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Synthesis and Properties of Diastereoisomers of Adenosine 5′-(*O*-1-Thiotriphosphate) and Adenosine 5′-(*O*-2-Thiotriphosphate)†

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ABSTRACT: The chemical synthesis of adenosine 5′-(*O*-1-thiotriphosphate) (ATPαS) and adenosine 5′-(*O*-2-thiotriphosphate) (ATPβS) is described. Both exist as a pair of diastereomers, A and B. The isomers of ATPαS can be distinguished on the basis of their different reaction rates with myokinase as well as nucleoside diphosphate kinase. With both enzymes, isomer A reacts fast whereas isomer B reacts considerably more slowly. Phosphorylation of a mixture of isomers of ADPαS with pyruvate or acetate kinase yields ATPαS, isomer A, whereas the phosphoryl transfer with creatine or arginine kinase yields isomer B. The isomers of ATPβS differ in their reactivity with myosin. Isomer A is readily hydrolyzed, whereas isomer B is not. However, isomer B reacts faster with nucleoside diphosphate kinase and ADP than isomer A. Phosphoryl transfer with pyruvate kinase onto ADPβS yields ATPβS; isomer A, with acetate kinase, isomer B.

It has been shown earlier that nucleoside 2′,3′-*O*,*O*-cyclothiophosphates exist in the form of two diastereomers which in favorable cases can be separated by crystallization (Saenger and Eckstein, 1970). The investigation of these diastereomers with ribonucleases has yielded valuable information on the mechanism of pancreatic ribonuclease A (Saenger et al., 1974) as well as ribonuclease T_1 (Eckstein et al., 1972). Some time ago we have reported the synthesis of nucleoside 5′-(*O*-1-thiotriphosphates) (Eckstein and Gindl, 1970) and their polymerization by DNA-dependent RNA polymerase. It was mentioned that such nucleoside 5′-(*O*-1-thiotriphosphates) should also exist in the form of two diastereomers which at that time could not be separated. We report in this publication the enzymatic synthesis of the two single diastereomers of adenosine 5′-(*O*-1-thiotriphosphate) as well as those of a new ATP analogue, adenosine 5′-(*O*-2-thiotriphosphate). The interactions of these diastereoisomers with some ATP- or ADP-requiring enzymes will be discussed.

Experimental Section

Materials and Methods

$^{35}SPCl_3$ was purchased from Radiochemical Center, Amersham (England); [^{14}C]ADP and [^{14}C]ATP were from New England Nuclear Corp. Alkaline phosphatase (calf intestine, 5 mg/ml, 350 U/mg), snake venom phosphodiesterase (10 mg/ml, 1.5 U/mg), acetate kinase (5 mg/ml, 170 U/mg), pyruvate kinase (rabbit muscle, 10 mg/ml, 200 U/mg), nucleoside diphosphate kinase (beef liver, 5 mg/ml, 80 U/mg), myokinase (rabbit muscle, 5 mg/ml, 360 U/mg), creatin kinase (rabbit muscle, 25 U/mg), lactate dehydrogenase (rabbit muscle, 5 mg/ml, 550 U/mg), and acetate kinase (*Escherichia coli,* 1 mg/ml, 170 U/mg) were purchased from Boehringer, Mannheim (Germany). Arginine phosphate was a product of Calbiochem. Myosin (rabbit muscle) and its subfragment 1 were kind gifts of Dr. H. J. Mannherz, Heidelberg. Arginine kinase (American lobster, 2 mg/ml, 190 U/mg) was a kind gift of Dr. M. Cohn, Philadelphia. Aquasol was purchased from New England Nuclear Corp.

^{31}P nuclear magnetic resonance spectra were recorded with a Bruker Physic HFX 60 spectrometer equipped with a Fourier transform unit Bruker-Data System B-NC 12 at approximately pH 10 with proton broad-band decoupling with dilute H_3PO_4 as external standard.

Optical densities were measured with a Zeiss PMQ II. For kinetic experiments it was equipped with a Servogor recorder.

Electrophoresis was performed on paper Schleicher & Schüll 2043b (washed) in 0.1 M triethylammonium bicarbonate (pH 7.5) or 0.05 M ammonium formate (pH 3.5) with 40 V/cm for 90 min. Thin-layer chromatography (TLC) was carried out on PEI-cellulose sheets (Polygram Cel-300 polyethylenimine, uv for TLC) (Macherey and Nagel, Düren, Germany) in 0.75 M KH_2PO_4 adjusted to pH 3.5 with concentrated HCl.

When radioactive compounds were used, the amount of radioactivity in a certain product was determined by cutting

† From the Max-Planck-Institut für Experimentelle Medizin, Göttingen, Abteilung Chemie, Germany. *Received September 2, 1975.* This work was supported by the Deutsche Forschungsgemeinschaft.
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out the appropriate uv-active spot from the PEI-cellulose chromatogram, placing it in a scintillation vial, and covering it with 1 ml of 1 N HCl for ca. 30 min. After addition of 10 ml of Aquasol, the samples were counted.

Chemical Syntheses

[^{35}S]*Adenosine 5'-(O-1-Thiotriphosphate)*. [^{35}S]Adenosine 5'-phosphorothioate (Murray and Atkinson, 1968) (7500 A_{260} units, 0.5 mmol) was converted to the pyridinium salt by passage over Merck I ion exchanger (pyridinium form). The solution was evaporated to dryness, tri-*n*-octylamine (0.22 ml, 0.5 mmol) and methanol (ca. 10 ml) were added, and the mixture was stirred until a clear solution was obtained. After evaporation, the residue was evaporated (three times) with dry dimethylformamide using an oil pump. The residue was dissolved in anhydrous dioxane (2 ml) and diphenyl phosphorochloridate (0.15 ml, 0.75 mmol) and tri-*n*-butylamine (0.25 ml, 1 mmol) were added. After stirring the mixture at room temperature for 3 h, the solvent was removed by evaporation, and anhydrous ether (10 ml) and petroleum ether (30 ml) were added to the residue, and the mixture was left at 0 °C for 30 min. The ether was decanted, the remaining material dissolved in anhydrous dioxane (1 ml), and the solution evaporated.

Tetrasodium pyrophosphate decahydrate (2.23 g, 5 mmol) was converted to the pyridinium salt by addition of tri-*n*-butylamine (2.43 ml, 10 mmol) and evaporation to dryness to the tri-*n*-butylammonium salt. After repeated evaporation with anhydrous pyridine (three times), the material was dissolved in anhydrous pyridine (3 ml) and added to the activated [^{35}S]adenosine 5'-phosphorothioate described above.

After stirring at room temperature for 2 h, ether (10 ml) was added to precipitate the product. The precipitate was dissolved in water and chromatographed on a DE-52 cellulose column (37 × 2.5 cm), with a linear gradient of 1.5 l. each of 0.05 M and 0.5 M triethylammonium bicarbonate. The product was eluted at ca. 0.33 M buffer, yield 1550 A_{260} units (0.1 mmol, 20%). For further purification this material was rechromatographed on a QAE-A 25 Sephadex column (1.5 × 25 cm) with a linear gradient of 800 ml each of 0.25 M and 0.5 M triethylammonium bicarbonate: yield 1200 A_{260} units (16%); for ^{31}P NMR spectrum, see Table I. The material was electrophoretically and chromatographically identical with material synthesized by the method described earlier (Eckstein and Gindl, 1970). The material was not degraded by snake venom phosphodiesterase but was degraded to AMPS[1] by alkaline phosphatase under conditions described for ATPβS.

[^{35}S]*Adenosine 5'-(O-1-Thiodiphosphate)*. The synthesis of this compound was carried out as described for [^{35}S]adenosine 5'-(*O*-1-thiotriphosphate) except that phosphate was added to the activated [^{35}S]adenosine 5'-phosphorothioate instead of pyrophosphate: yield 1410 A_{260} units (0.94 mmol, 18%); for ^{31}P NMR spectrum, see Table I.

Adenosine 5'-(O-2-Thiotriphosphate) (*ATPβS*). Adenosine 5'-(*O*-2-thiodiphosphate (1.5 mmol; Goody and Eckstein, 1971) was converted to its pyridinium salt by passage over Merck I ion exchanger (pyridinium form) in methanol–water (1:1, v/v), and the solution was evaporated to dryness using a rotary evaporator. Tri-*n*-octylamine (1.3 ml, ca. 3 mmol) and methanol (10 ml) were added to the residue, and the mixture was stirred until solution was obtained (ca. 30 min). After removal of solvent under reduced pressure, the residue was dissolved in dry pyridine (10 ml) and evaporated to dryness on a rotary evaporator using an oil pump. This process was repeated three times.

β-Cyanoethyl phosphate (Ba^{2+} salt, 854 mg, 3 mmol) was converted to its mono(tri-*n*-octylammonium) salt in a similar way to that described above, using 3 mmol of tri-*n*-octylamine. The salt was dried by repeated addition and reevaporation of dry dimethylformamide (10 ml) and then dissolved in dry dioxane (15 ml). Diphenyl phosphorochloridate (0.9 ml, 4.5 mmol) and tri-*n*-butylamine (0.45 ml) were added, and the solution was allowed to stand at room temperature for 3 h. After removal of dioxane under reduced pressure, ether (30 ml) followed by petroleum ether (60 ml; 60–80 °C) was added and, after shaking for a few minutes, the mixture was allowed to stand for 15 min in ice. The ether was then removed by decantation and the residue dissolved in dry dioxane (5 ml) which was then removed by evaporation under reduced pressure, and to the residue was added the ADPβS tri-*n*-octylammonium salt, prepared as described above dissolved in a mixture of dry hexamethyl phosphorotriamidate (4 ml) and dry pyridine (4 ml). The resulting solution was allowed to stand for 3 h at room temperature, and pyridine was then removed under reduced pressure. The remaining mixture was treated with 0.5 N sodium hydroxide (100 ml) for 1 h at room temperature, after which time the solution was neutralized by addition of Merck I ion exchanger (pyridinium form). The neutralized solution was applied to a column of DE-52-cellulose (ca. 50 × 4 cm) which was eluted with a linear gradient of triethylammonium bicarbonate (0.1–0.35 M, 2 × 2 l.). The product was eluted at about 0.25 M: yield 2750 A_{260} units (12%); for ^{31}P NMR spectrum, see Table I. This was slightly contaminated with ADPβS. A further 800 A_{260} units of product were obtained, more heavily contaminated with ADPβS. Pure ATPβS could be obtained by chromatography on DEAE-Sephadex A-25, using a gradient of triethylammonium bicarbonate (0.2–0.5 M) at 4 °C. The product behaved identically with regard to TLC on PEI-cellulose with ATPβS synthesized using pyruvate kinase.

Chemical Evidence for the Structure of ATPβS. (1) Reaction with Methyl Iodide. ATPβS (2 A_{260} units, chemically synthesized) in methanol (10 μl) was treated with 1 μl of methyl iodide. PEI-cellulose TLC showed progressive conversion to a product which moved much faster than ATPβS (R_f 0.75, ATPβS, R_f 0.17 in 0.75 M KH_2PO_4, pH 3.5). After 100 min, no residual ATPβS was detectable.

In a parallel experiment under identical conditions using ATPβS obtained from the pyruvate kinase reaction (see below), a compound having the same R_f as that obtained with chemically synthesized ATPβS was obtained. The reaction was judged qualitatively to occur at the same rate.

(2) Reaction with Ellman's Reagent. The reaction of Ellman's reagent with ATPβS was investigated in a manner similar to that described previously for other nucleoside thiophosphates (Goody and Eckstein, 1971). It was found that in pH 7.6 Tris-HCl buffer (0.1 M), ATPβS (both chemically and enzymatically prepared) reacted to a very small extent. Under the standard conditions used (ca. 0.5–0.6 A_{260} unit of ATPβS in 1 ml of buffer plus 250 μl of a

[1] The following abbreviations are used: AMPS, adenosine 5'-phosphorothioate; ATPαS, adenosine 5'-(*O*-1-thiotriphosphate); ADPαS, adenosine 5'-(*O*-1-thiodiphosphate); ATPβS, adenosine 5'-(*O*-2-thiotriphosphate); ADPβS, adenosine 5'-(*O*-2-thiodiphosphate); APCPP, adenosine 5'-(α,β-methylene)triphosphate.

solution of Ellman's reagent in the same buffer, saturated at 4 °C), there was little or no reaction. A small amount of yellow color was produced with some preparations of ATPβS which seemed to be due to ADPβS impurity. However, on addition of snake venom phosphodiesterase to the solution after addition of Ellman's reagent, under the conditions described, ca. 0.63 equiv of colored anion was produced with both chemically and enzymatically synthesized ATPβS. A similar effect was seen if alkaline phosphatase was used instead of phosphodiesterase, with the release of ca. 0.6 equiv of anion. Addition of both enzymes resulted in the release of 1.85 equiv in the case of chemically synthesized ATPβS and 1.9 equiv for the enzymatically synthesized product.

Enzymatic Degradations of ATPβS (Chemically Synthesized). (1) Approximately 5 A_{260} units of chemically synthesized ATPβS in 50 μl of 0.1 M Tris-HCl, pH 8.0, was treated with ca. 5 μg of alkaline phosphatase. After 30 min at room temperature, TLC on PEI-cellulose in 0.75 M KH_2PO_4 adjusted to pH 3.5 with concentrated HCl showed complete degradation to ADPβS.

(2) Approximately 5 A_{260} units of ATPβS in 5 μl of 0.1 M Tris-HCl, pH 8.5, was treated with snake venom phosphodiesterase (ca. 5 μg). After 1 h at room temperature, TLC on PEI-cellulose showed complete degradation to AMP.

(3) 3 A_{260} units of ATPβS was treated with 60 μg of myosin in a solution of total volume 50 μl containing 2 mM $CaCl_2$, 8 mM imidazole·HCl (pH 7.0) and 5 mM dithiothreitol. After 3 h at room temperature, TLC on PEI-cellulose in 0.75 M KH_2PO_4 (pH 3.5) showed that ca. 50% degradation to ADPβS had occurred. After 24 h no further degradation could be detected under these conditions.

Enzymatic Syntheses of Diastereomers

[^{35}S]Adenosine 5′-(O-1-Thiotriphosphate) (Isomer A). (a) Pyruvate Kinase. To an incubation mixture (total volume 17.15 ml) of 1.36 mM [^{35}S]ADPαS (chemically synthesized mixture of isomers A and B, 395 150 cpm/A_{260} unit), 3.65 mM $MgCl_2$, 0.85 mM dithiothreitol, 380 mM KCl, 38 mM Tris-HCl, pH 8.0, 2.0 mM phosphoenolpyruvate (K^+ salt), 2.40 mM NADH, and lactate dehydrogenase (200 μl) was added pyruvate kinase (100 μl). An aliquot of the reaction solution was placed in a cuvette (path length 1 mm) and the reaction followed at 340 nm at 22 °C. After 2 h, ca. 11 μmol of NADH had been oxidized and the reaction solution was chromatographed on a DEAE-Sephadex A-25 column (1.5 × 15 cm) with a linear gradient of 500 ml of each 0.1 M and 0.6 M triethylammonium bicarbonate. [^{35}S]ATPαS was eluted at 0.5 M and [^{35}S]ADPαS at 0.42 M buffer. The fractions were evaporated in vacuo and the buffer was removed by repeated (two times) evaporation with methanol, yield: [^{35}S]ATPαS, 150 A_{260} units (40%, 408 443 cpm/A_{260} unit); [^{35}S]ADPαS, 250 A_{260} units (46%, 395 203 cpm/A_{260} unit).

(b) Acetate Kinase. To an incubation mixture (total volume 1.3 ml) of 17 mM [^{35}S]ADPαS (mixture of isomers), 12 mM $MgCl_2$, 1.5 mM dithiothreitol, 4 mM Tris, pH 8.0, and 19 mM acetyl phosphate was added 1 μl of acetate kinase. The reaction was followed by TLC of 3-μl aliquots on PEI-cellulose following the disappearance of [^{35}S]ADPαS. After 40 min, about 43% of the starting material had been used up and the reaction was chromatographed on a DEAE-Sephadex column as described under (a), yield: 130 A_{260} units of [^{35}S]ATPαS and 180 A_{260} units of [^{35}S]ADPαS. Degradation with myokinase (Figure 1) showed this material to be the A isomer.

[^{35}S]Adenosine 5′-(O-1-Thiotriphosphate (Isomer B). (a) Myokinase. To an incubation mixture (total volume 5.25 ml) of 2.9 mM [^{35}S]ATPαS (mixture of isomers, 398 991 cpm/A_{260} unit), 5.8 mM AMP, 10 mM dithiothreitol, and 60 mM Tris-HCl, pH 8.0, was added myokinase (100 μl, dialyzed against 10 mM Tris-HCl, pH 8.0, for 2 h prior to use). Incubation temperature was 22 °C. The reaction was followed by TLC of 3-μl aliquots on PEI-cellulose. The disappearance of [^{35}S]ATPαS was monitored by measuring the radioactivity on the ATPαS spot as described in Methods. After 100 min, about 50% of [^{35}S]ATPαS had disappeared and the reaction solution was chromatographed on a DEAE-Sephadex column as described for isomer A, yield: 110 A_{260} units of [^{35}S]ATPαS (48%); 60 A_{260} units of [^{35}S]ADPαS (26%).

(b) Creatine Kinase. To an incubation mixture (total volume 6.4 ml) of 3.5 mM [^{35}S]ADPαS (mixture of isomers), 15 mM Tris-HCl, pH 8.9, 0.75 mM dithiothreitol, 1.5 mM $MgCl_2$, and 4.5 mM creatine phosphate was added 75 μl creatine kinase (10 mg/ml). The reaction was followed by TLC of 3-μl aliquots on PEI-cellulose following the disappearance of [^{35}S]ADPαS. After 45 min, about 50% had reacted and the reaction was chromatographed on a DEAE-Sephadex column as described for isomer A, yield: 80 A_{260} units of [^{35}S]ATPαS and 144 A_{260} units of [^{35}S]ADPαS. Degradation with myokinase (Figure 1) showed this material to be the B isomer.

(c) Arginine Kinase. To an incubation mixture (total volume 5 ml) of 2 mM [^{35}S]ADPαS (mixture of isomers), 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 20 mM $MgCl_2$, and 14 mM arginine phosphate was added 230 U of arginine kinase. The reaction was followed by TLC on PEI-cellulose. After incubation at 30 °C for 3 h the reaction was chromatographed on DEAE-Sephadex as described for isomer A, yield: 80 A_{260} units of [^{35}S]ADPβS and 80 A_{260} units of [^{35}S]ATPβS. Degradation with myokinase showed the latter to be isomer B (Figure 3).

Adenosine 5′-(O-2-Thiotriphosphate) (Isomer A). (a) Pyruvate Kinase. Conditions as described for isomer A of ATPαS using ADPβS (1.9 mM) instead of ADPαS. After approximately 4 h, the reaction solution was chromatographed on a DEAE-Sephadex A-25 column, yield: ATPβS, 310 A_{260} units (61%). The compound was identical by TLC with the chemically prepared material.

Adenosine 5′-(O-2-Thiotriphosphate) (Isomer B). (a) With Myosin. To an incubation mixture (total volume 1.6 ml) of 6.75 mM ATPβS (mixture of isomers), 3.12 mM KCl, 3.12 mM $CaCl_2$, and 6.25 mM imidazole-HCl buffer, pH 7.0, was added myosin (6 mg/ml, 200 μl). The reaction was followed by TLC on PEI-cellulose. After incubation for 2 h at 37 °C, the reaction solution was chromatographed on a DEAE-Sephadex column as described for isomer A. ATPβS was eluted at 0.41 M and ADPβS at 0.35 M buffer, yield: ATPβS, 82 A_{260} units (51%); ADPβS, 67 A_{260} units (42%(/ Reincubation of this ATPβS under identical conditions led to 8% cleavage to ADPβS.

(b) With Acetate Kinase. To an incubation mixture (total volume 7.00 ml) of 3.6 mM ADPβS, 7.2 mM $MgCl_2$, 72 mM Tris-HCl, pH 8.0, 0.55 mM dithiothreitol, and 28.5 mM acetate phosphate was added acetate kinase (500 μl). The reaction was followed by TLC on PEI-cellulose. After completion of the reaction (approximately 4 h), the reaction mixture was chromatographed on a DEAE-Sephadex col-

Table I: ^{31}P NMR Spectra of Thiophosphate Analogues.[a]

	α-Phosphorus	β-Phosphorus	γ-Phosphorus
ATP	+10.3 (d)	+20.0 (t)	+4.80 (d)
ATPαS	−42.40 (d)	+22.81 (t)	+6.09 (d)
ATPβS	+11.54 (d)	−29.48 (t)	+5.98 (d)
ADP	+10.58 (d)	+5.77 (d)	
ADPβS[b]	+11.45 (d)	−33.02 (d)	
ATPγS[b]	+11.0 (d)	+22.8 (t)	−33.3 (d)

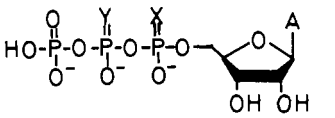
[a] Shift in δ (ppm) at pH ~10. [b] Prepared according to Goody and Eckstein (1971).

umn as described for isomer A, yield: ATPβS 290 A_{260} units (77%). The compound was identical by TLC with samples prepared chemically or by pyruvate kinase. It was not degraded by myosin under conditions described under (a).

(c) Mixture of Isomers of ATPβS with Creatine Kinase. To an incubation mixture (total volume 5.2 ml) of 5 mM ADPβS, 11 mM Tris-HCl, pH 8.9, 14 mM $MgCl_2$, 3 mM dithiothreitol, and 1 mM creatine phosphate was added 900 μl of creatine kinase (10 mg/250 μl). The reaction was followed by chromatography on PEI-cellulose. After 1.5 h at room temperature and further 2 h at 32 °C, the reaction solution was chromatographed on DEAE-Sephadex column as described for isomer A: yield 180 A_{260} units of ATPβS. This material was degraded to the extent of 70% by myosin indicating a 7:3 mixture of isomer A–isomer B.

Results

The synthesis of ADPαS and ATPαS by activation of AMPS with diphenyl phosphorochloridate and subsequent reaction with pyrophosphoric or phosphoric acid, respectively, yielded cleaner products and was more efficient than the method described earlier (Eckstein and Gindl, 1970). Attempts to synthesize ATPβS in a similar way by activation of ADPβS (Goody and Eckstein, 1971) with diphenyl phosphorochloridate and reaction with phosphoric acid failed. However, following a reaction scheme designed for



X = S, Y = O : $ATP_{\alpha}S$

X = O, Y = S : $ATP_{\beta}S$

the synthesis of ATPγS (Goody and Eckstein, 1971), activation of β-cyanoethyl phosphate and reaction with ADPβS led to the formation of ATPβS. In Table I the chemical shifts in the ^{31}P NMR of the various thiophosphate analogues are compared with those of ATP and ADP. It is clearly seen that the exchange of an oxygen by a sulfur results in a large downfield shift of that particular phosphate group. The ^{31}P NMR can, therefore, be taken as an additional analytical method for the localization of the thiophosphate group.

Incubation of the mixture of isomers of ATPαS with myokinase led to about 50% degradation (Figure 1). The remaining ATPαS was thought to be one isomer and was arbitrarily designated as isomer B. To obtain isomer A, phosphoryl transfer with pyruvate kinase on ADPαS was

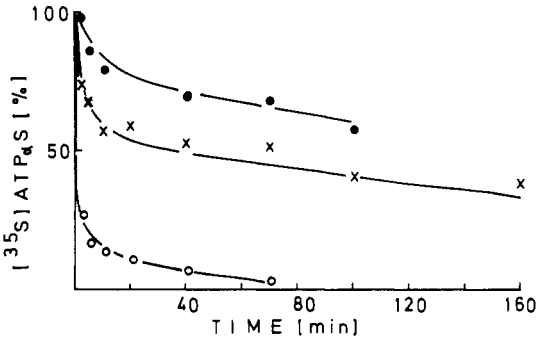


FIGURE 1: Degradation of [^{35}S]ATPαS isomers by myokinase. The incubation mixture (total volume 125 μl) contained 6 mM AMP, 8 mM dithiothreitol, 80 mM Tris-HCl, pH 8.0, 2.8 mM ATP analogue, and myokinase (1.7 mg/ml, 5 μl). Incubation temperature was 22 °C. Aliquots of 3 μl were chromatographed on PEI-cellulose and the uv-active spots corresponding to unreacted [^{35}S]ATP analogue cut out and counted as described in Materials and Methods. (●) ATPαS B; (○) ATPαS A; (X) ATPαS, mixture of isomers from chemical synthesis.

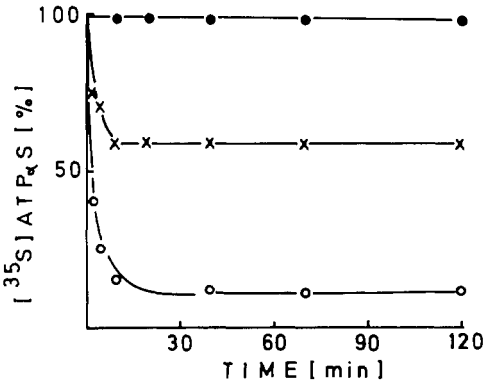


FIGURE 2: Phosphoryl transfer from [^{35}S]ATPαS isomers by nucleoside diphosphokinase. The incubation mixture (total volume 65 μl) contained 30 mM Tris-HCl, pH 8.0, 3 mM dithiothreitol, 2.3 mM $MgCl_2$, 15.25 mM GDP, 3.25 mM ATP analogue, and nucleoside diphosphokinase (5 mg/ml, 3 μl). Incubation temperature was 22 °C. Aliquots of 3 μl were chromatographed and the uv-active spots corresponding to unreacted [^{35}S]ATP analogue cut out and counted as described in Materials and Methods. (●) ATPαS B; (○) ATPαS A; (X) ATPαS, mixture of isomers from chemical synthesis.

tried. At most about 45% of ADPαS could be phosphorylated. The isolated ATPαS was readily degraded by myokinase and, therefore, thought to be isomer A (Figure 1). ATPαS obtained from ADPαS by reaction with acetate kinase was degraded by myokinase with the same rate as the A isomer of ATPαS, whereas the ATPαS produced by creatine kinase was as resistant as isomer B. A similar reaction pattern for the isolated isomers and their mixture was observed with nucleoside diphosphate kinase (Figure 2). The interaction of the isomers of ADPαS with pyruvate kinase was studied in more detail (Figure 3). The K_m value for isomer A of ADPαS was 1.0×10^{-3} M and V_{max} = 1.6 μmol min^{-1} mg^{-1} of protein. Under the same conditions, the values for ADP were $K_m = 3.3 \times 10^{-4}$ M and V_{max} = 50 μmol min^{-1} mg^{-1} of protein. The isomer B of ADPαS did not react but was a competitive inhibitor with $K_i = 1.5 \times 10^{-3}$ M (Figure 3).

Incubation of the mixture of isomers of ATPβS with myosin led to about 50% degradation. The reisolated ATPβS was arbitrarily named isomer B. To obtain the isomers separately, we tried three different enzymatic phosphoryl transfer reactions with ADPβS. The products of the pyruvate kinase as well as the creatine kinase reactions were

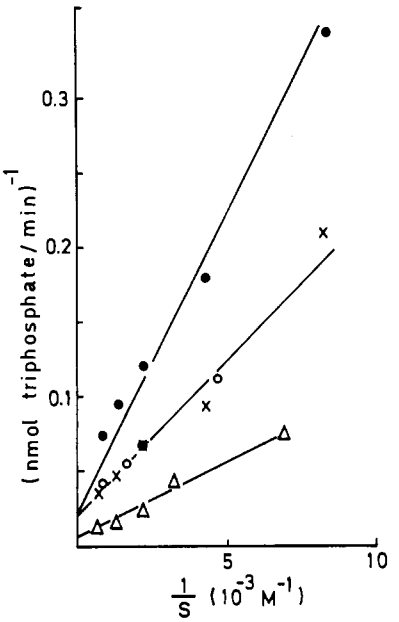


FIGURE 3: Pyruvate kinase reaction with isomers of ADPαS and ADPβS. The incubation mixtures (total volume 1.0 ml) contained 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, 0.16 mM phosphoenolpyruvate, 50 mM Tris-HCl, pH 8.0, 2.4 mM NADH, 10 μl of lactate dehydrogenase, and 30 mg of pyruvate kinase unless stated otherwise. (X) ADPαS A (from creatine kinase reaction); (O) ADPαS A (from myokinase reaction); (●) ADPαS A and ADPαS B (1.5 mM); (Δ) ADPβS with 7 mg of pyruvate kinase.

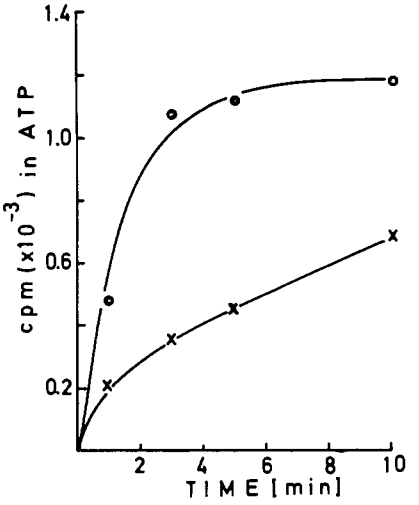


FIGURE 4: Phosphoryl transfer from ATPβS isomers by nucleoside diphosphate kinase. The reaction mixture (total volume 30 μl) contained ATPβS (2.7 mM), [^{14}C]ADP (8.4 mM), $MgCl_2$ (8.4 mM), dithiothreitol (7 mM), Tris-HCl, pH 8.0 (70 mM), and nucleoside diphosphate kinase (5 μl). Samples of 3 μl were applied at timed intervals to PEI-cellulose TLC plates, which were developed in 0.75 M KH_2PO_4 (pH 3.5). The areas corresponding to ATP were cut out and eluted in 1 N HCl (1 ml) for 30 min, and 0.5 ml of this solution was counted in 10 ml of Aquasol in the scintillation counter. ATPβS B (O); ATPβS A (X).

readily completely degraded by myosin as shown by TLC on PEI-cellulose and, therefore, thought to be isomer A. Under conditions as described in Figure 3 for the pyruvate kinase reaction, the K_m value for ADPβS was 2 mM and V_{max} = 24 μmol min^{-1} mg^{-1} of protein. Isomer B which was not degraded by myosin was obtained enzymatically by the reaction of ADPβS with acetate kinase.

Isomer B is a competitive inhibitor of myosin. Its inhibition constant for the S_1 fragment of myosin was measured with [γ-^{32}P]-α,β-methylene adenosine 5′-triphosphate (gift from H.-G. Mannherz, Heidelberg) as substrate. The reaction products were separated by chromatography on PEI-cellulose. The decrease in substrate as well as the increase in [^{32}P]phosphate was measured by PEI-cellulose TLC as described in Methods. Under the conditions used (40 mM imidazole-HCl, pH 7.0, 10 mM $MgCl_2$), the K_m for APCPP was determined from a Lineweaver–Burk plot to be 3.9×10^{-5} M, the K_i for ATPβS (isomer B) to be 7.6×10^{-5} M.

The rate of reaction of the two isomers of ATPβS with ADP in the presence of nucleoside diphosphate kinase was different (Figure 4), isomer B being the more reactive isomer.

Qualitatively similar results were obtained in the presence of GDP as judged by TLC on PEI-cellulose.

However, in the myokinase-catalyzed reaction in the presence of AMP, no difference in rate between the two isomers of ATPβS could be detected either by TLC on PEI-cellulose (2.7 mM ATPβS, 6 mM AMP) or in the coupled enzyme assay system involving pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, and NADH (6×10^{-5} M ATPβS, 1 mM AMP).

Discussion

Nucleoside phosphorothioate esters exist in form of pairs of diastereomers (for a review, see Eckstein, 1975). For nucleoside 2′,3′- and nucleoside 3′,5′-cyclic phosphorothioates, these diastereomers have either been separated (Saenger and Eckstein, 1970) or demonstrated by ^{31}P NMR (Eckstein et al., 1972; Eckstein et al., 1975). Particularly the isomers of uridine 2′,3′-cyclic phosphorothioate have been useful in elucidating some stereochemical aspects of the mechanism of RNase A (Saenger et al., 1974). In ATP and ADP the replacement at the phosphate side chain of an oxygen not involved in an ester bond and not on the terminal phosphate should also lead to the existence of diastereomers. Not unexpectedly, attempts to crystallize ATPαS, ADPαS, or ATPβS and to separate the diastereomers by fractional crystallization failed. However, the results with nucleoside 2′,3′-cyclic phosphorothioates and RNase A (Eckstein, 1968) as well as RNase T_1 (Eckstein and Gindl, 1970), where one diastereomer is either a better or the exclusive substrate, led us to try the separation of these diastereomers by enzymatic methods. A first indication of a difference in reactivity of ATPαS with kinases was obtained by the biphasic reaction of the chemically synthesized ATPαS with myokinase (Figure 1) and nucleoside diphosphate kinase (Figure 2) where about 50% reacted markedly faster than the rest.

A similar picture was obtained with myosin and ATPβS. The chemical synthesis of ATPαS, ADPαS, and ATPβS is expected to yield a mixture of approximately 1:1 of the diastereomers. A fast reaction of about 50% of the chemically synthesized ATPαS and ATPβS in these enzymatic reactions, therefore, suggests that one diastereomer reacts faster than the other. This suggestion is substantiated by the finding that ATPαS and ATPβS synthesized by pyruvate kinase are completely degraded whereas material synthesized by creatine (ATPαS) or acetate kinase (ATPβS) was hydrolyzed extremely slowly. These results taken together are probably sufficient indication for the presence and separation of these isomers. Support for this contention also comes from high-pressure liquid chromatography of the chemically synthesized ADPαS (Stahl et al., 1974) where two peaks with a ratio of about 48:52 can be identified, of which only

Table II: Stereospecificity of Kinases.[a]

Enzyme	ATPαS A	ATPαS B	ATPβS A	ATPβS B
Pyruvate kinase	+	−	+	−
Acetate kinase	+	*b*	−	+
Creatine kinase	*b*	+	+ (70%)	+ (30%)
Arginine kinase		+	ND	ND
Myokinase	+	*b*	+	+
Myosin	+	+	+	−
Nucleoside diphosphate kinase	+	−	ND	ND

[a] Conditions as described in Experimental Section. [b] With higher enzyme concentrations, formation or reaction of this isomer is also observed. ND means not determined.

one reacts readily with hexokinase. Attempts to distinguish between these diasteromers by NMR have failed so far. In Table II the stereospecificities of various kinases with respect to ATPαS and ATPβS are summarized qualitatively. This stereospecificity is not absolute for all enzymes. There are cases like acetate kinase and creatine kinase where at higher enzyme concentrations both isomers of ATPαS are formed. Here the difference in reaction rate between the diastereomers is not as pronounced as in, e.g., pyruvate kinase, where we could not see any reaction of the other isomer. For pyruvate kinase we have values for the Michaelis constants of both isomers of ADPαS. As shown under Results, isomer A has K_m = 1 mM and isomer B K_i = 1.5 mM. Both isomers of ATPαS also exhibit the same K_i value (1.3×10^{-4} M) in the inhibition of the ATP reaction with acetate kinase ($K_m = 3 \times 10^{-4}$ M). The apparent affinity of both isomers, at least for pyruvate and acetate kinase, is thus comparable and the inertness of one isomer is not due to weak binding but to the slowdown of a step following binding. Since it was conceivable that the isomers showed different affinities for the divalent metal ion necessary for these kinase reactions, we investigated the binding of Mn^{2+} by proton relaxation NMR (Stehlik, Goody, and Eckstein, unpublished). These experiments with ATPαS, ADPαS, ADPβS, and ATPβS showed not only that there was no significant difference in affinity between two isomers of a given compound but also that the analogues had affinities similar to those of the parent compounds (K_D for triphosphates 1.5–3 $\times 10^{-5}$ M, for diphosphates 1.5–3 $\times 10^{-4}$ M at pH 8.0). From the x-ray structural analysis of uridine 2′,3′-cyclic phosphorothioate (Saenger and Eckstein, 1970), as well as the Mg^{2+} salt of a pyridinium complex of diethyl phosphorothioate (Schwalbe et al., 1973), it is known that, at least in these crystals, these cations are associated with the oxygen and not the sulfur of the phosphorothioate.

This might help in understanding the different reactivities of the diastereomers with various kinases. Mg^{2+} forms a complex with ADP by interacting with the α- and β-phosphate (Cohn and Hughes, 1960). Although Mn^{2+} was shown also to interact via a water molecule with the adenine ring (Mildvan, 1970), such an interaction has so far not been demonstrated with Mg^{2+} but it cannot be excluded. For a number of kinases it has been shown that the nucleotide substrate is bound to the enzyme as such a metal complex. If the complexes of ADPαS with Mg^{2+} are formed by only involving an oxygen and not the sulfur of the α-phosphate group as suggested by the x-ray results, the positioning in space of the β-phosphate for the two isomers is quite different (Figure 5). In the case of the isomer with the *S* configuration, the β-phosphate points toward the 3′-carbon, whereas this position is taken by the α-phosphate for the isomer with the *R* configuration. Space-filling models show that the spatial requirements for these two complexes are similar. Depending on where the phosphate of the second substrate is situated, one of these two complexes will have a more favorable positioning of the β-phosphate for the acceptance of the phosphate of the second substrate to form ATPαS.

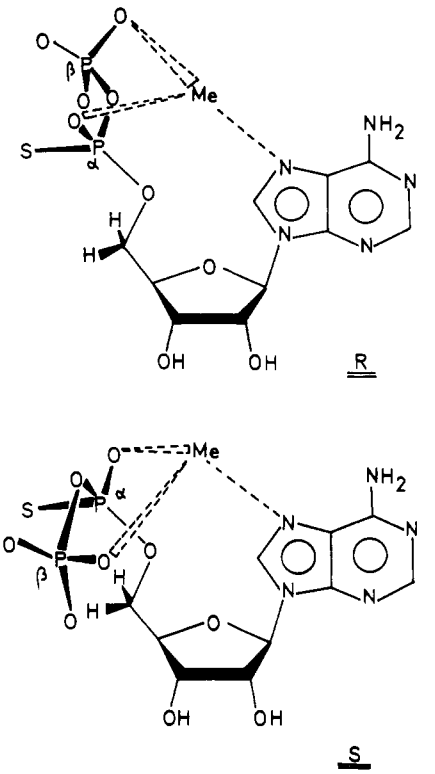


FIGURE 5: Structures of metal complexes of isomers of ADPαS.

An analogous explanation can be given for the stereospecific reactions with ADPβS which itself is not diastereomeric. If the sulfur of the β-phosphate is not involved in complex formation with the metal, two structures for such a complex can be drawn depending on which oxygen of the β-phosphate will chelate with the metal (Figure 6). Again depending on the positioning of the phosphate of the second substrate, one complex will be more reactive than the other leading to formation of one of the isomers of ATPβS. Since for ADPβS the two complexes are in equilibrium, a given enzyme can turn over all ADPβS to produce one isomer of ATPβS stereospecifically.

Some of the kinases discussed here form enzyme–substrate–metal complexes (creatine kinase, arginine kinase, myokinase), whereas pyruvate kinase forms an enzyme–metal–substrate complex (Mildvan, 1970). For the other enzymes mentioned (acetate kinase, nucleoside diphosphokinase, myosin), the type of ternary complex has not yet been analyzed. For the first class the interaction of the nucleotide–metal complex with the enzyme is well established but, for the pyruvate kinase, the role of the metal in binding the nucleotide is not clear as yet. However, the explanation given for the different reactivities of the isomers of ADPαS is not restricted to the formation of a nucleotide–metal

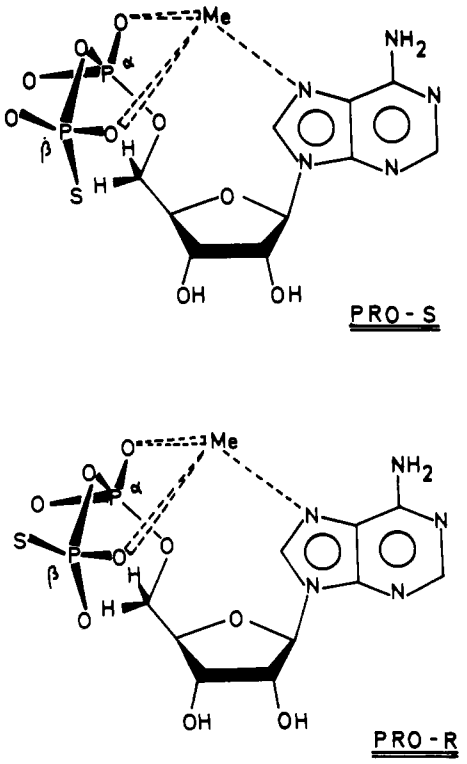


FIGURE 6: Structures of metal complexes of ADPβS.

complex. The role of the metal could also be fulfilled by positively charged groups of the protein. The argument would basically be the same.

Unfortunately, since not even the detailed structures of the Mg^{2+} or Mn^{2+} complexes of ATP or ADP are available, this argument has to be rather schematic.

The knowledge of the absolute configuration of the isomers would be of considerable interest since it would allow one to describe the stereochemistry of phosphate transfer (ATPβS) or of pyrophosphate transfer reaction (ATPαS) in absolute terms. We hope that with the availability of single isomers it might now be possible to crystallize one of them and to obtain the absolute configuration by x-ray diffraction. Until these configurations are known, these compounds can be useful in establishing certain relationships between, e.g., kinases, and they can be used to study some stereochemical aspects of certain enzymes at the α- or β-phosphate group at least in relative terms.

Acknowledgment

The authors thank Miss G. Witzel and Miss M. Kettler for expert technical assistance and B. Seeger for recording the NMR spectra.

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