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THE ENZYMATIC SYNTHESIS OF THIOPHOSPHATE ANALOGS OF NUCLEOTIDES

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SUMMARY

The enzymatic transfer of the thiophosphate group from a nucleoside 5'-*O*-(3-thiotriphosphate) to a nucleoside 5'-diphosphate with the aid of nucleosidediphosphate kinase (ATP: nucleosidediphosphate phosphotransferase, EC 2.7.4.6) is described. This method can be used for the synthesis of radioactively labelled nucleoside 5'-*O*-(3-thiotriphosphates). Thiophosphate transfer can also be achieved using adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3).

INTRODUCTION

We have recently reported the chemical synthesis of thiophosphate analogs of nucleotide anhydrides[1]. In the course of studies on the interactions of these analogs with various enzyme systems, it became necessary to develop methods for the synthesis of small quantities of radioactively labelled derivatives. Since application of the chemical procedure on such a small scale gave unsatisfactory yields of the desired products, the possibility of enzymatic synthesis was investigated. Of particular interest was labelled adenosine 5'-*O*-(3-thiotriphosphate) (Fig. 1) for studies with myosin ATPase (EC 3.6.1.3) (H. J. Mannherz, R. S. Goody and F. Eckstein, unpublished results), and the corresponding guanosine compound for investigations of its

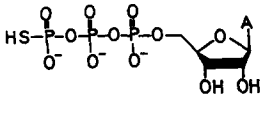

Fig. 1. Adenosine 5'-*O*-(3-thiotriphosphate).

Abbreviations: AMPS, adenosine 5'-*O*-phosphorothioate; ADPαS, adenosine 5'-*O*-(1-thiodiphosphate); ADPβS, adenosine 5'-*O*-(2-thiodiphosphate); ATPαS, adenosine-5'-*O*-(1-thiotriphosphate); A(U, G, s^6I, cl^6I) TPγS, adenosine (uridine, guanosine, 6-thioinosine, 6-chloroinosine) 5'-O-(3-thiotriphosphate); s^6IDP, 6-thioinosine 5'-*O*-diphosphate; cl^6IDP, 6-chloroinosine 5'-*O*-diphosphate; PEI-cellulose, polyethyleneimine-impregnated cellulose.

TABLE I

PROPERTIES OF NUCLEOTIDE ANALOGS WITH VARIOUS ENZYMES

Enzyme and source	*Compounds and properties*	*Ref.*
Alkaline phosphatase *(E. coli)*	ADPβS; competitive inhibitor, $K_i = 6.6 \cdot 10^{-5}$ M	1
	ATPγS; inhibitor	
DNA-dependent RNA polymerase *(E. coli)*	ATPγS; substrate; $K_m = 3.8 \cdot 10^{-5}$ M	*
	UTPγS; substrate	
Myosin ATPase (rabbit)	ATPγS; substrate with modified properties	**
Methionyl-tRNA synthetase (yeast)	ATPγS; substrate; $K_m = 6 \cdot 10^{-4}$ M	***
Phenylalanyl-tRNA synthetase *(E. coli)*	ATPγS; substrate	§
DNA polymerase II	dATPγS; substrate	§§
Snake venom phosphodiesterase	ATPγS; substrate	§§§
Polynucleotide phosphorylase *(M. lysodeicticus)*	ADPβS; substrate	§§§

* R. S. Goody, H. Sternbach and F. Eckstein, unpublished results.
** H. J. Mannherz, R. S. Goody and F. Eckstein, unpublished results.
*** F. Lawrence, J. P. Waller, R. S. Goody and F. Eckstein, unpublished results.
§ F. von der Haar, R. S. Goody and F. Eckstein, unpublished results.
§§ H. P. Vosberg, R. S. Goody and F. Eckstein, unpublished results.
§§§ R. S. Goody and F. Eckstein, unpublished results.

interaction with amino acid polymerization factors (A. Parmeggiani, R. S. Goody and F. Eckstein, unpublished results) (see Table I for further examples). The possibility of enzymatic synthesis of chemically sensitive compounds, for which the chemical procedure is not suitable, was also investigated.

MATERIALS AND METHODS

Enzymes

Rabbit muscle adenylate kinase (ATP: AMP phosphotransferase, EC 2.7.4.3) was prepared and assayed as described by Kress *et al.*[2]. A stock solution was prepared as follows: 10 mg of protein was dissolved in 0.5 ml of 0.2 M Tris–HCl–2 mM dithiothreitol, and dialysed exhaustively against distilled water. The protein solution was then diluted to 20 000 enzyme units/ml. It proved to be stable at 4 °C for about 6 weeks. Rabbit muscle adenylate kinase and pig muscle adenylate kinase were obtained from Boehringer and Söhne, Mannheim. Nucleosidediphosphate kinase (EC 2.7.4.6) from beef liver was obtained from the same firm.

Chemicals

ADP, ATP, GDP and UTP were obtained from Waldhof, Mannheim (Germany) and used without further purification. Thiophosphate analogs which were used as starting materials were prepared as previously described[1]. 6-Thioinosine 5′-*O*-diphosphate was prepared from the monophosphate by the method of Michelson[3]. The

monophosphate was prepared from 6-thioinosine (Waldhof) by the method of Yoshikawa[4]. 6-Chloroinosine 5′-*O*-diphosphate was prepared by the method of Michelson[3] from the monophosphate, which was prepared according to Brox *et al.*[5]. ^{3}H- and ^{14}C-labelled nucleotides were obtained from the Radiochemical Centre, Amersham, England or New England Nuclear Corporation. Polyethyleneimine-impregnated cellulose (PEI-cellulose) plates (Polygram CEL 380 PEI/UV) were obtained from Machery and Nagel, Düren (Germany).

General procedures for tests of substrate properties

Adenylate kinase

Standard conditions were: 50 μl incubation mixture; 5 mM each reactant; 5 mM $MgCl_2$; 30 mM Tris–HCl (pH 8.0); 5 mM dithiothreitol; 20 I.U. adenylate kinase; incubation first at 20 °C, and then at 37 °C if the reaction was slow. Aliquots of 3–5 μl were analysed by thin-layer chromatography on PEI-cellulose plates in 0.75 M KH_2PO_4 adjusted to pH 3.4 with concentrated HCl. When a radioactive label was used, the distribution of radioactivity in the products was determined by cutting out the appropriate spots from the PEI-cellulose chromatogram and counting directly in a Packard Tricarb scintillation counter.

Nucleosidediphosphate kinase

Standard conditions used were the same as for adenylate kinase, except that 4 I.U. of the enzyme were used. The reaction mixture was analysed in the same way as for adenylate kinase.

In one case (AMPS + ATP), since the reaction was not later attempted on a preparative scale, the distribution of reaction products was examined in greater detail by applying larger amounts of the reaction mixture in the form of a streak to PEI-cellulose plates, developing in the same buffer, and eluting the separated components with 1.5 M LiCl. The ultraviolet absorbance of each eluate was measured at the wavelength appropriate for the nucleobase, and the distribution of products was calculated.

RESULTS

The substrate properties of various mixtures of normal substrates and analogs are summarized in Table II.

Examples of the preparative use of the two enzymes

(a) Adenylate kinase

Preparation of [^{3}H]ATPγS. The reaction mixture contained: 0.2 mM [^{3}H]ADP (1.0 mCi); 1.8 mM ADP; 7.0 mM ADPβS; 3 mM $MgCl_2$; 5 mM dithiothreitol; 30 mM Tris–HCl (pH 8.0); 200 I.U. adenylate kinase (after Kress *et al.*[2]); total volume 500 μl. The reaction mixture was allowed to stand at 20 °C, and the course of the reaction was followed by PEI-cellulose thin-layer chromatography. After 2 h, the reaction was judged to be complete, and the mixture was separated by chromatography at 4 °C on DEAE-Sephadex A 25 (column size 8 cm × 1.6 cm), eluting with a linear gradient of triethylammonium bicarbonate (0.1–0.6 M over 1 l). A good separation of the five components present in the reaction mixture (see Table II) was obtained, ATPγS being eluted last. Yield: 10.8 $A_{259\ nm}$ units (0.72 μmole) of [^{3}H]ATPγS. The identity of this product was confirmed by chromatography on PEI-cellulose, where it

TABLE II

SUBSTRATE PROPERTIES OF VARIOUS ANALOGS

Reactants	*Enzyme*	*Time (h)*	*Temp. (°C)*	*Products**
AMP + ATPαS	Adenylate kinase**	2	20	ADPαS, ATPαS ATP, ADP
AMPS + ATP	Adenylate kinase	5	20***	ADPαS (11.6%) ATPαS (10.0%) AMPS, ADP (41%) ATP (27.5%) AMP (9.8%)
ADP + ADPβS	Adenylate kinase	0.5	20	ATPγS§, ATP AMP
ADPβS	Adenylate kinase	16	20	No reaction
ADP + UTPγS	Nucleosidediphosphate kinase	2	20	AMP, ATP, ATPγS; UDP
GDP + UTPγS	Nucleosidediphosphate kinase	2	20	GTPγS§ UDP
s^6IDP + UTPγS	Nucleosidediphosphate kinase	2	37	s^6ITPγS§ UDP
cl^6IDP + GTPγS	Nucleosidediphosphate kinase	2	37	cl^6ITPγS§ GDP
ADPβS + ATP	Nucleosidediphosphate kinase	16	20	No reaction

* Analysed by chromatography on PEI-cellulose (0.75 M KH_2PO_4, pH 3.5).

** For these experiments, only adenylate kinase prepared by the method of Kress *et al.*[2] was used.

*** After standing for 10 h at 20 °C, the same distribution of products was found.

§ Isolation described in the experimental section.

behaved identically with a sample of ATPγS prepared by the chemical synthesis, and by oxidation to the disulfide by H_2O_2.

When commercially available adenylate kinase was used in the above reaction, the reaction was judged qualitatively to proceed much more slowly, the enzyme from rabbit muscle being more effective than that from pig. This lower activity is probably partly due to the fact that these enzymes are supplied as suspensions in $(NH_4)_2SO_4$, which would lead to significant concentrations of inhibitory NH_4^+ in the reaction mixtures. This difficulty could presumably be eliminated by previous dialysis of the suspension.

(b) Nucleoside diphosphokinase

Preparation of [^{3}H]GTPγS. The reaction mixture contained: 3.2 mM [^{3}H]GDP (1.0 mCi); 6.7 mM GTPγS; 5 mM $MgCl_2$; 5 mM dithiothreitol; 20 mM Tris–HCl (pH 8.0); 20 I.U. nucleosidediphosphate kinase; total volume 200 μl. The mixture was left at 20 °C, and the reaction was followed by PEI-cellulose thin-layer chromatography. After 5 h, the reaction was complete, as shown by the even distribution of radioactivity between the two nucleotides. The mixture was separated by chromatography

on DEAE-Sephadex A 25 (column size 10 cm × 1.6 cm) eluting with a linear gradient of triethylammonium bicarbonate (0.1–0.6 M over 600 ml). Yield: 16.5 $A_{252\ nm}$ units (1.2 μmoles) of [^{3}H]GTPγS, eluted at approx. 0.5 M.

Preparation of s^{6}ITPγS. The reaction mixture contained: 30 mM s^{6}IDP, 10 mM UTPγS; 5 mM $MgCl_2$; 5 mM dithiothreitol; 20 mM Tris–HCl (pH 8.0); 80 I.U. nucleosidediphosphate kinase; total volume 1.0 ml. The reaction mixture was allowed to stand at 37 °C for 3 h, and was then separated by fractionation on DEAE-Sephadex A 25 (column size 15 cm × 2 cm), eluting with a linear gradient of triethylammonium bicarbonate (0.1–0.6 M over 1 l). Yield: 77 $A_{315\ nm}$, pH 7.0 units (3.3 μmoles) of s^{6}ITPγS, eluted after UTPγS.

Preparation of cl^{6}ITPγS. The reaction mixture contained: 6 mM cl^{6}IDP; 6 mM GTPγS; 5 mM $MgCl_2$; 5 mM dithiothreitol; 40 mM Tris–HCl, pH 8.0; 40 I.U. nucleosidediphosphate kinase; total volume 650 μl. The reaction mixture was allowed to stand 1 h at 37 °C, and was then separated by chromatography on DEAE-Sephadex A 25, using a linear gradient of triethylammonium bicarbonate (0.1–0.6 M over 1 l). Yield: 11.5 $A_{264\ nm}$ units (1.4 μmoles) of cl^{6}ITPγS, eluted between GDP and GTPγS. Eluted slightly after cl^{6}ITPγS was a small amount of a compound having a maximum absorption in the ultraviolet at 315 nm at pH 7.0. A similar diphosphate-like product eluted after cl^{6}IDP was also obtained. These are probably 6-thioinosine derivatives, arising from attack of thiophosphate on C^6 of the purine base, followed by loss of phosphate ion.

Detection of adenylate kinase activity in nucleosidediphosphate kinase

When the commercially available nucleosidediphosphate kinase was used to catalyse the transfer of thiophosphate from UTPγS to ADP, AMP and ATP were also obtained as products, in addition to the expected ATPγS (Table II). Incubation of 5 mM ADP with the enzyme under the conditions normally used for adenylate kinase led to the rapid formation of AMP and ATP detected by PEI-cellulose thin-layer chromatography. GDP was, however, not affected by the enzyme preparation even after longer incubation times. This evidence suggests the presence of adenylate kinase in the commercial preparation.

Removal of adenylate kinase from nucleoside diphosphokinase

The commercially available glycerol solution of nucleosidediphosphate kinase was dialysed overnight against 10 mM Tris (pH 9.0) buffer, and applied to a column of DEAE-cellulose (Whatman DE 52) previously equilibrated with a buffer containing 10 mM Tris–HCl (pH 9.0) and 5 mM NaCl[6]. On elution of the column with the same buffer, nucleoside diphosphokinase free of adenylate kinase was obtained in the first fractions.

Preparation of [^{3}H]ATPγS using nucleoside diphosphokinase

The reaction mixture contained: 0.55 mM [^{3}H]ADP (1.0 mCi); 5.5 mM ATPγS; 5.5 mM $MgCl_2$; 3 mM dithiothreitol; 25 mM Tris–HCl (pH 8.0), approx. 50 I.U. purified nucleosidediphosphate kinase; total volume 180 μl. The reaction mixture was allowed to stand at room temperature for 3 h, after which time PEI-cellulose thin-layer chromatography indicated that 85% of the [^{3}H]ADP had been converted to [^{3}H]ATPγS.

Chromatography on DEAE-Sephadex A 25 using a linear gradient of triethylammonium bicarbonate (0.2–0.5 M over 600 ml) gave pure [^{3}H]ATPγS. Yield: 13 $A_{259\ nm}$ units (0.87 μmole).

DISCUSSION

In the short time that thiophosphate analogs of nucleotides have been available, it has become apparent that they are potentially very useful compounds for the investigation of enzyme mechanism and structure. Current studies in our and other laboratories indicate that they may find application as affinity labels, *e.g.* for myosin ATPase (H. J. Mannherz, R. S. Goody and F. Eckstein, unpublished results) and ribosomal G-factor (A. Parmeggiani, R. S. Goody and F. Eckstein, unpublished results) and their properties as competitive inhibitors or substrates with modified behaviour are also of interest. In certain cases, it may be possible to bind heavy metal derivatives to the sulfur atom of such an analog to help in the elucidation of the structure of a macromolecule using X-ray diffraction. This has been demonstrated for tRNA containing a terminal AMPS residue[7].

For the types of studies outlined above, it is often desirable to have radioactively labelled derivatives. The use of enzymes involved in the transfer of phosphate groups between nucleotides is a standard technique for preparing radioactively labelled nucleoside triphosphates. This suggested a possible route for the preparation of labelled thiophosphate analogs of nucleotides, assuming that the appropriate enzymes have the ability to transfer thiophosphate groups. With the two enzymes chosen, adenylate kinase and nucleosidediphosphate kinase, it was easily possible to show that thiophosphoryl transfer can take place. It was possible to exploit this reaction to synthesize labelled analogs and thiophosphate analogs of chemically sensitive nucleosides.

For the preparation of γ-thiotriphosphates, nucleosidediphosphate kinase appears to be the more suitable enzyme. For example, in the synthesis of [^{3}H]GTPγS, the purification of the product involves a simple separation from [^{3}H]GDP. However, in the transfer of a thiophosphate group between nucleotides having different base residues, care must be taken to choose the nucleoside so that a separation of the mixture of thiophosphates obtained can be achieved. Usually, if a separation can be achieved on PEI-cellulose thin-layer chromatography, a column separation is possible on DEAE-Sephadex A 25. For use with adenosine-containing nucleotides, it is preferable to purify the commercially available enzyme to remove adenylate kinase impurity, which leads to the formation of a more complex reaction mixture.

Adenylate kinase can be used with adenosine nucleotide analogs. This has been demonstrated by the synthesis of [^{3}H]ATPγS and [^{14}C]ATPγS starting from ADPβS and [^{3}H]ADP or [^{14}C]ADP, respectively. Labelled ADPβS is also obtained in this reaction. Although the mixture obtained is relatively complex, the separation presents no special difficulties. Using an adenylate kinase, the best procedure for preparing [^{3}H]ATPγS would be to use [^{3}H]AMP with an excess of ATPγS, and to allow the label to be distributed evenly. The separation of ATPγS from the small quantities of ADP, ADPβS and ATP would then be relatively simple. However, the procedure using nucleosidediphosphate kinase free of adenylate kinase for the preparation of [^{3}H]-ATPγS is preferable on the grounds of ease of separation. It should be pointed out

that reaction of excess thiotriphosphate with radioactive diphosphate results in an even distribution of label between the two and a corresponding reduction of specific activity of the product.

It is of interest to note that no evidence was obtained for transfer of a phosphate group from ADP to ADPβS, although phosphorylation of AMPS by ATP in the presence of adenylate kinase was observed. It is of course possible that phosphate transfer to ADPβS does occur slowly, but the rate must be considerably slower than transfer of thiophosphate to ADP. It was also not possible to detect transfer of phosphate from a triphosphate to β-thiodiphosphate analogs in the presence of nucleoside-diphosphate kinase. These findings could be of importance for studies on systems where a diphosphate analog is required which is resistant to triphosphate regenerating systems.

Although detailed kinetic studies have not yet been carried out, it is clear from chromatographic assessment of the reaction rates that transfer of thiophosphate is considerably slower than transfer of phosphate with both enzymes. Although at this stage no interpretation of the reduced rate of transfer can be given, it is possible that this is simply a reflection of the lower susceptibility of the phosphorus towards nucleophilic attack which is characteristic of derivatives of thiophosphate.

In the case of the α-thiodi- and α-thiotriphosphates, since these compounds can exist as diastereomers due to the asymmetry of the α-phosphorus atom[8], the possibility of preferential synthesis of one isomer by adenylate kinase and nucleoside diphospho-kinase exists, due to stereochemical specificities of the active sites. This could lead to valuable information about their mechanism of action, in analogy to the results obtained from the hydrolysis of uridine 2′:3′-*O*,*O*-cyclophosphorothioate by pancreatic ribonuclease[9].

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