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S-Methylated Nucleoside Phosphorothioates as Probes of Enzyme Metal·Nucleotide Binding Sites†

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ABSTRACT: The S-methylated derivatives of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαSCH$_3$) have been prepared by the reaction of both diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) with methyl iodide. At physiological pH ATPαSCH$_3$ was unstable, decomposing predominantly to adenosine 5′-*O*-(*S*-methyl thiophosphate) (AMPSCH$_3$) and pyrophosphate. A minor degradation pathway also yielded ATP and methyl mercaptan. Greatly enhanced stability was observed at lower pH. The S_P diastereomer of ATPαSCH$_3$ was a substrate for hexokinase and acetate kinase, and both diastereomers were active with fructose-6-phosphate kinase. The products of these reactions were the appropriate sugar or acyl phosphate, AMPSCH$_3$, and inorganic phosphate, the latter two species arising from the breakdown of the transient intermediate 5′-*O*-(*S*-methyl 1-thiodiphosphate) (ADPαSCH$_3$). No measurable substrate activities were observed with creatine and phosphoglycerate kinase. These results are interpreted as meaning that creatine and phosphoglycerate kinase require Mg^{2+} coordination to the α-phosphate group during the enzyme-catalyzed reaction whereas the other three enzymes do not. Attempts to prepare adenosine 5′-*O*-(*S*-methyl 2-thiotriphosphate) (ATPβSCH$_3$) and ADPαSCH$_3$ by similar methods were unsuccessful with adenosine 5′-*O*-(*S*-methyl 2-thiodiphosphate) (ADPβS) and AMPSCH$_3$ being respectively isolated as the major products.

The determination of the structure of the metal·nucleotide complex active in a particular enzyme-catalyzed reaction has attracted a great deal of attention [see review, Eckstein (1980)]. One approach to this problem uses the diastereomers of ATPαS[1] and ATPβS in combination with hard and soft metal ions. This method relies on the preferential coordination of Mg^{2+} to oxygen and Cd^{2+} to sulfur in the phosphorothioate analogues (Jaffe & Cohn, 1978a, 1979). These authors found that only Mg·ATPβS B and Cd·ATPβS A showed appreciable activity with hexokinase. This reversal of diastereomeric selectivity arises because Mg·ATPβS B (Mg^{2+} to oxygen binding) and Cd·ATPβS A (Cd^{2+} to sulfur binding) have identical metal·nucleotide chelate structures and provides excellent evidence for Mg^{2+} coordination to the β-phosphate group of ATP during the hexokinase-catalyzed reaction. A lack of reversal in diastereomeric stereoselectivity (as was in fact observed with the ATPαS isomers) is most simply interpreted by a lack of metal coordination to the phosphate group under study although such an interpretation is by no means conclusive. For instance, within the constraints imposed at the active site of enzymes Mg^{2+} could be forced to bind to sulfur or Cd^{2+} to oxygen, leading to a lack of reversal of isomeric specificity even though metal ion binding to the phosphate group occurs. Many enzymes have now been studied by this technique, and although all combinations of reversal or lack of it have been observed, the most common pattern seems to be a reversal of stereoselectivity with the ATPβS diastereomers coupled with no reversal at the α phosphorus. This has been observed with all the nucleotidyl transferases so far tested [DNA polymerase (Burgers & Eckstein, 1979), RNA polymerase (Armstrong et al., 1979), phenylalanyl-tRNA synthetase (Connolly et al., 1980), methionyl-tRNA synthetase (Smith & Cohn, 1982)] and about half of the phosphoryl transferases thus studied [hexokinase (Jaffe & Cohn, 1979), myosin (Connolly & Eckstein, 1981), acetate kinase (Romaniuk & Eckstein, 1981)]. These results are usually interpreted as meaning that the β,γ-bidentate metal·nucleotide chelate is the active one in these enzyme-catalyzed reactions. This conclusion is based on the simple interpretation that lack of reversal at the α-phosphate group means no metal ion co-

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[1] Abbreviations: ATPαS A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); ATPαSCH$_3$ A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(*S*-methyl 1-thiotriphosphate); ADPαS A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ADPαSCH$_3$ A and B, the S_P and R_P diastereomers of adenosine 5′-*O*-(*S*-methyl 1-thiodiphosphate); ATPβSCH$_3$, adenosine 5′-*O*-(*S*-methyl 2-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ADPβSCH$_3$, adenosine 5′-*O*-(*S*-methyl 2-thiodiphosphate); AMPS, adenosine 5′-*O*-(thiophosphate); AMPSCH$_3$, adenosine 5′-*O*-(*S*-methyl thiophosphate); N^1-CH$_3$AMPS, N^1-methyladenosine 5′-*O*-(thiophosphate); N^1-CH$_3$AMPSCH$_3$, N^1-methyladenosine 5′-*O*-(*S*-methyl thiophosphate); TEAB, triethylammonium bicarbonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NAD, nicotinamide adenine dinucleotide; EPR, electron paramagnetic resonance.

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ordination at this locus and also on the much greater thermodynamic stability of β,γ-bidentate metal·nucleotide complexes as compared to β-monodentate ones. In order to try to strengthen this conclusion, we have prepared the two diastereomers of adenosine 5′-*O*-(*S*-methyl 1-thiotriphosphate) (ATPαSCH$_3$). These compounds are triesterified at the α-phosphate group with the two nonbridging oxygen atoms present in ATP replaced by a P–SCH$_3$ and a P=O moiety. Due to triesterification, the P=O grouping is unable to form a resonance P–O$^-$ species. We have assumed that metal ions will bind extremely weakly, if at all, to the α-phosphate group in these derivatives. This means that enzymes that require metal coordination to the α-phosphate group for activity should not use these analogues as substrates. Conversely, enzymatic activity would argue against metal binding to this phosphate group. Clearly, experiments of this kind can help in the interpretation of results obtained with the normal phosphorothioate analogues, particularly when the results seen with the ATPαS diastereomers are independent of the nature of the metal ion. Thus, this paper describes the synthesis and chemical properties of ATPαSCH$_3$ A and B and also their interaction with five kinases.

Materials and Methods

Acetate kinase (*Escherichia coli*, 5 mg/mL, 200 units/mg), aldolase (rabbit muscle, 10 mg/mL, 9 units/mg), creatine kinase (rabbit muscle, 380 units/mg, lyophilized powder), fructose-6-phosphate kinase (rabbit muscle, 10 mg/mL, 60 units/mg), glucose-6-phosphate dehydrogenase (yeast, 5 mg/mL, 350 units/mg), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 10 mg/mL, 80 units/mg), glycerol-3-phosphate dehydrogenase (rabbit muscle, 10 mg/mL, 170 units/mg), hexokinase (yeast, 140 units/mg, lyophilized powder), phosphoglycerate kinase (yeast, 10 mg/mL, 450 units/mg), and triosephosphate isomerase (yeast, 10 mg/mL, 10000 units/mg) were obtained from Boehringer Mannheim (Mannheim, West Germany). These enzymes were dialyzed, at 4 °C, against two changes of 2 L of 10 mM K-Hepes, pH 7, containing 1 mM EDTA and 0.5 mM dithiothreitol prior to use. ADPβS was purchased from Boehringer Mannheim and purified by chromatography over a DEAE-Sephadex A-25 (20 × 2 cm) column with gradient elution of 750 mL each of 0.1 M and 0.5 M TEAB, pH 8. ATP was obtained from Pharma-Waldhof (Düsseldorf, West Germany) and N^1-methyladenosine from Sigma (München, West Germany). Methyl iodide was purchased from Merck (Darmstadt, West Germany). ODS-Hypersil (5-μm particle size) was obtained from Shandon Southern Products Ltd. (Runcorn, England), and Nucleosil 10SB (10-μm particle size) was the product of Macherey-Nagel (Düren, West Germany). All other enzyme substrates, buffer substances, and general reagents were the best quality available from Boehringer Mannheim, Merck, or Serva (Heidelberg, West Germany) and were used as supplied.

All HPLC experiments were performed with a Waters Associates (Model 6000 A) liquid chromatograph fitted with a 254-nm absorbance detector (Model 440). For the purification of ATPαSCH$_3$ A or B (10 μmol scale synthesis), a 24 × 0.6 cm column packed with ODS-Hypersil and eluted isocratically with 50 mM tetramethylammonium acetate, pH 4, containing 2% methanol was used. The flow rate was 2 mL/min and ATPαSCH$_3$ A and B had retention times of 23 and 18 min, respectively. For the purification of N^1-CH$_3$AMPSCH$_3$ (30 μmol scale synthesis), an identical column eluted with 5 mM potassium phosphate, pH 6, containing 2.5% methanol was used. At a flow rate of 2 mL/min this compound eluted after 20 min. For the analysis of ATPαSCH$_3$ (A or B) and AMPSCH$_3$ an ODS-Hypersil column eluted isocratically with 50 mM potassium phosphate, pH 6, containing 10% methanol was utilized. At a flow rate of 3 mL/min ATPαSCH$_3$ A, ATPαSCH$_3$ B, and AMPSCH$_3$ had retention times of 4, 3, and 8 min, respectively. ATP and ATPαS A or B were not retained on the ODS-Hypersil column with this buffer. For the determination of ATP and ATPαS A or B contamination in ATPαSCH$_3$ A or B preparations, the above system eluted isocratically with 50 mM potassium phosphate, pH 6, was used. At a flow rate of 2 mL/min ATP, ATPαS A, and ATPαS had retention times of 2.5, 4, and 5 min, respectively. This buffer, however, did not elute the S-methylated nucleotides, and thus for a complete analysis of ATPαSCH$_3$ A or B, both buffer systems had to be used. The 50 mM potassium phosphate pH 6 buffer was also used to resolve the substrates ATPαS A or B (retention times as above) from the products ADPαS A or B (retention times 5.5 and 6.5 min, respectively) in the experiments with acetate kinase. For the resolution of ATP from ADP in the studies with acetate and creatine kinase and also the separation of ATPαS A or B from ADPαS A or B with creatine kinase, a 20 × 0.6 cm column packed with Nucleosil 10SB and eluted isocratically at 4 mL/min with 200 mM potassium phosphate plus 300 mM potassium acetate, pH 4.5, was used. The observed retention times were as follows: ATP, 6 min; ADP, 4 min; ATPαS A, 10.5 min; ADPαS A, 5.5 min; ATPαS B, 12 min; ADPαS B, 6 min.

The ^{31}P NMR spectra of ATPαSCH$_3$ A or B were recorded at 109.32 MHz on a Bruker WH 270 spectrometer in the quadrature detection mode with ^{1}H broad-band decoupling. All other ^{31}P NMR spectra were measured at 81.01 MHz on a Bruker WP 200 SY again with quadrature detection and ^{1}H broad-band decoupling. The parameters used were as follows: pulse width 10–25 μs; acquisition time 1.8 s; pulse delay 0–2 s; sweep width 2500–4000 Hz; line broadening 0.5 Hz; number of transients 50–3000. Positive δ values are chemical shifts in parts per million downfield from external phosphoric acid (85%). ^{1}H NMR spectra were measured at 200 MHz with the Bruker WP 200 SY spectrometer with (trimethylsilyl)propanesulfonic acid as internal standard.

The concentrations of all adenine nucleotides were measured at pH 7 on a Zeiss PMQ II spectrophotometer and by using an E_{260}^{1mM} = 15.4 mol^{-1} cm^2 (Bock et al., 1956). The concentrations of N^1-methyladenine nucleotides were determined at pH 7 by using an E_{257}^{1mM} = 14.6 mol^{-1} cm^2 (Jones & Robins, 1963). UV spectra were recorded on a Shimadzu UV-200 spectrophotometer equipped with a Shimdadzu U-125 MU recorder.

AMPS was prepared by the method of Murray & Atkinson (1968). ATPαS A was synthesized by enzymatic phosphorylation of AMPS (Sheu & Frey, 1977; Jaffe & Cohn, 1978b). ATPαS β was prepared from the chemically synthesized mixture of the A and B isomers of ADPαS (Eckstein & Goody, 1976) by using creatine kinase and back-digestion of the A isomer with hexokinase (Yee et al., 1979). ADPαS A and B were prepared from diastereomerically pure ATPαS A and B, respectively, by using hexokinase. The reaction mixture contained in 10-mL total volume 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 100 mM glucose, and 2.5 mM ATPαS A or B. A total of 200 units of hexokinase was added, and after 1-h incubation at room temperature the dinucleotide products were purified by chromatography over DEAE-Sephadex A-25 and gradient elution with 750 mL each of 0.1 and 0.5 M TEAB, pH 8. Essentially quantitative yields of products, which eluted at about 0.35 M TEAB, were obtained. Although

hexokinase is stereoselective for ATPαS A, the large amounts of enzyme used here were sufficient to completely degrade ATPαS B. ATPβS A and B were prepared by the procedure of Yee et al. (1979).

Preparation of $AMPSCH_3$ and $ADP\beta SCH_3$. A total of 250 μmol of AMPS (disodium salt) was suspended in 5 mL of a water–pyridine mixture (50:50 v/v). A 2.5-mmol (157-μL) aliquot of methyl iodide was added and the mixture stirred in the dark, at room temperature, for 3 h. The solution was dried down by rotary evaporation at 35 °C and the oil obtained dissolved in 50 mL of water and adjusted to pH 8 with triethylamine. The crude product was purified by ion-exchange chromatography on a 20 × 2.5 cm column of DEAE-Sephadex A-25 eluted with a gradient of 1.5 L each of 10 and 200 mM TEAB, pH 8. Fractions (which eluted at about 80 mM buffer) containing product were pooled and evaporated at 35 °C. Excess buffer was removed by repeated coevaporation from methanol and the product finally dissolved in 5 mL of water, adjusted to pH 7, and stored frozen at −20 °C. Yields of 70% were obtained. $ADP\beta SCH_3$ was similarly obtained in 55% yield starting from ADPβS and by using a TEAB gradient of between 20 and 400 mM. This product eluted at about 180 mM buffer.

Preparation of $ATP\alpha SCH_3$ A and B. A total of 10 μmol of diastereomerically pure ATPαS A or B (triethylammonium salt) was evaporated to dryness by using an oil pump and dried down twice from 1 mL of absolute dimethylformamide and then twice from 1 mL of anhydrous methanol. The product was dissolved in 1 mL of dry methanol and after the addition of 500 μmol (34 μL) of methyl iodide set aside, in the dark, for 2 h. The crude product was then evaporated with an oil pump and routinely dissolved in 0.5 mL of 50 mM acetic acid adjusted to pH 4 with tetramethylammonium hydroxide and containing 2% methanol and purified by HPLC as described above. The fractions containing product were immediately placed on ice or concentrated to 1–2 mL at 20 °C with a vacuum pump and then stored as above. Yields were typically 70%. When the UV or ^1H NMR spectra of these compounds were to be measured, the HPLC column was eluted with 20 mM potassium phosphate adjusted to pH 4 with HCl and containing 1% (v/v) methanol.

Attempted Synthesis of $ADP\alpha SCH_3$ and $ATP\beta SCH_3$. The attempted synthesis of these two compounds was as for $ATP\alpha SCH_3$ but starting from ADPαS and ADPβS, respectively. The products obtained were not purified but, after being dried down, dissolved in 0.5 mL of 50 mM acetic acid (adjusted to pH 4 with tetramethylammonium hydroxide) for immediate analysis by HPLC.

Synthesis of N^1-Methyladenosine 5′-O-(Thiophosphate) (N^1-CH_3AMPS). N^1-Methyladenosine (281 mg, 100 μmol) was dried for 20 h at 40 °C, over P_2O_5 in vacuo, and then dissolved in 20 mL of dry trimethyl phosphate with gentle heating from a Bunsen burner. After it was cooled, 26 μL (250 μmol) of thiophosphoryl chloride was added and the mixture left at room temperature for 20 h. The crude product was partially purified by selective precipitation with barium acetate as given for the corresponding adenosine compound (Eckstein et al., 1975) with due allowance for scale down. Final purification was by ion-exchange chromatography. N^1-CH_3AMPS (Ba^{2+} salt) was converted to the pyridinium salt on a Merck I ion-exchange resin and the solution obtained concentrated to 10 mL, adjusted to pH 8 with 1 M TEAB, and applied to a column of DEAE-Sephadex A-25 (30 × 3 cm). The column was developed with a gradient of 750 mL each of 25 and 150 mM TEAB, pH 8. The fractions containing product (eluting at around 70 mM TEAB) were pooled, dried down, coevaporated from methanol, and stored frozen, in solution at −20 °C. Yields of 40% were obtained. The product appeared ≥95% pure by HPLC on (1) ODS-Hypersil eluted with 50 mM potassium phosphate, pH 6 (retention time 2.9 min) and (2) Nucleosil 10SB eluted with 50 mM potassium phosphate, pH 7.8, containing 100 mM potassium acetate (retention time 3 min). ^{31}P NMR showed a single peak at −43.74 ppm, and ^1H NMR gave a typical adenosine spectrum with an additional peak (3 H) at 3.97 ppm due to the N^1-CH_3 group.

Synthesis of N^1-Methyladenosine 5′-O-(S-Methyl thiophosphate) (N^1-$CH_3AMPSCH_3$). N^1-CH_3AMPS (30 μmol, triethylammonium salt) was evaporated to dryness and dissolved in 100 μL of H_2O and 100 μL of methanol. A 4-μL (60-μmol) aliquot of methyl iodide was added, and 2 h later, the mixture was dried down, evaporated twice from water, and finally dissolved in 0.5 mL of H_2O. Purification was by reverse-phase HPLC as described above, and the product-containing fractions were pooled and stored frozen at −20 °C. Yields were 70%.

Chemical Decomposition of $ATP\alpha SCH_3$ (A or B). A 150 μM solution of $ATP\alpha SCH_3$ A dissolved in a buffer consisting of 100 mM citrate, 100 mM phosphate, and 100 mM borate adjusted to pH values of between 1 and 10 with HCl or KOH and containing either 10 mM $MgCl_2$ or 5 mM EDTA was incubated at 25 °C. At times aliquots were withdrawn and analyzed by HPLC for residual $ATP\alpha SCH_3$ A and product $AMPSCH_3$. A more limited but otherwise identical study at pH values of 4, 6, and 7 was performed with $ATP\alpha SCH_3$ B. The stability of $ATP\alpha SCH_3$ A and B was also determined in 200 mM $(CH_3)_4N$-Mes, pH 6, 200 mM $(CH_3)_4N$-Mops, pH 7, and 200 mM $(CH_3)_4N$-Bicine, pH 8, each containing 50 mM KCl and 10 mM $MgCl_2$. In this case, 500 μM solutions of the two diastereomers were incubated at 25 °C and aliquots assayed for residual $ATP\alpha SCH_3$ (A or B) and products $AMPSCH_3$ and ATP at various times. The decomposition products formed in the Mes pH 6 and Bicine pH 8 buffer and also in 100 mM potassium phosphate, pH 7.5, were also checked by ^{31}P NMR. Here 5 μmol of nucleotide was incubated at 25 °C in the appropriate buffer for 15 h, evaporated to dryness, and redissolved in 0.5 mL of 0.2 M triethanolamine chloride, pH 8, containing 0.2 M EDTA and 50% D_2O, and the ^{31}P NMR spectra were measured.

The activities of hexokinase, fructose-6-phosphate kinase, phosphoglycerate kinase, and acetate kinase toward the S-methylated nucleotides were tested at 25 °C in 200 mM $(CH_3)_4N$-Mes, pH 6, containing 200 μM $ATP\alpha SCH_3$ (A or B), 10 mM $MgCl_2$, and either 20 mM glucose, 200 mM potassium acetate, 20 mM 3-phosphoglycerate, or 20 mM fructose 6-phosphate. Hexokinase (280 μg), acetate kinase (112 μg), phosphoglycerate kinase (190 μg), or fructose-6-phosphate kinase (29 μg) was added to the appropriate solution, and the mixtures were assayed, after 5 min, by HPLC for unchanged $ATP\alpha SCH_3$ (A or B) and $AMPSCH_3$. Three sets of controls consisting of (1) replacement of the 10 mM $MgCl_2$ with 10 mM EDTA, (2) omission of cosubstrate, and (3) omission of enzyme were simultaneously carried out. Creatine kinase was similarly tested in 200 mM potassium glycine, pH 9.0, as buffer with 20 mM creatine and 2.2 mg of enzyme. The products of the particular enzyme nucleotide combinations showing activity in the above tests were also studied by ^{31}P NMR. Solutions contained, in a total volume of 2 mL, 200 mM $(CH_3)_4N$-Mes, pH 6, 20 mM $MgCl_2$, 2.5 mM $ATP\alpha SCH_3$, and either 100 mM glucose, 200 mM potassium acetate, or 5 mM fructose 6-phosphate. Approxi-

mately 2 mg of hexokinase, acetate kinase, or fructose-6-phosphate kinase (the combinations tested were hexokinase and acetate kinase with the A diastereomer of ATPαSCH$_3$ and fructose-6-phosphate kinase with both diastereomers) was added as appropriate, and the solutions were incubated for 1 h at 25 °C. After this time the solutions containing hexokinase or fructose-6-phosphate kinase were heated at 90 °C for 5 min and the precipitated protein was removed by centrifugation. The supernatants were evaporated to dryness and the ^{31}P NMR spectra measured as above. Due to the lability of acetyl phosphate, the heating step was not used in studies with acetate kinase.

The K_m and V_{max} values of ATP, ATPαS (A and B), and ATPαSCH$_3$ (A and B) with hexokinase, fructose-6-phosphate kinase, and acetate kinase were determined as follows. Hexokinase was assayed by coupling the reaction product glucose 6-phosphate to NADP reduction via glucose-6-phosphate dehydrogenase. Solutions contained, in a 1-mL total volume, 200 mM $(CH_3)_4$N-Mes, pH 6, 20 mM glucose, 20 mM $MgCl_2$, 50 mM KCl, 1 mM citrate, 1 mM NADP, and 10 units of glucose-6-phosphate dehydrogenase. A total of 2 μg of hexokinase was used when ATP and ATPαS A were studied, 20 μg for ATPαS B, 500 μg for ATPαSCH$_3$ A, and 5 mg for ATPαSCH$_3$ B. The reactions were initiated by nucleotide addition (amounts given under Results), and the increase in absorbance was measured at 25 °C and 340 nm. Fructose-6-phosphate kinase was assayed by coupling fructose 1,6-diphosphate to NADH oxidation via aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase. Solutions contained, in a 1-mL total volume, 200 mM $(CH_3)_4$N-Mes, pH 6, 10 mM fructose 6-phosphate, 10 mM $MgCl_2$, 50 mM KCl, 1 mM AMP, 0.5 mM NADH, and 0.27 unit of aldolase, 30 units of triosephosphate isomerase, and 5 units of glycerol-3-phosphate dehydrogenase. The amounts of fructose-6-phosphate kinase used were 0.75 μg (ATP and ATPαS A), 1.5 μg (ATPαS B and ATPαSCH$_3$ A), and 15 μg (ATPαSCH$_3$ B). The reactions were initiated by nucleotide addition, and the increase in absorbance at 340 nm was measured as for hexokinase. Acetate kinase was directly assayed by HPLC. The solutions contained in a 500-μL total volume 200 mM $(CH_3)_4$N-Mes, pH 6, 200 mM potassium acetate, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol. Acetate kinase (0.125 μg for ATP, 0.25 μg for ATPαS A, 2 μg for ATPαS B, 5 μg for ATPαSCH$_3$ A, and 500 μg for ATPαSCH$_3$ B) was added, and the mixtures were preincubated at 25 °C for 1 h. The reactions were initiated by nucleotide addition. Aliquots were withdrawn at various times and added to an equal volume of ice-cold 0.2 N HCl containing 50% methanol. This completely quenched the reaction, and all the nucleotides studied were reasonably stable in this solution. The amounts of substrate remaining and product produced were determined directly by HPLC. All kinetic constants were obtained from plots of substrate concentration/velocity against substrate concentration.

The velocities observed with creatine kinase and phosphoglycerate kinase were determined at the single nucleotide concentration of 200 μM. Phosphoglycerate kinase was assayed by coupling the reaction to NADH oxidation with glyceraldehyde-3-phosphate dehydrogenase. The solutions contained (total volume 1 mL) 200 mM $(CH_3)_4$N-Mes, pH 6, 20 mM 3-phosphoglycerate, 10 mM $MgCl_2$, 0.5 mM NADH, and 10 units of glyceraldehyde-3-phosphate dehydrogenase. Phosphoglycerate kinase (0.1 μg for ATP, 0.15 μg for ATPαS A, 38 μg for ATPαS B, and 380 μg for ATPαSCH$_3$ A or B) was added, and after a 5-min preincubation at 25 °C, 200 μM nucleotide was added. The decrease in absorbance was observed at 340 nm. Creatine kinase was assayed by HPLC in a 200-μL total volume containing 200 mM potassium glycine, pH 9, 20 mM creatine, and 10 mM $MgCl_2$. Creatine kinase (1.25 μg for ATP, 630 μg for ATPαS A, 42 μg for ATPαS B, and 2.2 mg for ATPαSCH$_3$ A or B) was added, and after a 5-min incubation at 25 °C, the reaction was initiated by nucleotide addition to 200 μM final concentration. For ATP and ATPαS A or B, aliquots were withdrawn at various times and added to an equal volume of 100 mM EDTA. This stopped the reaction, and the amount of product formed was then determined by HPLC (see above). For ATPαSCH$_3$ A or B, aliquots were directly analyzed by HPLC after 5 min.

Table I: ^{31}P NMR Parameters and HPLC Retention Times for a Variety of Nucleotides Used in This Study[a]

nucleotide	^{31}P NMR parameters α-P	β-P	γ-P	HPLC retention times[b] (min)
AMP	4.34 (s)			4.5 (1)
AMPS	42.93 (s)			3.5 (1)
AMPSCH$_3$	22.91 (s)			8.0 (2)
N^1-CH$_3$AMPS	43.74 (s)			
N^1-CH$_3$AMPSCH$_3$	22.90 (s)			3.5 (2)
ADP	−10.58 (d)	−5.77 (d)		2.5 (1)
ADPβS	−11.45 (d)	33.02 (d)		2.2 (1)
ADPβSCH$_3$	−12.03 (d)	9.81 (d)		6.0 (1)
ATP	−10.77 (d)	−22.44 (t)	−10.23 (d)	1.8 (1)
ATPαS A	43.05 (d)	−24.12 (dd)	−10.83 (d)	2.4 (1)
ATPαSCH$_3$ A	22.59 (d)	−23.69 (dd)	−10.26 (d)	4.5 (2)
ATPαS B	42.90 (d)	−24.18 (dd)	−10.86 (d)	2.8 (1)
ATPαSCH$_3$ B	21.88 (d)	−23.76 (dd)	−10.34 (d)	3.2 (2)

[a] The ^{31}P NMR spectra were measured at pH 8 except for the triphosphates and their derivatives where pH 4 was used: s = singlet, d = doublet, t = triplet, and dd = doublet of doublets. [b] HPLC was performed as detailed under Materials and Methods on a 24 × 0.6 cm column of ODS-Hypersil eluted isocratically with (1) 50 mM potassium phosphate, pH 6, or (2) 50 mM potassium phosphate, pH 6, containing 10% methanol.

Results

The methylation of phosphorothioate nucleotide analogues with methyl iodide to yield S-methylated derivatives proceeds easily and specifically although the stability of the products formed differs sharply (Figure 1). Derivatives bearing a terminal *S*-methyl thiophosphate group are stable as is exemplified by the formation of AMPSCH$_3$, N^1-CH$_3$AMPSCH$_3$, and ADPβSCH$_3$. For the characterization and determination of the purity of these compounds, we have relied principally on ^{31}P NMR and reverse-phase HPLC. As is shown in Table I and has previously been observed (Eckstein & Goody, 1976; Jaffe & Cohn, 1978b), substitution of a phosphorothioate for a phosphate group causes a large downfield shift of 40–50 ppm. Further modification from a phosphorothioate to an *S*-methyl thiophosphate group brings about an upfield shift of about 20 ppm. Furthermore, AMPSCH$_3$, N^1-CH$_3$AMPSCH$_3$, and ADPβSCH$_3$ appeared pure by ^{31}P NMR (N^1-CH$_3$AMPSCH$_3$ contained inorganic phosphate due to it being purified by HPLC in phosphate buffer) with no other resonances of ≥5% intensity being observed. All three products also appeared ≥95% pure by reverse-phase HPLC with the retention times and the particular system used being given in Table I. A particular worry with these studies was adenosine base methylation with methyl iodide. Adenosine is reactive toward methyl iodide and many other methylating agents with the

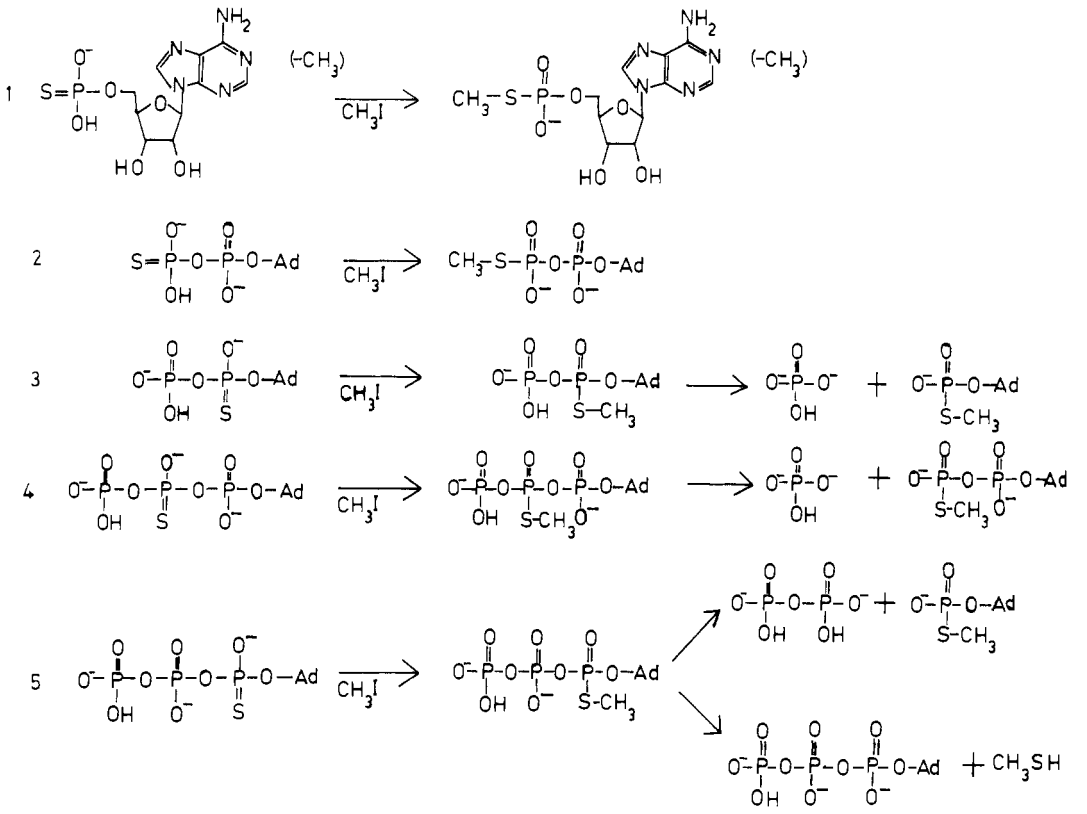

FIGURE 1: Reactions of various nucleoside phosphorothioates with methyl iodide and products of any further decomposition of S-methylated nucleotides so formed. (1) Reaction of AMPS (or N^1-CH_3AMPS) with methyl iodide to AMPSCH_3 (or N^1-CH_3AMPSCH_3). (2) Reaction of ADPβS with methyl iodide to ADPβSCH_3. (3) Reaction of ADPαS with methyl iodide to ADPαSCH_3 and further decomposition to AMPSCH_3 and phosphate. (4) Reaction of ATPβS with methyl iodide to ATPβSCH_3 and further decomposition to ADPβS and phosphate. (5) Reaction of ATPαS with methyl iodide to ATPαSCH_3, which can be isolated at pH 4. At higher pH values ATPSCH_3 decomposes predominantly ($\simeq$95%) to AMPSCH_3 and pyrophosphate with a minor pathway ($\simeq$5%) leading to ATP and methyl mercaptan. Ad = adenosyl.

main product being N^1-methyladenosine (Wacker & Ebert, 1959; Jones & Robins, 1963; Broom et al., 1964). In order to eliminate the possibility of base methylation at the susceptible N-1 position, we have synthesized N^1-CH_3AMPSCH_3 as well as the desired product AMPSCH_3. The ^{1}H NMR spectrum of AMPSCH_3 was typical for that of adenosine nucleotides but contained an additional signal at 2.11 ppm (doublet, J = 13.7 Hz, 3 H). This chemical shift and coupling constant are characteristic of the PSCH_3 group (Eckstein et al., 1974) and provide further proof of identity. The proton spectrum of N^1-CH_3AMPSCH_3 was similar to that of AMPSCH_3 with an additional resonance at 3.95 ppm (singlet, 3 H) due to the N^1-methyl group. The absence of this very easily detectable signal and indeed any other singlets in the spectrum of AMPSCH_3 provides good evidence that base methylation does not occur under the conditions used. Furthermore, both these compounds are easily distinguishable by reverse-phase HPLC (Table I). AMPSCH_3 had a typical adenosine UV spectrum with λ_{max} 259 nm and λ_{min} 228 nm at pH 7. On its own, this is not a sufficient criterium of purity as N^1-methyladenosine derivatives have similar UV spectra (Jones & Robins, 1963; Griffin & Reese, 1963). However, this does eliminate almost all other base-methylated products.

The reaction of ATPαS A or B with methyl iodide also produced the S-methylated derivatives. Both diastereomers appeared $\geq$95% pure by ^{31}P NMR and reverse-phase HPLC (Table I), and additionally, both had typical adenosine UV spectra. These reactions were performed in methanol rather than the pyridine–water mixtures used to methylate AMPS and so it is once again important to eliminate the possibility of base methylation. The ^{1}H NMR spectra of ATPαSCH_3 A or B were not altogether satisfactory. Typical adenosine spectra were obtained although the signal for the furthest upfield (the base H-8) proton was somewhat broadened. The doublet at 2.11 ppm (J = 13.7 Hz, 3 H) was present, indicating the presence of the PSCH_3 group. Additionally, there is an ethyl multiplet at 1.33 and 3.25 ppm due to triethylamine that occurs because the nucleotides are prepared as the triethylammonium salts. The exchange of triethylamine for another cation such as sodium by ion-exchange chromatography was not possible for the ATPαSCH_3 preparations. Exchange before methylation leads to an extremely low yield of product due to the insolubility of NaATPαS in methanol. Exchange after reaction and purification leads to extensive decomposition of the product. Another set of multiplets at 1.05 and 1.40 ppm is due to an unidentified contaminant. Most importantly, no singlets that would arise if base methylation had occurred were observed at 3.95 ppm or anywhere else in the spectrum. Further proof of the lack of methylation at the susceptible N-1 position comes from the decomposition of ATPαSCH_3 at neutral pH values. The product nucleotide was chromatographically identical with AMPSCH_3 and not the easily resolved N^1-CH_3AMPSCH_3.

As it was our intention to use the ATPαSCH_3 derivatives as enzyme substrates, their purity has been very carefully evaluated by HPLC. Freshly prepared samples of ATPαSCH_3 A or B are usually 98% pure and contain about 2% AMPSCH_3. The levels of ATP and ATPαS A or B are $\leq$0.1%. Solutions of these analogues were kept on ice and used within 8 h of purification during which time the levels of AMPSCH_3 rise to 4% and ATP or ATPαS A or B to 0.25%.

The stability of ATPαSCH_3 A is highly dependent upon pH as is shown in Figure 2. In the citrate–phosphate–borate buffer used in this experiment the nucleotide was stable at pH

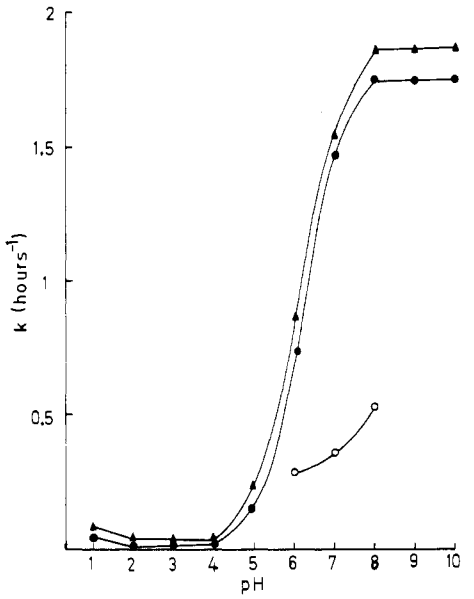

FIGURE 2: Decomposition of ATPαSCH$_3$ as a function of pH. 200 μM ATPαSCH$_3$ A was incubated in 100 mM each of citrate, phosphate, and borate containing 50 mM EDTA (●) or 10 mM $MgCl_2$ (▲) and adjusted to the appropriate pH values with HCl or KOH. 500 μM levels of ATPαSCH$_3$ A were also incubated in Mes, pH 6, Mops, pH 7, or Bicine, pH 8, each containing 50 mM KCl and 10 mM $MgCl_2$ (○). Aliquots were assayed, at various time intervals, for residual ATPαSCH$_3$ A, and the rates of decomposition, which were all pseudo first order, are based on the disappearance of this nucleotide. ATPαSCH$_3$ B behaved identically.

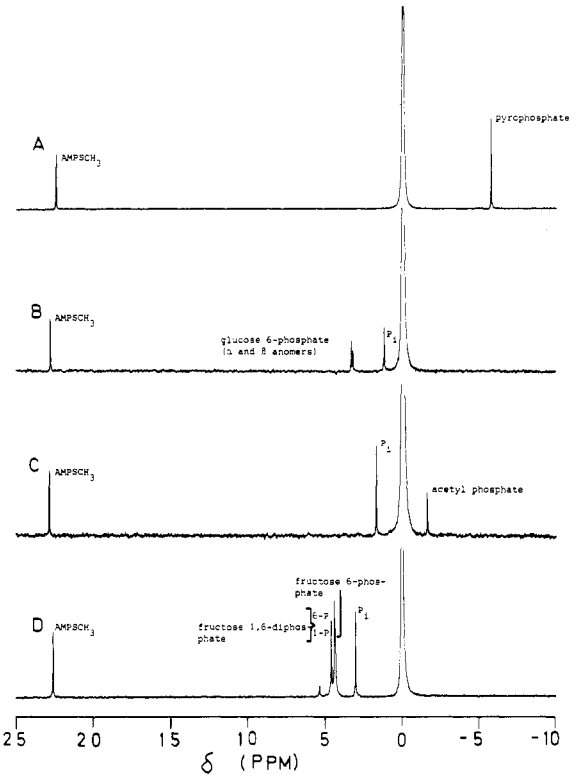

FIGURE 3: ^{31}P NMR spectra of products of hydrolysis and enzymatic transformation of ATPαSCH$_3$. (A) Hydrolysis products formed from ATPαSCH$_3$ A in Bicine buffer, pH 8. Identical results were obtained in Mes buffer, pH 6, and 100 mM KH_2PO_4, pH 7.5, and with ATPαSCH$_3$ B in all three buffer systems. (B) Products of the hexokinase-catalyzed reaction of ATPαSCH$_3$ A. (C) Products of the acetate kinase catalyzed reaction of ATPαSCH$_3$ A. (D) Products of the fructose-6-phosphate kinase catalyzed reaction of ATPαSCH$_3$ A. Similar results were seen with this enzyme and ATPαSCH$_3$ B. The large peak at 0 ppm is 85% H_3PO_4 external standard. The differences in the position of the P_i peak in spectra B–D are due to pH variation.

4 and below but above these values decomposed at an increasing rate that reached a maximum at pH 8. The presence or absence of Mg^{2+} had little effect on the rates of breakdown. Similar results were observed for ATPαSCH$_3$ B at pH 4, 6, and 7. The results shown in Figure 2 were obtained by measuring the disappearance of ATPαSCH$_3$ A or B and the concomitant appearance of the main UV-active decomposition product (95% of the product) AMPSCH$_3$. The decomposition rates of ATPαSCH$_3$ A or B and the products formed have been more carefully evaluated in buffers likely to be useful for enzymatic work, namely, 200 mM Mes, Mops, or Bicine respectively adjusted to pH 6, 7, and 8 with tetramethylammonium hydroxide and each containing 50 mM KCl and 10 mM $MgCl_2$. In all three buffers greater stability than that in the citrate–phosphate–borate system was observed, and the differences between the pH values, although still apparent, were less extreme (see Figure 2). Careful HPLC analysis of the UV-absorbing products showed that while AMPSCH$_3$ was the major product, ATP was also formed. The relative amount of products formed was slightly pH dependent with ratios of 96:4, 94:6, and 93:7 being found at pH 6, 7, and 8, respectively. ^{31}P NMR analysis of the products of the reactions at pH 6 and 8 showed two resonances (Figure 3) assigned to AMPSCH$_3$ and inorganic pyrophosphate by reference to standard tables (Gadian et al., 1979) and also by running the spectrum a second time in the presence of added AMPSCH$_3$ and pyrophosphate. Identical decomposition products were observed in 100 mM potassium phosphate, pH 7.5. ATP was not observed in these NMR experiments presumably due to its low abundance and the relative insensitivity of the NMR method. Although the coproduct in the pathway leading to ATP has not been identified, the typical unpleasant odor of solutions of ATPαSCH$_3$ aged at neutral pH values suggests methyl mercaptan.

The reaction of ADPαS or ATPβS with methyl iodide gave rise only to AMPSCH$_3$ and ADPβSCH$_3$, respectively. The identity of these products was determined by coinjection with standard AMPSCH$_3$ and ADPβSCH$_3$ (prepared and characterized as above) by using reverse-phase HPLC. Presumably, the initial products formed, ADPαSCH$_3$ or ATPβSCH$_3$, are extremely unstable and rapidly loose their terminal phosphate group to give the products observed (Figure 1).

The reactions of ATPαSCH$_3$ have been studied in detail with five kinases. Wherever possible, 200 mM $(CH_3)_4N$-Mes, pH 6, has been used as buffer to minimize the decomposition of the S-methylated nucleotides. This high buffer concentration (pK = 6.15) is necessary to ensure a constant pH as the modified nucleotides are purified in 50 mM tetramethylammonium acetate buffer, pH 4. The inactivity of creatine kinase in the direction of creatine phosphate formation at pH 6 meant that potassium glycine, pH 9, had to be used in this case. Controls established that no time-dependent enzyme inactivation occurred under the conditions used. Enzyme activity was investigated by HPLC (Figure 4) and ^{31}P NMR (Figure 3). Figure 4B shows that when hexokinase is incubated with ATPαSCH$_3$ A in the presence of glucose and $MgCl_2$, HPLC analysis indicates complete conversion to AMPSCH$_3$. Identical results with those of Figure 4B were obtained with acetate kinase and ATPαSCH$_3$ A and fructose-6-phosphate kinase with ATPαSCH$_3$ A and B, providing that cosubstrate and $MgCl_2$ were present. Very little AMPSCH$_3$ was formed (Figure 4C) with hexokinase and acetate kinase with ATPαSCH$_3$ B as well as by creatine and phosphoglycerate kinase with both diastereomers. Controls

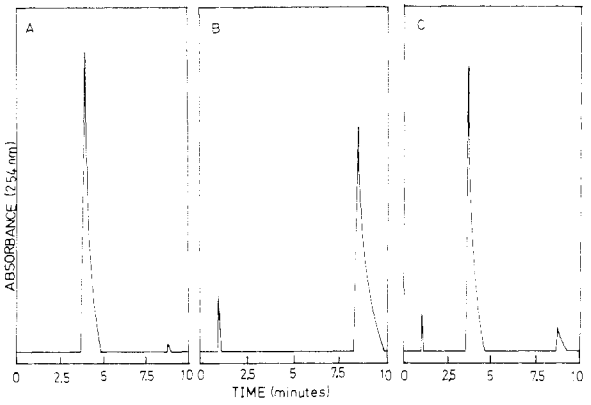

FIGURE 4: HPLC analysis of hexokinase-catalyzed transformation of ATPαSCH$_3$: (A) Freshly prepared ATPαSCH$_3$ A; (B) products formed on incubating ATPαSCH$_3$ A with hexokinase in the presence of glucose and $MgCl_2$; (C) products formed when ATPαSCH$_3$ A was incubated with hexokinase in the absence of glucose, when $MgCl_2$ was replaced with EDTA, or when hexokinase was omitted. The peaks at 4 and 9 min are ATPαSCH$_3$ A and AMPSCH$_3$, respectively. The early eluting peaks in panels B and C are due to UV impurities present in the buffers used.

in which the $MgCl_2$ was replaced by EDTA, the cosubstrate was omitted, or the enzyme was omitted also resulted in very little AMPSCH$_3$ formation. The simplest interpretation of these results is that ATPαSCH$_3$ A is a substrate for hexokinase, acetate kinase, and fructose-6-phosphate kinase and that the B diastereomer is also active with fructose-6-phosphate kinase. In all cases the immediate products are the appropriate sugar or acyl phosphate and ADPαSCH$_3$. However, ADPαSCH$_3$ rapidly decomposes to AMPSCH$_3$ (detected by HPLC) and P_i. The strict dependence of this process on Mg^{2+} and cosubstrate strongly suggests that it is a true enzyme-mediated process. The small amounts of AMPSCH$_3$ formed in all the other cases are most likely due to background decomposition of ATPαSCH$_3$.

The above hypothesis has been confirmed by ^{31}P NMR experiments. When ATPαSCH$_3$ A was incubated with hexokinase, equimolar amounts of glucose 6-phosphate, AMPSCH$_3$, and P_i were formed (Figure 3). Similarly with acetate kinase, acetyl phosphate, AMPSCH$_3$, and P_i were produced. The spectrum obtained with fructose-6-phosphate kinase and ATPαSCH$_3$ A (the B diastereomer gave identical results) is somewhat complicated by the excess of the cosubstrate fructose 6-phosphate used, but nevertheless, fructose 1,6-diphosphate, AMPSCH$_3$, and P_i are clearly present. The identification of the resonances shown in Figure 3 are based on literature results (Gadian et al., 1979). However, the chemical shifts of the above compounds are very pH sensitive particularly around pH 7 and so a second spectrum was always run following the addition of 5 μmol of the appropriate sugar or acyl phosphate, AMPSCH$_3$, and P_i. In all cases no extra signals appeared in the second spectrum, confirming peak assignment.

The K_m and V_{max} values obtained for hexokinase, fructose-6-phosphate kinase, and acetate kinase with ATP, ATPαS A or B, and ATPαSCH$_3$ A or B are shown in Table II. In all cases the reactions were initiated by addition of nucleotide in order to minimize ATPαSCH$_3$ decomposition. As acetate kinase is reversibly inactivated by low temperatures (Anthony & Spector, 1971), it was reactivated by preincubation in the assay mixture for 1 h prior to nucleotide addition. No allowance was made for any ATP or ATPαS A or B contamination in the S-methylated nucleotides. The lack of substrate activity measured with ATPαSCH$_3$ B and hexokinase and also the lack of activity seen with both diastereomers and phosphoglycerate kinase (also tested with a coupled assay sensitive to ATP, see below) suggest that rate contributions due to contamination can be neglected. Hexokinase and fructose-6-phosphate kinase have been assayed by commonly used coupled assays and require little comment. In the absence of a convenient coupled assay for acetate kinase this enzyme has been tested directly by HPLC. Clearly, ADP, ADPαS A, and ADPαS B production from ATP, ATPαS A, and ATPαS B, respectively, gave a direct representation of enzymatic activity. However, the acetate kinase assay with ATPαSCH$_3$ A based on AMPSCH$_3$ production depends on the assumption that the initial reaction (ATPαSCH$_3$ + acetate → ADPαSCH$_3$ + acetyl phosphate) is rate limiting and the second reaction (ADPSCH$_3$ → AMPSCH$_3$ + P_i) is fast. This has been tested by showing that AMPSCH$_3$ formation is proportional to the amount of acetate kinase added, providing between 1 and 10 μg of enzyme was used.

Table II: K_m and V_{max} Values Obtained for Hexokinase, Fructose-6-phosphate Kinase, and Acetate Kinase with ATP, ATPαS A or B, and ATPαSCH$_3$ A or B[a]

	hexokinase		fructose-6-phosphate kinase		acetate kinase	
	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
ATP	60	41	60	27	125	69
ATPαS A	68	26	40	23	110	44
ATPαSCH$_3$ A	150	0.12	400	16	700	17
ATPαS B	500	2.5	50	12	115	9
ATPαSCH$_3$ B			1200	1.4		

[a] Nucleotide levels varied between 0.1 and 5 times the K_m values observed.

Although no activity was observed with phosphoglycerate kinase and creatine kinase and the S-methylated derivatives, we have nevertheless assayed them (together with ATP and ATPαS A and B) in order to get maximal possible velocities on the basis of the sensitivity of the assay procedures used. At 200 μM nucleotide the following velocities (in μmol min^{-1} mg^{-1}) were observed with phosphoglycerate kinase: ATP, 180; ATPαS A, 80; ATPαS B, 0.35; ATPαSCH$_3$ A or B, ≤0.001. With creatine kinase the following rates (in μmol min^{-1} mg^{-1}) at 200 μM nucleotide were observed: ATP, 12; ATPαS A, 0.02; ATPαS B, 0.12; ATPαSCH$_3$ A or B, ≤0.0003.

Discussion

The reaction of adenosine phosphorothioates with methyl iodide leads to methylation of the sulfur atom and formation of an *S*-methyl thiophosphate group. Under the conditions used in this study the reaction is very selective with no detectable base modification taking place. By this procedure both diastereomers of ATPαSCH$_3$ have been prepared. The purification of ATPαSCH$_3$ A or B is based on their high affinity for the octadecyl reverse-phase material ODS-Hypersil. Thus these derivatives are retained on this material in 50 mM potassium phosphate, pH 6, containing 10% methanol, conditions under which almost all other adenosine nucleotides are rapidly eluted. This property seems to occur only in derivatives having an *S*-methyl thiophosphate group at the α position, AMPSCH$_3$ and N^1-CH$_3$AMPSCH$_3$ being also retained whereas ADPβSCH$_3$ was rapidly eluted.

The stability of ATPαSCH$_3$ A or B is very pH dependent. In 50 mM tetramethylammonium acetate, pH 4 (the buffer used to purify these compounds), the two derivatives could be

stored frozen at −20 °C for 3 or 4 days with very little decomposition. However, both compounds were routinely used on the day of preparation. At physiological pH values much lower stability was observed with half-lives in the order of hours. The main decomposition pathway (95%) gave $AMPSCH_3$ and pyrophosphate while a minor pathway (5%) gave ATP and methyl mercaptan. $ATP\alpha SCH_3$ can be considered analogous to pyrophosphate derivatives, being diesterified at one phosphate and monoesterified at the other. Such compounds have been prepared (Dudek & Westheimer, 1959; Brown & Hamer, 1960). Brown & Hamer (1960) reported a half-life of 30 h for trimethyl pyrophosphate at pH 8.8 although detailed studies were not carried out and no degradative mechanisms proposed. Several mechanisms can be suggested for the decomposition of $ATP\alpha SCH_3$ to $AMPSCH_3$ and pyrophosphate. At present, we favor an S_N2 mechanism with water attack at the triesterified α phosphorus, the most electrophilic site in the phosphate chain, with elimination of pyrophosphate. The rate increase seen as the buffer system is changed from Mes, pH 6, through Mops, pH 7, to Bicine, pH 8, is compatible with this mechanism in terms of a simple increase in OH^- concentration. $ATP\alpha SCH_3$ is less stable in the citrate–phosphate–borate buffer system than in Mes, Mops, or Bicine at the same pH values. The decomposition of tetramethyl pyrophosphate is known to be increased in the presence of phosphate (Avison, 1955; Brown & Hamer, 1960; Samuel & Silver, 1961). This is attributed to nucleophilic attack on one of the diesterified phosphorus atoms of tetramethyl pyrophosphate to give dimethyl phosphate and the extremely unstable P^1-dimethyl pyrophosphate. This rapidly decomposes to give a second molecule of dimethyl phosphate and regenerate phosphate. A similar mechanism could operate with $ATP\alpha SCH_3$ with phosphate attack at the α-phosphate group yielding initially $ADP\alpha SCH_3$ and pyrophosphate. The $ADP\alpha SCH_3$ would then rapidly decompose giving $AMPSCH_3$ and P_i. This would explain the lowered stability of these derivatives in the presence of phosphate and also tend to favor S_N2 attack at the α phosphorus of $ATP\alpha SCH_3$ by water as the usual hydrolytic mechanism. Clearly, further studies, especially with $H_2{}^{18}O$ and $P^{18}O_4$, are needed to confirm the mechanism. The minor pathway leading to ATP is most likely due to attack at the α phosphorus by one of the γ-phosphate oxygens, leading to the elimination of methyl mercaptan and the formation of a cyclic trimetaphosphate derivative. The reaction of this species with water then yields ATP. Support for such a mechanism comes from the appearance of oxygen-18 in the γ phosphorus of ATP when $ATP\alpha S$ is activated with *N*-bromosuccinimide (Connolly et al., 1982) or cyanogen bromide (Frey, 1982) in $H_2{}^{18}O$.

The compounds $ADP\alpha SCH_3$ and $ATP\beta SCH_3$ are too unstable to be isolated. However, the formation of $AMPSCH_3$ and $ADP\beta SCH_3$ when $ADP\alpha S$ and $ATP\beta S$ are methylated suggests that these two compounds are initially formed. Additionally, $ADP\alpha SCH_3$ must also be transiently generated during enzymatic phosphorylations with $ATP\alpha SCH_3$ as substrate. However, here again only $AMPSCH_3$ and P_i are detected. $ADP\alpha SCH_3$ can be considered analogous to P^1-diethyl pyrophosphate. Brown & Hamer (1960) have argued that P^1-diethyl pyrophosphate decomposes via metaphosphate giving, in H_2O, diethyl phosphate and phosphate. The metaphosphate mechanism was preferred because the phosphate group (which carries two negative charges) was thought unlikely to react with water in an S_N2-type mechanism at the extremely fast rate observed. Additionally P^1-diethyl pyrophosphate prepared under anhydrous conditions gave rise to trimetaphosphate presumably by polymerization of the metaphosphate formed. Thus, we propose that on methylation of $ADP\alpha S$ or $ATP\beta S$ with methyl iodide, $ADP\alpha SCH_3$ and $ATP\beta SCH_3$ are formed but rapidly further react to give $AMPSCH_3$, $ADP\beta SCH_3$, and inorganic phosphate via a metaphosphate mechanism. $ADP\alpha SCH_3$ generated enzymatically presumably undergoes an identical reaction.

The enzymatic reactions of $ATP\alpha SCH_3$ A and B have been investigated with hexokinase, acetate kinase, fructose-6-phosphate kinase, creatine kinase, and phosphoglycerate kinase. The lack of activity with these analogues could be due to two causes. First, the analogue might not be able to form the correct metal·nucleotide chelate required by the enzyme. Second, even if the correct metal·nucleotide complex was formed, this in itself might not be a substrate due to the bulky $PSCH_3$ group and, hence, steric considerations. Although all ATP-dependent enzymes use Mg·ATP as the true substrate, the structure of this chelate is still a matter of some controversy. The most widely used method to study this question has been ^{31}P NMR. However, ^{31}P NMR chemical shift data have not resolved the question of whether Mg·ATP exists as a β,γ-bidentate chelate (Cohn & Hughes, 1962) or an α,β,γ-tridentate chelate (Kuntz & Swift, 1973; Gupta & Mildvan, 1977; Bishop et al., 1981). Although metal ion interaction with the β- and γ-phosphate groups is well established, the main argument centers on whether or not the metal ion also binds to the α-phosphate group. In an elegant study with ^{17}O NMR Huang & Tsai (1982) demonstrated that Mg^{2+} interacts with the α-, β-, and γ-phosphate groups in ATP. Although interaction at the α-phosphate was clearly demonstrated, the effects seen were smaller than those observed at the other two phosphate groups, and it was not possible to decide whether Mg·ATP was only an α,β,γ-tridentate chelate or a mixture of this chelate with β,γ-bidentate and other structures. Despite this controversy, all the above authors implicitly assume that this binding is electrostatic in character and therefore of the type $O{=}P{-}O^-{\cdots}Mg^{2+}$ rather than $^-O{-}P{=}O{\cdots}Mg^{2+}$. Support for this comes from Brintzinger (1961), who, on the basis of the binding constants observed between various metal ions, ATP^{4-}, and other anionic species, proposed that metal·ATP chelates exist as hydrated ion pairs. Further evidence for the predominantly ionic nature of this interaction comes from the higher affinity of ATP^{4-} than that of $HATP^{3-}$ for metal ions (Smith & Alberty, 1956a,b) and the poor metal binding properties of $ATP\gamma F$ (three negative charges) as compared to those of ATP (Haley & Yount, 1972). On the basis of the above observations, we propose that $Mg{\cdot}ATP\alpha SCH_3$ exists as the β,γ-bidentate chelate in solution. The α-phosphate group in these compounds is triesterified, and the P=O group cannot therefore form a resonance $P{-}O^-$ structure and so is unlikely to make an ionic interaction with Mg^{2+}. As metal ion complexation to the α-phosphate is precluded, the controversy found in ATP of metal ion interaction with the α-phosphate does not occur and so the bidentate chelate structure is proposed.

A test of this hypothesis is that the two diastereomers of $ATP\alpha SCH_3$ should not show activity with enzymes that require metal ion binding at the α-phosphate. On the basis of the reversal of diastereomeric selectivity seen with creatine kinase and $ATP\alpha S$ A and B in the presence of Mg^{2+} and Cd^{2+}, Burgers & Eckstein (1980) proposed metal coordination to the α-phosphate group of ATP at some stage of the catalytic cycle. This was confirmed with Mn^{2+} EPR measurements in the presence of ^{17}O-labeled nucleotides (Reed & Leyh, 1980). With 3-phosphoglycerate kinase (Jaffe et al., 1982) a similar

reversal of diastereomeric specificity is seen with the ATPαS isomers, again leading to the conclusion that the active metal·nucleotide chelate involves an α-phosphate group metal ion coordination. Neither of these two enzymes showed measurable activity with ATPαSCH$_3$ A or B, results in agreement with the proposition put forward above. However, the lack of activity seen with the diastereomers of ATPαSCH$_3$ may be due to simple steric effects rather than the inability to form the correct metal·nucleotide chelate.

Much more information can be obtained when substrate activity is observed with the ATPαSCH$_3$ derivatives. Both hexokinase (Jaffe & Cohn, 1979) and acetate kinase (Romaniuk & Eckstein, 1981) show the most common pattern of activity with the phosphorothioates in the presence of Mg^{2+} or Cd^{2+}, i.e., a reversal of diastereomeric selectivity at the β phosphorus with no reversal at the α phosphorus. For the reasons given in the introduction this is interpreted in terms of an active β,γ-bidentate Mg·ATP chelate although a metal ion interaction with the α-phosphate cannot be rigorously excluded. Phosphofructokinase has not been studied by this technique, but on the basis of the activity observed with the exchange inert Cr·ATP analogues, the β,γ-bidentate Mg·ATP complex has also been proposed to be the active one (Dunaway-Mariano & Cleland, 1980). All three enzymes show activity with at least one diastereomer of ATPαSCH$_3$. As these nucleotides cannot interact with metal ions via the α-phosphate group, the use of a metal nucleotide chelate involving this mode of binding would not seem to be possible with these three enzymes. This confirms the simple interpretation that both hexokinase and acetate kinase use β,γ-bidentate complexes proposed from the phosphorothioate results. It is fortunate that the phosphorothioate and *S*-methyl phosphorothioate methods are complementary. A positive reversal of stereoselectivity with ATPαS A and B should be accompanied by a lack of reactivity with ATPαSCH$_3$ A or B whereas when no reversal is observed, activity with the ATPαSCH$_3$ isomers is a possibility. The activities seen with phosphofructokinase are also in accord with the results obtained with the Cr·ATP analogues.

Further information can be obtained from the K_m and V_{max} values given in Table II. With hexokinase on replacing ATP by ATPαS B the K_m increases and the V_{max} decreases both by 1 order of magnitude. Additional modification to ATPαSCH$_3$ B causes a complete loss of substrate activity. This suggests that the *pro-R* oxygen atom at the α phosphorus makes an important interaction, probably a hydrogen bond, with the enzyme surface. Thus on going from ATP to ATPαS B this interaction is weakened, resulting in poorer substrate properties, and on modification to ATPαSCH$_3$ B this interaction cannot occur, accounting for the lack of catalysis. In contrast, ATP and ATPαS A have similar K_m and V_{max} values, indicating that the *pro-S* oxygen atom is less important for good substrate properties. ATPαSCH$_3$ A binds almost as well as ATP and ATPαS A (K_m increases about 2-fold), but the V_{max} is reduced by about 2 orders of magnitude. One possible explanation for this is that ATPαSCH$_3$ A can bind normally to the enzyme but that the bulky SCH$_3$ group prevents the conformational change in the enzyme·glucose·nucleotide ternary complex that is necessary for efficient catalysis (Koshland, 1955; Bennett & Steitz, 1978; Anderson et al., 1978). With acetate kinase, ATP and ATPαS A had similar K_m and V_{max} values, and ATPαSCH$_3$ A was also a substrate although with an increased K_m and a decreased V_{max}. The simplest interpretation here is that no specific interaction between the *pro-S* oxygen at the α phosphorus group and the enzyme surface occurs but that this oxygen is subject to steric interactions with the enzyme. The increase in bulk on going from oxygen to sulfur is too small to cause a significant increase in steric interference, but on forming the much larger SCH$_3$ group steric hindrance becomes serious enough to make ATPαSCH$_3$ A a much poorer substrate. Similar arguments apply with ATP, ATPαS B, and ATPαSCH$_3$ B although the *pro-R* oxygen would seem to be more important than the *pro-S* oxygen for good substrate properties as shown by the total lack of activity of ATPαSCH$_3$ B. Phosphofructokinase is remarkably tolerant to modification at the α phosphorus of ATP. ATP and ATPαS A or B had K_m and V_{max} values close enough to indicate a lack of any specific reaction between the enzyme and this phosphorus. Although both ATPαSCH$_3$ A and B were substrates, the K_m values were increased and the V_{max} values decreased, probably for steric considerations. Once again modification of the *pro-R* oxygen resulted in greater loss of good substrate ability than that of the *pro-S* oxygen.

The discussion above has shown that ATPαSCH$_3$ A and B can be used to probe metal·nucleotide chelate structures and also as general probes of nucleotide binding sites. However, the chemical properties of ATPαSCH$_3$ suggest two further uses that we had not considered at the outset of this project. First, the decomposition of these compounds is pH sensitive in the range that enzymes show activity, i.e., pH 7. It should be possible to bind these analogues to enzyme active sites and then study this decomposition. To a first approximation, this will give a value for the apparent pH at the active site. Second, and perhaps more interestingly, ATPαSCH$_3$ is a substrate for some kinases and presumably initially liberates ADPαSCH$_3$ at the active site. In theory, this compound could further decompose at the active center, liberating bound metaphosphate and AMPSCH$_3$. The very reactive metaphosphate would then have the possibility of reacting with nucleophiles at the enzyme active site. Although such inactivation was not observed in control experiments, a wide variety of conditions were not tried. For these reasons we feel that both the chemical and enzymatic properties of ATPαSCH$_3$ merit further study.

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Mechanism of Action of Thrombin on Fibrinogen. Direct Evidence for the Involvement of Phenylalanine at Position P_9[†]

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ABSTRACT: The following peptides were synthesized by classical methods in solution: Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-6) and Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-7). The rates of hydrolysis of the Arg–Gly bond in these peptides by thrombin were measured, and the rate for the Phe-containing peptide F-6 was found to be much larger than that for F-7. Previous work [van Nispen, J. W., Hageman, T. C., & Scheraga, H. A. (1977) *Arch. Biochem. Biophys. 182*, 227] has demonstrated the importance of Phe-Leu at positions P_9-P_8 of the Aα chain of fibrinogen for the thrombin–fibrinogen interaction. This work demonstrates that the presence of Leu (P_8) alone is insufficient to account for the enhanced hydrolysis rates and that the presence of Phe (P_9) is essential for normal action of thrombin on the Aα chain of fibrinogen.

From the observation that the amino acid sequence in a portion of the fibrinogens of many species is strongly conserved, Blombäck (1967) had suggested that Phe at position P_9 of the Aα chain of fibrinogen[1] is essential for normal thrombin action. This was supported, in part, by van Nispen et al. (1977), who showed that Phe-Leu at positions P_9-P_8 in the peptide Ac-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg-$NHCH_3$ (F-3) greatly increased the rate of

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[1] Abbreviations: AcONP, 4-nitrophenyl acetate; CNBr Aα, the N-terminal CNBr fragment of the Aα chain of fibrinogen; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Ac, acetyl; HOAc, acetic acid; OBu^t, *tert*-butyloxy; DEAE-Sephadex, diethylaminoethyl-Sephadex. The abbreviations used for the amino acid residues and the notation of peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1972). The positions of residues in peptide substrates are described by the nomenclature of Schechter & Berger (1967) wherein residues on the N-terminal side of the Arg–Gly bond are designated as P_1, P_2, etc., and those on the C-terminal side are designated as P_1', P_2', etc. (see Table I).

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