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ENZYMATIC SYNTHESIS OF ADENOSINE 5′-*O*-(3-[^{35}S]-THIOTRIPHOSPHATE)

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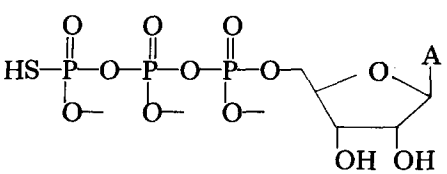
Summary

A method for the enzymatic synthesis of adenosine 5′-*O*-(3-[^{35}S]thiotriphosphate) is described. The method involves the thiophosphate exchange of adenosine 5′-*O*-(3-thiotriphosphate) with [^{35}S]thiophosphate with the aid of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase in the presence of 3-phosphoglycerate. The method is also applicable for the synthesis of guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate).

Introduction

ATP analogues that are hydrolyzed slowly, or not at all, by ATPases can be useful tools for the study of enzyme mechanism [1,2]. With this goal in mind we have recently synthesized ATPγS [3] which has been used for the study of



Adenosine 5′-*O*-(3-thiotriphosphate).

certain aspects of muscle contraction [4]. In the course of these and other studies it has become of increasing interest to follow the fate of the thiophosphate group in more detail. For such purposes the preparation of [^{35}S]ATPγS

Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

had become necessary. The enzymatic thiophosphate exchange reaction following the procedure for the ATP-phosphate exchange with glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase [5] was therefore undertaken. Because changes in this procedure are necessary to obtain a reasonable exchange with thiophosphate it is reported here in detail.

Materials

ATPγS and GTPγS were purchased from Boehringer, Mannheim (Germany). Glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle, 10 mg/ml, 80 units/mg), 3-phosphoglycerate kinase (from yeast, 10 mg/ml), 450 units/mg) and 3-phosphoglycerate were also products from Boehringer, Mannheim (Germany). [^{35}S]Thiophosphate (Na_3 salt, 10 Ci/mol) was obtained from the Radiochemical Centre, Amersham (England) or New England Nuclear. Myosin was a kind gift from H.J. Mannherz, Heidelberg.

Results

Although the commercial samples of ATPγS contained approx. 32% of ADP, the material was used without purification. It will be shown below that the presence of ADP did not interfere with the exchange reaction. ATPγS concentrations were corrected for the content of ADP. Incubation of [^{35}]thiophosphate with ATPγS, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and 3-phosphoglycerate led to incorporation of [^{35}S]thiophosphate into ATPγS (Fig. 1). After purification of the reaction mixture by ion-exchange

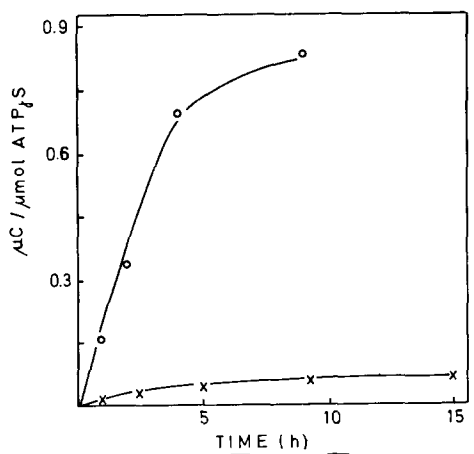


Fig. 1. Kinetics of the incorporation of [^{35}S]thiophosphate into ATPγS. A solution (X———X) (total volume 4.75 ml) containing 7.5 mM ATPγS, 8 mM $MgCl_2$, 80 mM Tris · HCl, pH 8.0, 0.5 mM dithiothreitol, 8 mM 3-phosphoglycerate, 14 mM [^{35}S]thiophosphate, 2.1 I.U. of 3-phosphoglycerate kinase per ml and 0.75 I.U. of glyceraldehyde phosphate dehydrogenase per ml was incubated at 37°C. At time intervals 550 μl were removed and chromatographed on a DEAE-Sephadex A-25 column (1.5 × 20 cm) with a linear gradient of 500 ml each of 0.1 and 0.6 M triethylammonium bicarbonate. Fractions of about 13 ml were collected. A typical elution pattern is given in Fig. 2. The nucleotide-containing fractions were pooled, evaporated, reevaporated with methanol to remove the buffer and the specific activity determined. In a second experiment (○———○) the solution (total volume 7.2 ml) had the same composition except ATPγS was 1.8 mM and 1.45 ml were chromatographed each time.

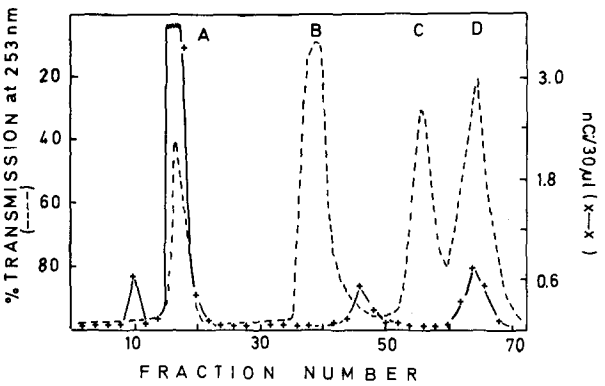


Fig. 2. Example of the purification of the reaction products by chromatography on DEAE-Sephadex A-25. Conditions as described in Fig. 1 after incubation for 9 h. - - - - - -, transmission; X———X, radioactivity. A, AMP plus thiophosphate; B, ADP; C, ATP; D, ATPγS.

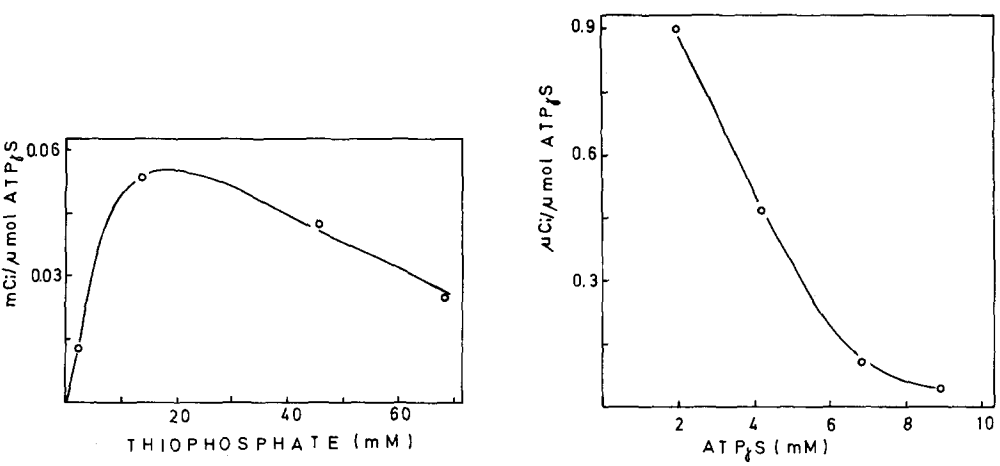


Fig. 3. Dependence of [^{35}S]thiophosphate exchange on [^{35}S]thiophosphate concentration. Solutions (total volume 1.2 ml) with compositions as described in Fig. 1 but with varying [^{35}S]thiophosphate concentrations were incubated for 6 h at 37°C, chromatographed and the specific activity determined.

Fig. 4. Dependence of [^{35}S]thiophosphate exchange on ATPγS concentrations. Solutions (total volume 1.5 ml) with compositions as described in Fig. 1 but with 11 mM [^{35}S]thiophosphate and with varying ATPγS concentrations were incubated for 9 h at 37°C, chromatographed and the specific activity determined.

TABLE I

DEPENDENCE OF [^{35}S]THIOPHOSPHATE EXCHANGE ON Mg^{2+} CONCENTRATION

Solutions (total volume 1.85 ml) with compositions as in Fig. 1 but with 4 mM ATPγS and varying concentrations of Mg^{2+} were incubated for 12 h at 37°C, chromatographed and the specific activity determined.

Mg^{2+} (mM)	[^{35}S]ATPγS (mCi/mol)
—	1.8
6	570
30	250
60	50

chromatography (Fig. 2) ATPγS was identified by thin-layer chromatography on polyethyleneimine-impregnated cellulose [3], electrophoresis at pH 3.5 [3] and its cleavage to ADP and [^{35}S]thiophosphate by myosin [4]. No exchange reaction was observed in the absence of enzyme. In addition to the expected nucleotides, as well as AMP, ADP and ATPγS, also some ATP was detected in the reaction mixture after long incubation times (Fig. 2). The exchange reaction was dependent on the thiophosphate (Fig. 3) as well as the Mg^{2+} concentration (Table I). There is also a marked dependence on the ATPγS concentration (Fig. 4). High concentrations of ATPγS inhibit the exchange reaction and slow down the rate of exchange (Fig. 1).

Discussion

As an ATP analogue that is hydrolysed only slowly by ATPases such as myosin [2,4], ATPγS might be a useful analogue for the study of other ATP-dependent enzymes. For such studies the compound should be radioactively labelled. Although labelling in the nucleoside part has been described earlier [6], the availability of the ^{35}S-labelled material is desirable for many studies, in particular when the fate of the thiophosphate group is to be followed. This becomes important in cases where the thiophosphate group can be transferred by kinases to other nucleotides [6] or where a thiophosphorylated enzyme can be formed. An interesting example for a thiophosphorylated enzyme has recently been described by Gratecos and Fischer [7]. With the aid of ATPγS they could thiophosphorylate muscle phosphorylase *b* to the thio analogue of phosphorylase *a* which is as active as phosphorylase *a*. However, this thiophosphorylase *a* cannot be hydrolysed by phosphorylase phosphatase. A similar observation has been reported for the light chains of myosin after thiophosphorylation with ATPγS [8].

That a modification of the phosphate-ATP exchange reaction [5] might be a feasible approach for the synthesis of [^{35}S]ATPγS was apparent from the result by De Sousa et al. [9] who showed that thiophosphate can be a substrate for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. In the presence of ADP they identified ATPγS as the major reaction product by thin-layer chromatography and by oxidation to the disulfide. The thiophosphate-ATPγS exchange reaction is an inefficient process. The elution diagram (Fig. 2) shows that the ^{35}S label is far from being evenly distributed between thiophosphate and ATPγS. At low ATPγS concentration there is a marked increase in the exchange (Fig. 4). By decreasing the ATPγS concentration even further than indicated in Fig. 4 one can probably obtain [^{35}S]ATPγS with higher specific activity than 0.90 Ci/mol. Also, [^{35}S]thiophosphate with a higher specific activity is now obtainable.

Analysis of the reaction described in Fig. 1 for the low concentration of ATPγS showed that there was no ATP to be found upon chromatography of the reaction mixture up to 1 h incubation time. After that ATP appeared and increased at the expence of ATPγS. After 4 h, 0.66 μmol of ATP and 1.8 μmol of ATPγS were isolated. In a control experiment it was ascertained that ATPγS is stable for at least 11 h under the incubation conditions, decomposing neither to ADP nor ATP. The explanation for the appearance of ATP is probably an

ATPγS-phosphate exchange, the latter being produced by slow decomposition of thiophosphate to phosphate. Under the reaction conditions it was found that approx. 10% of [^{35}S]thiophosphate decomposed in about 8 h as followed by thin-layer chromatography.

The exchange reaction can also be carried out starting with ATP since the actual exchange takes place at the 1,3-diphosphoglycerate level and it is immaterial whether this compound is derived from ATP or as 1-thiophospho-3-phosphoglycerate from ATPγS. Using conditions as in Fig. 1, the yield of ATPγS is smaller by a factor of about 8 than starting with ATPγS. Here too, the amount of ADP was considerable, similar to the amount of ATP produced. This result taken together with the results from the experiments starting with ATPγS indicate that the equilibrium between ATPγS and ADP is more on the side of ADP than in the phosphate exchange reaction with ATP.

In experiments with GTPγS or GTP under the conditions described for ATPγS, [^{35}S]GTPγS with the same specific activity as [^{35}S]ATPγS was synthesized. The best method of storage for these analogues is at −20°C or lower as shock-frozen aqueous solutions. Samples have been kept for more than a year under these conditions without either loss of sulfur or decomposition to the nucleoside diphosphate. Storage of lyophilized samples is not recommended even at low temperatures.

It is hoped that the availability of these labelled analogues will make ATPγS and GTPγS useful tools for the study of ATP- and GTP-dependent enzymes.

Acknowledgement

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