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Supplementary Material for

Purple Acid Phosphatase: A Di-iron Enzyme that Catalyzes a Direct Phospho Group Transfer to Water

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Enzymes. Purple acid phosphatase (PAP) was isolated from bovine spleen.[1] The enzyme was stored at 4° C and was stable for weeks. All other enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and were rid of small molecules by ultrafiltration using Centricon-10 units (Amicon, Danvers, MA). The enzymes were subjected to repeated concentration and dilution into 100 mM triethanolamine·HCl buffer, pH 7.6, containing EDTA (1 mM) and, when glyceraldehyde-3-phosphate dehydrogenase was present, dithiothreitol (1 mM).

Chemicals.[2] Triethanolamine, ATPγS (tetralithium salt), NAD^+ (sodium salt), NADH, pyruvic acid, 2-mercaptoethanol and EDTA (tetrasodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol, ADP (disodium salt), ATP (trisodium salt), fructose 1,6-bisphosphate (disodium salt), AMP (disodium salt), and glucose 6-phosphate (disodium salt) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). AG 1-X8 and Chelex 100 were purchased from Bio-Rad Laboratories (Richmond, CA). DE-52 was purchased from Whatman BioSystems (Maidstone, UK). 2-(4-Morpholino)-ethane sulfonic acid (MES) was purchased from Eastman Kodak (Rochester, NY). Sodium periodate was

purchased from Mallinckrodt, Inc. (Paris, KY). Potassium hydroxide, Celite, triethylamine, pyridine, methanol, acetonitrile, dichloromethane, 1,4-dioxane, *p*-toluenesulfonic acid, sulfur, and sodium potassium tartrate were purchased from Fisher Chemical Co. (Fairlawn, NJ). The $H_2{}^{17}O$ was purchased from Monsanto Research Laboratories (Miamisburg, OH). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Pyridine, tri-*n*-butylamine, tri-*n*-octylamine, and dichloromethane were distilled from calcium hydride before use. DMF was distilled *in vacuo* from barium oxide directly onto activated 4Å molecular sieves (Union Carbide, South Plainfield, NJ). Dioxane was distilled from sodium. Triethylamine was distilled just prior to preparation of TEAB buffer. Diphenyl chlorophosphate was distilled *in vacuo* immediately prior to use. Phosphorus trichloride was distilled under argon immediately prior to use.

HPLC. A Waters Associates (Milford, MA) system, consisting of one M-6000 pump, one M-45 pump, a model 680 gradient controller, and a U6K injector, was used. Isocratic elution of a C_{18} Bondpak column (Waters) with 15% acetonitrile/water was used to monitor the conversion of [5'-^{18}O]2',3'-isopropylideneadenosine into [5'-^{18}O]adenosine; detection was by $A_{254\ nm}$ using a model 441 absorbance detector (Waters). Isocratic elution of a column (HR 5/5) of Mono Q (Pharmacia-LKB, Piscataway, NJ) with 0.7 M ammonium phosphate buffer, pH 4, was used to monitor the conversion of *S*P-ATP$\alpha^{17}O_2$,α^{18}O,αS into 2',3'-methoxymethylideneadenosine[3]; detection was by $A_{254\ nm}$.

Spectroscopy. ^{31}P NMR spectra were recorded on a Bruker WM-300 instrument; chemical shifts are referenced to external 85% phosphoric acid (downfield is positive). ^{1}H NMR spectra were recorded on Bruker AM-300 or AM-400 instruments. Enzymatic reactions were monitored and chromatography fractions were assayed enzymatically (when indicated) by monitoring the change in absorbance at 340 nm due to production or consumption of NADH. Adenosine and adenosine nucleotides were detected in column fractions and quantitated using the $A_{260\ nm}$ ($\varepsilon_{260\ nm}$ = 15000 $M^{-1}cm^{-1}$). A Perkin-Elmer 554, Hewlett-Packard 8452A, or Cary 3 spectrophotometer was used.

Determination of the Site of Hydrolytic Attack. ATPγS (8.7 mg, 16 μmol) was dissolved in 250 mM MES·KOH buffer, pH 6.2, (400 μL) containing $H_2{}^{18}O$ (200 μL, 95% ^{18}O) and PAP (30 μg, 750 pmol). The reaction was monitored by ^{31}P NMR. At 75% reaction (3 h), D_2O (1 mL) was added, and the pH was raised to 13 with KOH (one small pellet). An incubation without PAP showed no siginificant hydrolysis of ATPγS. The ^{31}P NMR spectrum showed the products of the reaction to be AMP, inorganic phosphate, and inorganic thiophosphate. Both the inorganic thiophosphate and the inorganic phosphate displayed isotopic shifting due to the presence of ^{18}O.[4] The two inorganic thiophosphate resonances were at 32.22 ppm ([$^{16}O_3$]thiophosphate) and 32.19 ppm ([$^{16}O_2{}^{18}O$]thiophosphate) and were present in the ratio 1.1:1 (quantitation by peak intensities), corresponding to 53% ^{16}O and 47% ^{18}O. Based upon the volume of ^{18}O added, the predicted composition is 52.5% [^{16}O]thiophosphate and 47.5% [$^{16}O_2$,^{18}O]thiophosphate.

[^{18}O]Benzoic acid. Freshly distilled benzoyl chloride (580 μL; 5 mmol) was dissolved in dioxane (1 mL), and [^{18}O]H_2O (1 g; 95% ^{18}O) was added. The solution was sealed, placed in a dessicator, and incubated for 40 h in a water bath at 85° C. HCl and water were removed from the resultant white solid by evaporation of three portions of added dioxane under reduced pressure. The isotopic content was determined by mass spectrometry (EI ionization): *m/s* (M^+) 124 (20.6%), 125 (6.7%), 126 (65.0%), 127 (7.7%); these data correspond to 13% ^{16}O and 87% ^{18}O. The yield was quantitative.

5'-O-Benzoyl-2',3'-isopropylidene[5'-^{18}O]adenosine.[5] 2',3'-Isopropylideneadenosine (1.23 g, 4 mmol) and triphenylphosphine (1.31 g, 5 mmol) were dried by evaporation under reduced pressure of two portions of added dioxane; these dried reagents were suspended in dioxane (10 mL). The [^{18}O]benzoic acid (5 mmol) was dissolved in dioxane (15 mL) containing diethyl azodicarboxylate (787 μL; 5 mmol). This solution was added dropwise over 1 h to the suspension of 2',3'-isopropylideneadenosine and triphenylphosphine, and the reaction mixture was stirred under argon. After 20 h, thin layer chromatography over silica gel eluting with

dichloromethane-methanol (9:1 (v/v)) showed complete conversion of 2',3'-isopropylidene-adenosine to 5'-*O*-benzoyl-2',3'-isopropylideneadenosine. Methanol (2 mL) was added, and the solvent was removed by evaporation under reduced pressure. The product was purified by flash chromatography over silica gel, eluting with dichloromethane:methanol (19:1 (v/v)). Fractions containing impurities were again subjected to chromatography. Fractions containing pure product were combined, and the solvent was removed by evaporation under reduced pressure. Methanol was removed by repeated evaporation of added dichloromethane under reduced pressure. The yield of 5'-*O*-benzoyl-2',3'-isopropylidene[5'-^{18}O]adenosine was 1.66g (4 mmol; 100%). Mass spectrometric analysis (fast atom bombardment) was consistent with complete retention of ^{18}O.[6]

^{1}H NMR (300 MHz; $CDCl_3$) δ: 1.42 (3H, s); 1.64 (3H, s); 4.49 (1H, m); 4.63 (2H, m); 5.20 (1H, dd, J = 6 Hz, 3 Hz); 5.60 (1H, dd, J = 6 Hz, 3 Hz); 6.82 (2H, br s); 7.34 (2H, dd, J = 1 Hz, 7 Hz); 7.49 (1H, td, J = 1 Hz, 7 Hz); 7.89 (2H, dd, J = 1 Hz, 7 Hz); 7.94 (1H, s); 8.31 (1H, s).

[5'-^{18}O]Adenosine. The 5'-*O*-benzoyl-2',3'-isopropylidene[5'-^{18}O]adenosine was dissolved in methanol (10 mL). Ammonia (15 M, 5 mL) was added, and the solution was stirred for 20 h. A white precipitate readily dissolved upon addition of dichloromethane, and thin layer chromatography over silica gel eluting with dichloromethane-methanol (9:1 (v/v)) showed that all starting material had been cleanly converted into 2',3'-isopropylideneadenosine. The solvent was removed by evaporation under reduced pressure, and traces of ammonia and water were removed by evaporation of added methanol and dichloromethane under reduced pressure. The resultant white solid was suspended in 10% acetic acid/water and brought to reflux under argon; all of the solid dissolved. After 9 h, analysis by reverse-phase HPLC (as described above) revealed that the formation of [5'-^{18}O]adenosine was essentially complete. The reaction mixture was diluted with water (100 mL) and washed with diethyl ether (2 x 50 mL). The solvent was removed by evaporation under reduced pressure, and the resultant solid was dissolved in water (300 mL). The adenosine was then purified by chromatography over AG 1-X8 (hydroxide form), eluting with a linear gradient (790 mL + 1000 mL)[7] of methanol in water (0-100%).[3] Product-bearing fractions were identified by $A_{260\ nm}$; they were combined, and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in water, and this solution was lyophilized.

Mass spectrometric analysis (fast atom bombardment) was consistent with complete retention of ^{18}O.[6] The yield of [5'-^{18}O]adenosine was 0.97 g (3.6 mmol; 90%).

2',3'-methoxymethylidene[5'-^{18}O]adenosine. [5'-^{18}O]adenosine (0.97 g, 3.6 mmol) and *p*-toluenesulfonic acid was dried by evaporation of added DMF (2 x 10 mL) under reduced presure. These reagents were then dissolved in DMF (10 mL), and trimethylorthoformate (600 μL, 5.5 mmol) was added; the solution was stirred under argon. After 27 h, thin layer chromatography over silica gel eluting with dichrolomethane-methanol (9:1 (v/v) revealed that the reaction was complete. Sodium methylate (0.34 g; 6.3 mmol) was added, and the solvent was removed by evaporation under reduced pressure. The resultant cloudy syrup was filtered through Celite, and the filter cake was washed exhaustively with dichloromethane-methanol (19:1 (v/v)). The solvent was removed by evaporation under reduced pressure, and the resultant syrup was subjected to flash chromatography over silica gel eluting with dichloromethane-methanol (19:1 (v/v)). The solvent was removed by evaporation under reduced pressure, with DMF added as needed to maintain the product in solution. After all dichloromethane had been removed, traces of methanol were removed by evaporation of added DMF (2 x 5 mL) under reduced pressure. The yield of 2',3'-methoxymethylidene[5'-^{18}O]adenosine was 1.03 g (3.3 mmol; 92%).

^{1}H NMR (400 MHz; DMSO) δ: 3.24 (3H, d, J = 0.7 Hz), 3.37 (3H, d, J = 0.7 Hz), 3.55 (m, 4H), 4.21 (1H, dd, J = 6 Hz, 8 Hz), 4.28 (1H, dd, J = 6,8), 4.99 (1H, dd, J = 3 Hz, 7 Hz), 5.08 (1H, dd, J = 3 Hz, 6 Hz), 5.18 (1H, dd, J = 6 Hz, 6 Hz), 5.24 (1H, J = 6 Hz, 6 Hz), 5.44 (1H, dd, J = 3 Hz, 6 Hz), 5.49 (1H, dd, J = 3 Hz, 6 Hz), 6.09 (1H, s), 6.16 (1H, d, J = 3 Hz), 6.19 (1H, s), 6.27 (1H, d, J = 3 Hz), 7.40 (2 H, br s), 8.17 (1H, s), 8.18 (1H, s), 8.36 (1H, s), 8.40 (1H, s).

Adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate. This synthesis is shown in Figure 1. The 2',3'-methoxymethylidene[5'-^{18}O]adenosine (1.0 g, 3.3 mmol) was suspended in dichloromethane (20 mL) containing tri-*n*-butlyamine (1.0 mL, 4.2 mmol), and the mixture was cooled in a dry ice-acetone bath. Phosphorus trichloride (320 μL, 3.7 mmol) was added, and the reaction was stirred under argon; the dry ice-acetone bath was not replenished. After 4 h, the solution was at room temperature, and all solid had dissolved. The solvent was removed by evaporation under reduced pressure. The resultant yellow oil was suspended in pyridine (10 mL), and [^{17}O]H_2O (300 μL; 17 mmol; 20.7% ^{16}O, 48.6% ^{17}O, 30.7% ^{18}O) was added. A gummy

precipitate formed then redissolved as the reaction proceeded. After 14 h, the solvent was removed by evaporation under reduced pressure. The resultant yellow oil was dried by evaporation of added pyridine (10 mL) under reduced pressure and then suspended in pyridine (20 mL). Bis(trimethylsilyl)acetamide (2.7 mL, 11 mmol) was added, and all material immediately entered solution. Elemental sulfur (0.18 g; 5.6 mmol atom) was added. After 2.5 h, the reaction was complete as judged by ^{31}P NMR. Water (5 mL) was added, and the solvent was removed by evaporation under reduced pressure. The resultant solution was diluted with 1 M ammonia (20 mL), and the cloudy solution (pH 9.4) was passed through a 0.2 μm filter (Nalge Company, Rochester, NY). The filtrate was reduced in volume by evaporation under reduced pressure, and the pH was adjusted to 2.0 with hydrochloric acid. The solution clouded at pH 3.6, then it slowly cleared. After 1 h, the solution was adjusted to pH 7.8 with ammonia. The solution was then subjected to chromatography over DE-52 (HCO_3^- form), eluting with a linear gradient (1 L + 1 L) of TEAB, pH 8 (50-250 mM; 4° C). Fractions bearing adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate were identified by the absorbance at 260 nm. Colorimetric assay using 5,5'-dithiobis(2-nitro)benzoic acid (DTNB) was also performed (Richard & Frey, 1982), allowing the exclusion of fractions bearing AMP and the identification of fractions bearing inorganic thiophosphate. Product-bearing fractions were combined, and solvent was removed by evaporation under reduced pressure. Buffer salts were removed by evaporation of two volumes of added water under reduced pressure; the pH was kept basic by addition of ammonia as needed. The yield of adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate was 1.6 mmol (47%). ^{31}P NMR (121 MHz, H_2O) δ: 44.77, 44.76, 44.74, 44.72 (all broad).

Sp-ADPα$^{17}O_2$,α^{18}O,αS (adenosine 5'-(1-thio[1-$^{17}O_2$,^{18}O]diphosphate)).[8] Adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate (510 μmol) was added to 20 mM triethanolamine·HCl buffer, pH 8.0, (500 mL) containing KCl (10 mM), $MgCl_2$ (3 mM), glucose 6-phosphate (3 mM), dithiothreitol (3 mM), phosphoenolpyruvate (6 mM), ATP (0.1 mM), and NADH (0.1 mM). A solution (2 mL) in 100 mM triethanolamine·HCl buffer, pH 8.0, containing adenylate kinase (5000 U), pyruvate kinase (5000 U), lactate dehydrogenase (500 U), and glucose-6-phosphate

dehydrogenase (500 U) was then added. After 4 h, the conversion of adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate into *S*P-ATP$\alpha^{17}O_2$,$\alpha^{18}O$,αS (adenosine 5'-(1-thio[1-$^{17}O_2$,^{18}O]triphosphate)) was complete as judged by ^{31}P NMR, and the enzymes were removed from the reaction mixture by ultrafiltration through a PM-10 membrane (Amicon). The filtrate was subjected to chromatography over DE-52 (HCO_3^- form), eluting with a linear gradient (1L + 1L) of TEAB, pH 8 (100-500 mM). Fractions bearing *S*P-ATP$\alpha^{17}O_2$,$\alpha^{18}O$,αS were identified by $A_{260\ nm}$ and worked up as before. The yield combined yield of *S*P-ATP$\alpha^{17}O_2$,$\alpha^{18}O$,αS and ATP was 490 μmol (92%). ^{31}P NMR (121 MHz, H_2O) δ: 43.1 (br d, J = 30 Hz), -5.96 (d, J = 22 Hz), -22.83 (dd, J = 28 Hz, 21 Hz).

The *S*P-ATP$\alpha^{17}O_2$,$\alpha^{18}O$,αS (490 μmol) was dissolved in 20 mM triethanolamine·HCl buffer, pH 8.0 (20 mL) containing $MgCl_2$ (10 mM) and glucose (250 mM). Hexokinase (170 U) in 100 mM triethanolamine·HCl buffer, pH 8.0 (100 μL) was added. After 1 h, the conversion of *S*P-ATP$\alpha^{17}O_2$,$\alpha^{18}O$,αS into *S*P-ADP$\alpha^{17}O_2$,$\alpha^{18}O$,αS was complete as judged by ^{31}P NMR. Hexokinase was removed by ultrafiltration using Centriprep-10 units (Amicon). The filtrate was subjected to chromatography over DE-52 (HCO_3^- form), eluting with a linear gradient (1L + 1L) of TEAB, pH 8.0 (100-400 mM). Fractions bearing *S*P-ADP$\alpha^{17}O_2$,$\alpha^{18}O$,αS were identified by $A_{260\ nm}$ and were worked-up as described for the other nucleotides. The yield of *S*P-ADP$\alpha^{17}O_2$,$\alpha^{18}O$,αS was 395 μmol (78% from adenosine 5'-[$^{17}O_2$,^{18}O]phosphorothioate; contaminated with 2% ADP). ^{31}P NMR (121 MHz, H_2O) δ: 41.18 (br d, J = 32 Hz), -6.33 (J = 33 Hz).

Sp-2',3'-methoxymethylidene-ATPγ$^{17}O_2$γ^{18}OγS (2',3'-methoxymethylideneadenosine 5'-(3-thio[3-$^{17}O_2$,^{18}O]triphosphate)).[8] 2',3'-Methoxymethylidene-AMP (0.48 mmol) was dissolved in DMF (10 mL) containing tri-*n*-octylamine (420 μL, 960 μmol), and the solvent was removed by evaporation under reduced pressure. The residue was dried by evaporation of added dioxane (2 x 5 mL) under reduced pressure then lyophilization of added dioxane (5 mL). The white solid was dissolved in dioxane (5 mL) containing tri-*n*-butylamine (340 μL, 1.4 mmol), and diphenyl chlorophosphate (100 μL, 0.48 mmol) was added. After 1.5 h, the solvent was

removed by evaporation under reduced pressure. The resultant oil was stirred with petroleum ether-diethyl ether (4:1 (v/v), 30 mL). After 0.5 h, the ether was decanted, and traces of ether were removed from the residue by evaporation of added dioxane (10 mL) under reduced pressure.

*S*P-ADP$\alpha^{17}O_2$,α^{18}O,αS (395 μmol) was suspended in DMF-pyridine (2:1 (v/v)) containing tri-*n*-octylamine (350 μL, 790 μmol), and the solvent was removed by evaporation under reduced pressure. The residue was dried by evaporation under reduced pressure of added pyridine-DMF (2:1 (v/v), 10 mL) then added pyridine (2 x 5 mL). The residue was suspended in pyridine (3 mL) and added to the activated 2',3'-methoxymethylidene-AMP. After 1.5 h, the reaction reaction was complete as judged by ^{31}P NMR. The solvent was removed by evaporation under reduced pressure, and the residue was partitioned (in the reaction flask) between diethyl ether (10 mL) and water (25 mL). The pH of the aqueous phase was adjusted from 5.4 to 8 with KOH (1 M). The resultant cloudy solution was washed with diethyl ether (10 mL), and the solvent was removed from the aqueous phase by evaporation under reduced pressure. The residue was dissolved in water (20 mL), and the pH was adjusted to 11 with KOH (1 M). This solution was washed with diethyl ether, and the solvent was removed from the aqueous phase by evaporation under reduced pressure.

The residue was dissolved in water (20 mL), and sodium periodate (0.1 g, 470 μmol) was added. After 0.5 h, the pH was adjusted to 10 with KOH (1 M), and the solution was incubated at 50° C. After 2 h, 2-mercaptoethanol (350 μL, 5 mmol) was added, and the pH was adjusted to 7.6 with HCl (1 M). The solution was subjected to chromatography as with *S*P-ATP$\alpha^{17}O_2$,α^{18}O,αS . The yield of *S*P-2',3'-methoxymethylidene-ATP$\gamma^{17}O_2\gamma^{18}O\gamma$S was 174 μmol (44%). ^{31}P NMR (121 MHz, H_2O) δ: 33.68 (br d, J = 31 Hz), -11.05 (d, J = 20 Hz), -11.06 (d, J = 19 Hz), -23.23 (dd, J = 19 Hz, 31 Hz).

*PAP-catalyzed hydrolysis of S*P*-2',3'-methoxymethylidene-ATP$\gamma^{17}O_2\gamma^{18}O\gamma$S.* The *S*P-2',3'-methoxymethylidene-ATP$\gamma^{17}O_2\gamma^{18}O\gamma$S (174 μmol) was dissolved in 100 mM MES·KOH buffer, pH 6.0, (90 mL) containing KCl (2 M), EDTA (5 mM), dithiothreitol (5 mM), and sodium potassium tartrate (5 mM). PAP (1.6 mg, 40 nmol) in 100 mM MES·KOH (200 μL)

was then added. The disappearance of ATPγS had been monitored by HPLC (under conditions described above) in a trial reaction, but S_P-2',3'-methoxymethylidene-ATPγ^{17}O$_2$γ^{18}OγS elutes as a very broad peak, so the appearance of 2',3'-methoxymethylideneadenosine 5'-phosphate (in the void volume) was monitored. Since this method of monitoring is not rigorously quantifiable, the reaction was continued for 10 times the length of the trial incubation to ensure complete hydrolysis. Therefore, after 1 h, the pH of the reaction mixture was adjusted to pH 9 with KOH (1 M), and one third of solution was diluted with water (to a final volume of 2 L) and subjected to chromatography over AG 1-X8 (HCO_3^- form), eluting with a linear gradient (500 mL + 500 mL) of TEAB, pH 8 (100-300mM). Fractions containing inorganic [^{16}O,^{17}O,^{18}O]thiophosphate were identified by colorimetric assay with DTNB. These fractions were combined, and solvent was removed by evaporation under reduced pressure. Buffer salt was removed by evaporation of one portion of added water under reduced pressure then lyophilization of added water. The yield of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was 20 μmol (34%). The ^{31}P NMR spectrum revealed contamination by 2',3'-methoxymethylideneadenosine 5'-phosphate (24 mol%; 2.85 ppm) and inorganic phosphate (7 mol%; 1.45 ppm). ^{31}P NMR (121 MHz, H_2O, pH 11) δ: 37.6 (br s).

Incorporation of [^{16}O,^{17}O,^{18}O]thiophosphate into S_p-ATPβS (adenosine 5'-(2-thiotriphosphate)).[9] One half of the total inorganic [^{16}O,^{17}O,^{18}O]thiophosphate (10 μmol) was dissolved in 14 mM triethanolamine·HCl buffer, pH 8.2, (7 mL) containing D_2O (30%), fructose 1,6-bisphosphate (38 mM), ADP (30 mM), NAD^+ (0.7 mM), $MgCl_2$ (16 mM), pyruvate (100 mM), and dithiothreitol (2 mM). A solution (0.14 mL) in 100 mM triethanolamine·HCl buffer, pH 8.0, containing glyceraldehyde-3-phosphate dehydrogenase (400 U), phosphoglycerate kinase (3400 U), aldolase (5 U), triosephosphate isomerase (50 U), and lactate dehydrogenase (55 U) was added. After 2.5 h, EDTA (150 μL, 1 M) was added, and the conversion of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate into ATPγS (adenosine 5'-(3-thiotriphosphate)) was complete as judged by ^{31}P NMR; the presence of inorganic phosphate in the initial solution resulted in the presence of ATP. Adenylate kinase (3250 U), KCl (22 μL, 3.7 M), AMP (0.4 g, 800 μmol) and $MgCl_2$ (300 μL, 1 M) were added. After 5 h, EDTA was added,

and the conversion of ATPγS into ADPβS (adenosine 5'-(2-thiodiphosphate))was complete as judged by ^{31}P NMR. Enzymes were removed by ultrafiltration using Centricon-10 units. EDTA (120 μL, 1 M) was added to the filtrate, and the solution was subjected to chromatography over DE-52 (HCO_3^- form), eluting with a linear gradient (500 mL + 500 mL) of TEAB, pH 8, (100-500 mM). Fractions were assayed both by $A_{260\ nm}$ and by enzymatic ADP assay.[10] ADPβS eluted as a shoulder following the ADP peak; the late ADP-bearing fractions were combined, and solvent was evaporated under reduced pressure. The ^{31}P NMR spectrum revealed 27% ADPβS and 73% ADP. Since the amount of ADPβS was small (5.6 μmol), the ADP was retained as carrier for the labeled compound. ^{31}P NMR (121 MHz, H_2O) δ: 32.90, 32.87, 32.62, 32.60 (isotopomeric d, J = 36 Hz;), -11,43 (d, J = 34 Hz).

The ADPβS resulting from the incorporation of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was dissolved in 20 mM triethanolamine·HCl, pH8.0 (5.2 mL), and the following were added: inorganic phosphate (42 μL, 1 M), fructose 1,6-bisphosphate (1.1 mL, 0.19 M), pyruvate (47 mg, 430 μmol), NAD^+ (200 μL, 10 mM), dithiothreitol (21 μL, 1 M), $MgCl_2$ (42 μL, 1 M), and D_2O (1 mL). A solution (70 μL) in 100 mM triethanolamine·HCl buffer, pH 8.0, containing glyceraldehyde-3-phosphate dehydrogenase (100 U), phosphoglycerate kinase (350 U), lactate dehydrogenase (80 U), triosephosphate isomerase (50 U), and aldolase (4 U) was added. After 2.5 h, EDTA (100 μL, 1 M) was added, and the conversion of ADPβS into S_p-ATPβS was complete as judged by ^{31}P NMR. Enzymes were removed by ultrafiltration using Centricon-10 units. The filtrate was subjected to chromatography over DE-52 (HCO_3^- form), eluting with a linear gradient (500 mL + 500 mL) of TEAB, pH 8 (200-500 mM). Fractions containing nucleotides were identified by $A_{260\ nm}$; these fractions were combined, and solvent was removed by evaporation under reduced pressure. Buffer salt was removed by evaporation of two portions of added water under reduced pressure; the pH was kept above 8 at all times by addition of KOH (1 M) as needed. The residue was dissolved in 75 mM EDTA buffer, pH 9.0, containing D_2O (50%) and dithiothreitol (20 mM). This solution was passed over a column (0.25 mL) of Chelex 100 (sodium form) that had been pre-equilibrated with the same buffer. The column was

eluted with the same buffer (2 x 0.25 mL), and the combined eluates were subjected to ^{31}P NMR analysis.

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Footnotes

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[2]Abbreviations: AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; ATPγS, adenosine 5'-(3-thio)triphosphate; NAD^+, β-nicotinamide adenine dinucleotide (oxidized form); NADH, β-nicotinamide adenine dinucleotide (reduced form); EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate buffer; U, unit (the amount of enzyme required to convert one micromole of substrate into product in one minute).

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[6]Fast atom bombardment mass spectrometry is not rigorously quantitative.

[7]The difference in volumes was mandated by the difference in density between water and methanol.

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Figure 1: The synthesis of AMP$\alpha^{17}O_2$,α^{18}O,αS. O = ^{16}O, O = ^{17}O, and O = ^{18}O; ***A*** = adenyl group. *a*: PCl_3, $H_2\phi$; *b*: bis(trimethylsilyl)acetamide; *c*: sulfur; *d*: H_2O, pH 2. For simplicity, all multiple bonds and charges on the phospho groups are omitted.

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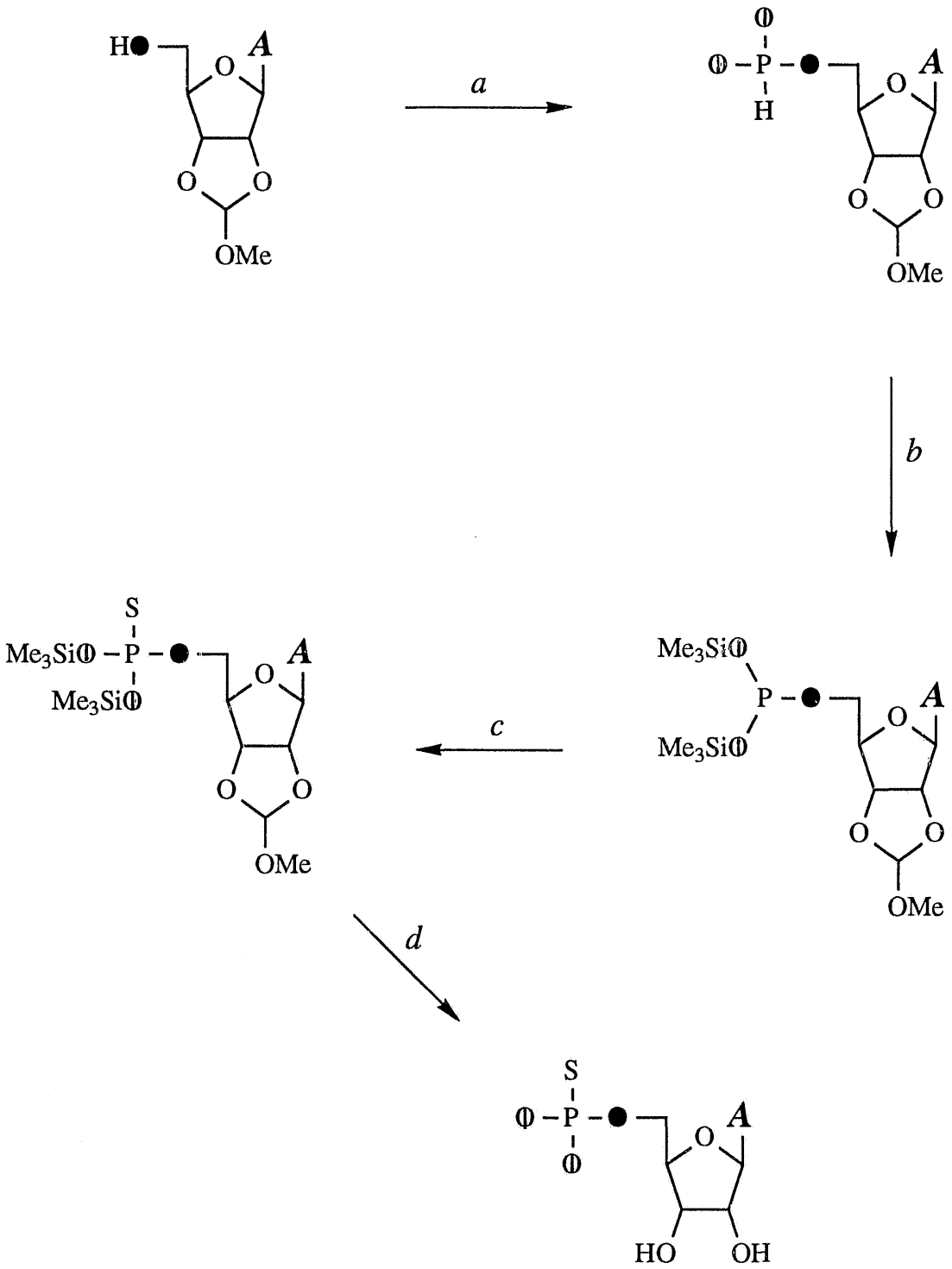

Fig. 1 Supplementary Material: Mueller et al.