cyclase [12]. Instead, ATPαS which is a substrate for the majority of the investigated phosphotransferases, is ineffective as substrate for phenylalanyl-tRNA-synthetase [13], being an inhibitor for adenylate cyclase [12];

(iii) Unlike other phosphotransferases, which show a greater specificity for one of the stereoisomers of ATPαS, phosphofructokinase makes no distinction between the two forms of the analog, whether substrates or allosteric effectors,

(iv) A comparison of the effect of the above analogs with the effect of sATP and sAMP, in which the sulfur atom is a part of the base structure, shows net differences as regards the allosteric effects, which depend primarily on the integrity of the adenine ring. sATP is a good substrate for phosphofructokinase [11] but it does not bind to the allosteric site. In much the same way, sAMP does not reactivate the enzyme inhibited with excess ATP.

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