Mevalonate-5-diphosphate Decarboxylase: Stereochemical Course of ATP-Dependent Phosphorylation of Mevalonate 5-Diphosphate†

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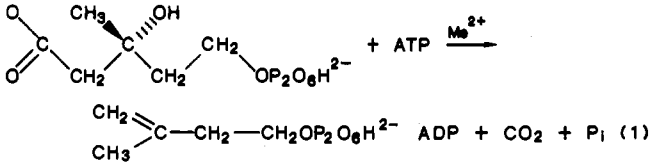
ABSTRACT: Chicken liver mevalonate-5-diphosphate decarboxylase catalyzes the reaction of mevalonate 5-diphosphate (MVADP) with ATP to produce isopentenyl diphosphate, ADP, CO_2, and inorganic phosphate. The overall reaction involves an anti elimination of the tertiary hydroxyl and carboxyl groups. To investigate the mechanism for transfer of the terminal phosphoryl group of ATP to the C-3 oxygen of MVADP, we have carried out the reaction using stereospecifically labeled (S_P)-adenosine 5′-*O*-(3-thio[3-$^{17}O_2$,^{18}O]triphosphate) ([γ-$^{17}O_2$,^{18}O]ATPγS) in place of ATP. The configuration of the [^{17}O,^{18}O]thiophosphate produced was found to be R_P, corresponding to overall inversion of configuration at phosphorus in the thiophosphoryl group transfer step. This result is consistent with the direct transfer of the thiophosphoryl group from (S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS to MVADP at the active site. Our result does not indicate the involvement of a covalent thiophosphoryl-enzyme on the reaction pathway.

Chicken liver mevalonate-5-diphosphate decarboxylase [ATP:5-diphosphomevalonate carboxylase; ATP:5-diphosphomevalonate carboxy-lyase (dehydrating), EC 4.1.1.33] catalyzes the reaction of mevalonate 5-diphosphate (MVADP)[1] with ATP to produce isopentenyl diphosphate, ADP, CO_2, and inorganic phosphate according to eq 1.



The overall reaction involves both decarboxylation at C-1 and dehydration of C-3 of MVADP. Previous studies by Lindberg et al. (1961) with a partially purified enzyme from yeast showed that when [3-^{18}O]MVADP and ATP were used as substrates [^{18}O]inorganic phosphate was produced. They postulated the transient involvement of a triphosphorylated MVA derivative on the reaction pathway. Attempts to obtain further proof of this intermediate have, however, been unsuccessful[2] [Hellig and Popjak, as quoted in Cornforth et al. (1966)]. Experiments in extracts using samples of mevalonate labeled stereospecifically with deuterium at C-2 [(2*R*)-[2-^{2}H]MVA and (2*S*)-[$^2H_1$]MVA)] demonstrated that the decarboxylation of MVADP proceeds by an anti elimination of the tertiary hydroxyl and carboxyl groups (Cornforth et al., 1966). It is not known from earlier work whether transfer of the terminal phosphoryl group of ATP to the C-3 oxygen of MVADP proceeds by a single-displacement or a double-displacement mechanism. In a single-displacement mechanism the enzyme catalyzes direct transfer of the phosphoryl group from ATP to MVADP, whereas in a double displacement the enzyme mediates transfer by covalent catalysis via an intermediate phosphoryl enzyme. Kinetic evidence suggests a sequential binding mechanism involving a compulsory ternary complex [E·MVADP·ATP] in the reaction as shown in eq 1 (Alvear et al., 1982). The kinetic data, therefore, exclude the possibility of a *free* phosphoryl-enzyme intermediate. However, the kinetic studies do not bear on the possibility that a phosphoryl-enzyme might exist as a catalytically important central complex (e.g., E–P·MVADP·ADP) in the reaction mechanism.

To obtain direct information about whether the phosphoryl transfer step proceeds by a single- or double-displacement mechanism, we have carried out the reaction using stereospecifically labeled [S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS in place of ATP and determined the absolute configuration of the [^{17}O,^{18}O]-inorganic thiophosphate produced using the stereoanalytical method developed by Webb and Trentham (1980). Our results show that the product is (R_P)-[^{17}O,^{18}O]thiophosphate, demonstrating that thiophosphoryl group transfer proceeds with overall inversion of configuration at phosphorus.

Experimental Procedures

Materials

Mevalonate-5-diphosphate decarboxylase was purified from chicken liver as described by Cardemil and Jabalquinto (1985).

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[1] Abbreviations: [α-$^{17}O_2$,5′-^{18}O]AMPαS, adenosine 5′-[^{18}O]*O*-(1-thio[1-$^{17}O_2$]phosphate); (S_P)-[α-$^{17}O_2$,5′-^{18}O]ADPαS, adenosine 5′-[^{18}O]*O*-(1-thio[1-$^{17}O_2$]diphosphate) with the *S* configuration about P_α; ADP, adenosine 5′-*O*-diphosphate; [β-^{17}O,^{18}O]ADPβS, adenosine 5′-*O*-(2-thio[2-^{17}O,2-^{18}O]diphosphate); (S_P)-[α-$^{17}O_2$,5′-^{18}O]ATPαS, adenosine 5′-[^{18}O]*O*-(1-thio[1-$^{17}O_2$]triphosphate) with the *S* configuration about P_α; (R_P)- and (S_P)-[β-^{17}O,^{18}O]ATPβS, adenosine 5′-(2-thio[2-^{17}O,2-^{18}O]triphosphate) having the *R* and *S* configurations about the β-phosphorus; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); [γ-^{17}O,^{18}O]ATPγS, adenosine 5′-*O*-(3-thio[3-^{17}O,^{18}O]triphosphate); (S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS, adenosine 5′-*O*-([2-^{17}O]-3-thio[3-^{17}O,3-^{18}O]-triphosphate) with ^{17}O bridging P_β and P_γ and the *S* configuration about P_γ; MVADP, mevalonate 5-diphosphate; MVA, mevalonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Me_2SO, dimethyl sulfoxide; DCC, *N*,*N*′-dicyclohexylcarbodiimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

[2] A. M. Jabalquinto and E. Cardemil, unpublished results.

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All other enzymes, coenzymes, and biochemicals were purchased from Sigma Chemical Co. or Boehringer-Mannheim and used without further purification. DEAE-Sephadex A-25 was purchased from Sigma Chemical, and Chelex-100, sodium form, Bio-Gel P-2 (200–400 mesh), Dowex 50, hydrogen form (100–200 mesh, 4% cross linked), and Dowex 1, chloride form (100–200 mesh, 2% cross-linked) were from Bio-Rab Laboratories. The latter resin was converted to the hydroxide form, just prior to use, by washing with 1 N NaOH until free of chloride ion (negative silver nitrate test) and then with water to neutral pH. Me_2SO, triethyl phosphate, and pyridine were dried over CaH_2 and then distilled. Dichloroacetic acid was dried over $MgSO_4$ before distillation. Methanol was distilled over magnesium methoxide, formed in situ. Thiophosphoryl trichloride, DCC, and tri-*n*-octylamine were vacuum distilled before use. Triethylamine was redistilled before use. 2′,3′-Isopropylideneadenosine was dried in vacuo at 110 °C for 24 h. Reagent-grade salts were purchased from commercial suppliers and used without further purification. Both $H_2{}^{17}O$ (containing 52.8% ^{17}O, 37.8% ^{18}O, and 9.4% ^{16}O) and $H_2{}^{18}O$ (containing 95.2% ^{18}O, 2.6% ^{17}O, and 2.2% ^{16}O) were purchased from Monsanto Research Corp., Mound Laboratory. ATPγS was obtained from Boehringer Mannheim and purified by column chromatography on DEAE-Sephadex A-25 as described by Goody and Eckstein (1971).

Methods

Thin-layer chromatographic analysis of nucleotides was carried out by using fluorescent-indicating Eastman 13181 silica gel plates, with 1-propanol–concentrated NH_3–water in the ratios 6:3:1 as the mobile phase. Adenine nucleotides were visualized as fluorescence-quenched spots under an ultraviolet lamp. Nucleotides containing sulfur were stained by the vapor of I_2.

Nucleotides were purified by anion-exchange chromatography through columns of DEAE-Sephadex A-25 in the HCO_3^- form. The columns were eluted with linear gradients of triethylammonium bicarbonate at 4 °C. Nucleotides in pooled column fractions were desalted by rotary evaporation in vacuo using a Buchi apparatus with a bath temperature no higher than 30 °C. The dried residues initially obtained were twice dissolved in small volume of methanol and again evaporated to remove final traces of triethylammonium bicarbonate. The nucleotides were finally dissolved in a minimum volume of water or triethylammonium bicarbonate and stored at −15 °C.

Mevalonate-5-diphosphate decarboxylase was assayed by the spectrophotometric assay method, as described by Cardemil and Jabalquinto (1985). The progress of the reaction between ATPγS or (S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS and MVADP catalyzed by MVADP decarboxylase was monitored by removing aliquots from the reaction solution and measuring ADP formed by the coupled actions of pyruvate kinase and lactate dehydrogenase. Thionucleotides with terminal thiophosphate groups and thiophosphate were qualitatively monitored in column eluates by their reaction with DTNB, as described by Richard and Frey (1982). Adenine nucleotide concentrations were calculated from measurements of A_{260} by using the extinction coefficient 15×10^3 M^{-1} cm^{-1}.

HPLC analysis was performed on a Waters Model 440 liquid chromatography system equipped with a ultraviolet detector and a Waters Novapak C_{18} column (3.9 mm × 15 cm).

^{31}P NMR Spectral Measurements. ^{31}P NMR spectra were recorded at 25 °C on a Nicolet 200-MHz instrument with quadrature detection. The spectrometer was field frequency locked on the resonance of deuterium in the solvent. Chemical shifts are reported relative to external 85% phosphoric acid. For observing effects of ^{18}O on the P_β signals of ATPβS and ADPβS, the decoupler was not used. The samples for high-resolution ^{31}P NMR were prepared by dissolving the compounds in deionized water and percolated through a small column of Chelex-100 (sodium form) in a Pasteur pipet. The column was washed with 5 mL of deionized water and the effluent lyophilized to dryness. The residue was dissolved in 0.6 mL of a 1 mM solution of EGTA in ^2H_2O, pD 9.0, and filtered through a 0.45-μm filters directly into the NMR tube. In the calculations of percentages of the ^{18}O-containing species, the areas under the peaks were determined by the triangulation method. The reported values are in excellent agreement with those obtained by computer integration.

Mass Spectral Analysis. Mass spectral analysis were recorded on a Kratos GC/MS 25 equipped with an Al Tech RSL-100 column (10 m) and a DS 55 data system. The ionization energy was 70 eV. Multiple scans were made of *m/e* 28–830, and at least four scans were used for the calculations of the enrichments.

The isotopic enrichment of adenosine was determined by volatilizing samples (2 μL of a solution in methanol containing 10% water) using a direct inlet probe. Unlabeled adenosine gave a M and M + 1 peak (~11%). Therefore, for the calculation of the ^{18}O enrichment the intensities of *m/e* 267 and 268 were compared with *m/e* 269 and 270.

Mass spectral analysis of the triethyl phosphate obtained from [α-$^{17}O_2$,5′-^{18}O]AMPαS were recorded on samples without prior GC purification (Iyengar et al., 1984). Samples (5 μL) were injected into the GC/MS at an initial column temperature of 40 °C followed by a hyperbolic temperature increase to 100 °C. The triethyl phosphate emerged from the column at ~3.5 min. The intensities of the base peak (*m/e* 99) and the molecular fragment (*m/e* 155) corresponding to $H_4PO_4^+$ and $(C_2H_5)_2H_2PO_4^+$, respectively, were used to determine the isotopic enrichments.

Synthesis of [5′-^{18}O]Adenosine. [5′-^{18}O]Adenosine was synthesized by a modification of the procedure of Webb and Trentham (1981). DCC (15 mmol) and 2′,3′-isopropylideneadenosine (5 mmol) were stirred with 22 mL of dry Me_2SO until the solids dissolved. Upon addition of dichloroacetic acid (0.2 mL), a white precipitate formed, and the heterogeneous mixture was stirred at room temperature. The progress of the reaction was monitored by HPLC analysis using a Novapak C_{18} column eluted with 15% acetonitrile in water at a flow rate of 0.7 mL/min. 2′,3′-Isopropylideneadenosine, 2′,3′-isopropylideneadenosine 5′-aldehyde, and adenosine emerged with retention times of 3, 8, and 13 min, respectively. After 90 min all the starting materials had been consumed and only 2′,3′-isopropylideneadenosine 5′-aldehyde and <5% adenosine could be detected. The reaction was quenched by the dropwise addition of a solution of oxalic acid (1.25 g) in 0.5 mL of methanol. Vigorous frothing due to carbon dioxide formation occurred. The resulting mixture was left stirring for 30 min at room temperature. The white precipitate of dicyclohexylurea was removed by filtration and washed with cold methanol (5 mL). The filtrate and washings were reduced to approximately 2 mL by rotary evaporation, combined with aqueous acetic acid (10% by volume, 200 mL), and stirred at 100 °C for 60 min. HPLC analysis of an aliquot using a Novapak C_{18} column eluted with 4% acetonitrile in water at a flow rate of 0.7 mL/min showed predominantly adenosine 5′-aldehyde, retention time 4 min with trace amounts of adenosine, retention time 11 min, and 2′,3′-iso-

propylideneadenosine 5′-aldehyde, retention time 7 min. The mixture was cooled on ice and filtered and the precipitate washed with cold aqueous acetic acid (10 mL). The volume of the combined filtrate and washings was reduced to approximately 20 mL by rotary evaporation followed by lyophilization to dryness. The residue was dried in vacuo over P_2O_5 overnight. The crude product (4 mmol, based on A_{260} measurement) was dissolved in 80 mL of dry methanol and 3.5 mL of $H_2{}^{18}O$ (95.2% ^{18}O) and stirred at 37 °C for 48 h. [5′-^{18}O]Adenosine 5′-aldehyde was then reduced to [5′-^{18}O]adenosine in a nitrogen atmosphere in a glovebag by the slow addition of solid sodium borohydride (760 mg, 20 mmol). The solution was stirred at room temperature for 30 min. HPLC analysis, using the system described earlier, indicated that the reduction had gone to completion. The reaction mixture was diluted to 500 mL, adjusted to pH 2.0 with 1 N HCl, and applied to a 1.5 × 30 cm column of Dowex 50 (100–200 mesh) in the hydrogen form. The column was washed with water (200 mL) and then eluted with 140 mM ammonia. All material absorbing at 260 nm eluted as a single band and was pooled (80 mL). The pooled fractions were diluted to 200 mL and adjusted to pH 10.0 with concentrated ammonia. The crude product was applied to a 1.5 × 30 cm column of Dowex I-X2 (100–200 mesh) in the hydroxide form. The column was washed with 300 mL of 30% methanol in water and then eluted with 50% methanol in water. Fractions of 15 mL volume were collected. [5′-^{18}O]Adenosine appeared in fractions 20–40 (1.2 mmol) and was >95% pure by HPLC analysis. The exact ^{18}O enrichment as determined by mass spectral analysis was 74.88 ± 0.45% ^{18}O. The site of the label was confirmed by thiophosphorylation of a portion of the adenosine and quenching the reaction with unlabeled water. ^{31}P NMR analysis of the resulting [5′-^{18}O]AMPαS indicated that the 5′-position contained all the ^{18}O.

Synthesis of [α-$^{17}O_2$,5′-^{18}O]*AMPαS*. The procedure used was similar to that reported by Richard and Frey (1982). [5′-^{18}O]Adenosine (440 μmol, dried in vacuo at 110 °C for 24 h) was dissolved in 25 mL of triethyl phosphate by carefully heating the suspension over a bunsen burner. Tri-*n*-octylamine (190 μL, 420 μmol) was added and the solution cooled in an ice–water bath. After addition of thiophosphoryl trichloride (43 μL, 420 μmol), the mixture was stirred at 40 °C for 75 min. Pyridine (0.8 mL) and $H_2{}^{17}O$ (0.13 mL, 52% ^{17}O) were added. A white precipitate formed, and the mixture was left stirring at room temperature overnight. The resulting [α-^{17}O$_2$,5′-^{18}O]AMPαS was purified by chromatography through a 2.5 × 30 cm column of DEAE-Sephadex A-25 preequilibrated with 0.05 M triethylammonium bicarbonate and eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.05 to 0.25 M, with a total volume of 4 L. [α-^{17}O$_2$,5′-^{18}O]AMPαS (260 μmol, 60% yield) eluted at 0.2 M salt. The exact ^{17}O,^{18}O content was determined by degrading 2 μmol of the product to [^{17}O,^{18}O]thiophosphate followed by GC/MS analysis (Iyengar et al., 1984). The observed values were within experimental error of the calculated enrichments.

Synthesis of (S_P)-[γ-$^{17}O_2$,^{18}O]*ATPγS*. The [α-^{17}O$_2$,5′-^{18}O]AMPαS isolated from the preceding reaction was first converted to (S_P)-[α-^{17}O$_2$,5′-^{18}O]ATPαS by stereospecific phosphorylation with phosphoenolpyruvate and a catalytic amount of ATP catalyzed by the coupled actions of adenylate kinase and pyruvate kinase as described by Sheu and Frey (1977). Hexokinase was then used to convert (S_P)-[α-^{17}O,5′-^{18}O]ATPαS to (S_P)-[α-^{17}O$_2$,5′-^{18}O]ADPαS in an overall 82% yield from [α-^{17}O$_2$,5′-^{18}O]AMPαS. (S_P)-[α-^{17}O$_2$,5′-^{18}O]ADPαS was then coupled with 2′,3′-methoxymethylidene-AMP by the Michelson phosphoanhydride synthesis. The product, (S_P)-P^1-[5′-^{18}O]adenosine-5′ P^3-2′,3′-methoxymethylideneadenosine-5′ 1-thio[1-$^{17}O_2$]triphosphate, was degraded by periodate-base elimination of the unprotected adenosyl moiety and deprotection of the other nucleoside, producing (S_P)-[γ-^{17}O$_2$,^{18}O]ATPγS. The procedure described by Richard and Frey (1978) was followed, except that periodate used to open the ribose ring of the unprotected adenosine was removed by the addition of a 10-fold molar excess of 2-mercaptoethanol and not by the addition of ethylene glycol and Na_3PSO_3. Just prior to use, [^{17}O,^{18}O]ATPγS was passed through a 1 × 68 cm column of Bio-Gel P-2 (200–400 mesh) equilibrated and eluted with water. Pooled fractions were lyophilized to dryness.

Enzymatic Reaction of ATPγS with MVADP, in the Presence of Metal Ions. The reaction of ATPγS with MVADP in the presence of MVADP decarboxylase and several metal ions was carried out at 30 °C in 0.13-mL solutions containing 3.6 mM ATPγS, 4.7 mM MVADP, 83 mM Tris-HCl buffer at pH 7, 0.16 unit of MVADP decarboxylase (specific activity 4.52 units/mg of protein), and 3.8 mM solution of the appropriate metal chloride. The progress of the reaction was followed by determining the ADP produced, in 10-μL aliquots, as described under Methods.

Enzymatic Conversion of (S_P)-[γ-$^{17}O_2$,^{18}O]*ATPγS and MVADP to* (R_P)-[^{17}O,^{18}O]*Thiophosphate*. [^{17}O,^{18}O]Inorganic thiophosphate was produced by the action of MVADP decarboxylase on (S_P)-[γ-^{17}O,^{18}O]ATPγS and MVADP in a 14-mL reaction mixture consisting of 3.6 mM (S_P)-[γ-^{17}O$_2$,^{18}O]ATPγS, 4.7 mM MVADP, 4 mM $MnCl_2$, 5 mM 2-mercaptoethanol, 100 mM Tris-HCl buffer at pH 7.0, and 9.5 units of MVADP decarboxylase (specific activity) 4.72 units/mg of protein) at 30 °C. The production of ADP and the activity of the MVADP decarboxylase (10-μL aliquots) was monitored throughout the course of the reaction as described under Methods. After 15 h, 69% of the theoretical yield of ADP had been produced, while the decarboxylase retained full activity. At this point, the reaction mixture was passed through Amicon ultrafiltration cones, diluted to approximately 30 mL with 50 mM triethylammonium bicarbonate, and applied to a 1.5 × 30 cm column of DEAE Sephadex A-25 that had been equilibrated with 0.1 M triethylammonium bicarbonate. The column was eluted with a 2-L linear gradient of the same buffer increasing in concentration from 0.1 to 0.6 M. Fractions of 16 mL were collected, and [^{17}O,^{18}O]thiophosphate was detected in fractions 25–36. The recovery of [^{17}O,^{18}O]thiophosphate as determined by DTNB assay was 14.6 μmol.

Incorporation of [^{17}O,^{18}O]*Thiophosphate into* [β-^{17}O,^{18}O]*ADPβS*. The [^{17}O,^{18}O]thiophosphate isolated from the above reaction was first converted to [γ-^{17}O$_2$,^{18}O]ATPγS according to the procedure of Webb (1982) and the compound purified by column chromatography using DEAE-Sephadex A-25. The terminal thiophosphoryl group of the purified [γ-^{17}O$_2$,^{18}O]ATPγS was then stereospecifically transferred to AMP by using adenylate kinase and the conditions described by Sheu et al. (1984). The [β-^{17}O,^{18}O]ADPβS (11.2 μmol) isolated was pure by HPLC and TLC analysis.

Synthesis of (R_P)-[β-^{17}O,^{18}O]*ATPβS*. [β-^{17}O,^{18}O]ADPβS (5.6 μmol) was enzymatically phosphorylated to (R_P)-[β-^{17}O,^{18}O]ATPβS with acetate kinase according to the procedure of Sheu et al. (1984). The isolated (R_P)-[β-^{17}O,^{18}O]ATPβS (4.5 μmol) was >95% pure by HPLC and TLC analysis but considerable degradation (30–40%) to [β-^{17}O,^{18}O]ADPβS

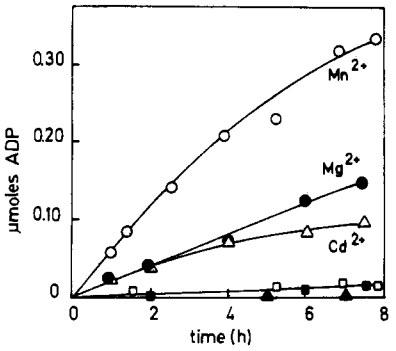


FIGURE 1: Time course of the reaction of ATPγS with MVADP in the presence of several different metal ions. The incubation media contained 0.47 μmol of ATPγS, in the conditions described under Experimental Procedures. Control experiments in the presence of Mn^{2+} (□), Mg^{2+} (■) or Cd^{2+} (▲) contained the enzyme plus all the components except MVADP.

occurred during preparation of the sample for ^{31}P NMR analysis.

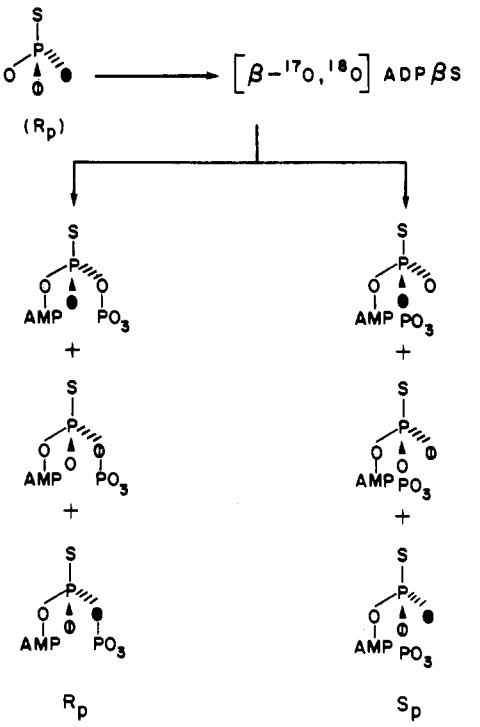
Synthesis of (S_P)-[β-^{17}O,^{18}O]*ATPβS*. [β-^{17}O,^{18}O]ADPβS (5.6 μmol) was enzymatically phosphorylated to (S_P)-[β-^{17}O,^{18}O]ATPβS with phosphoglycerate kinase by the procedure of Webb (1982). After column chromatography using DEAE-Sephadex A-25, (S_P)-[β-^{17}O,^{18}O]ATPβS (3.75 μmol) and unreacted [β-^{17}O,^{18}O]ADPβS (1.8 μmol) were isolated. The latter compound was then rephosphorylated at the *pro-S* position with phosphoglycerate kinase. The combined sample of (S_P)-[β-^{17}O,^{18}O]ATPβS (5.08 μmol) was >95% pure by TLC and HPLC analysis. However, considerable degradation (30–40%) to [β-^{17}O,^{18}O]ADPβS occurred during the preparation of the sample for ^{31}P NMR analysis.

Results and Discussion

The ability of ATPγS to behave as an alternative substrate in the MVADP decarboxylase reaction is illustrated in Figure 1. For this substrate Mn^{2+} is superior as the activating ion relative to Mg^{2+} or Cd^{2+}. This contrasts with the previously reported effects of metal ions activating the reaction of ATP, where Mg^{2+} and Mn^{2+} were shown to be equally effective (Alvear et al., 1982). The molecular basis for this difference is not clear at the present time but may be a consequence of the metal ion binding properties of S and O.

The reaction of chiral (S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS with MVADP in the presence of chicken liver MVADP decarboxylase proceeded at approximately 1% the rate observed with ATP. After 15 h, when 69% of the starting material was consumed, the chiral [^{17}O,^{18}O]thiophosphate was purified by DEAE-Sephadex column chromatography. The configuration of [^{17}O,^{18}O]thiophosphate, was determined by the procedure described by Webb and Trentham (1980). Reaction of [^{17}O,^{18}O]thiophosphate with glyceraldehyde 3-phosphate and ADP in the presence of glyceraldehyde-3-phosphate dehydrogenase, NAD^+, phosphoglycerate kinase, and required cofactors produced [γ-^{17}O,^{18}O]ATPγS. Further reaction of [γ-^{17}O,^{18}O]ATPγS with AMP in the presence of adenylate kinase and Mg^{2+} produced [β-^{17}O,^{18}O]ADPγS. The configuration at P_β was known to be the same as that of the starting [^{17}O,^{18}O]thiophosphate, since the two phosphotransfer steps catalyzed by phosphoglycerate and adenylate kinases both proceeded with inversion of configuration (Webb & Trentham, 1980; Richard & Frey, 1978). To determine the configuration at P_β, the [β-^{17}O,^{18}O]ADPβS was subjected to stereoselective enzymatic phosphorylation in two samples, one at the *pro-R* oxygen using acetate kinase and the other at the *pro-S* position with phosphoglycerate kinase, giving the structures shown in

Scheme I



Scheme I. The (R_P)- and (S_P)-[β-^{17}O,^{18}O]ATPβS were then separately analyzed by ^{31}P NMR. The stereoanalytical method depends on the nuclear quadrupole moment of ^{17}O which broadens the ^{31}P–^{17}O signals such that they are not observed in the ^{31}P NMR spectrum (Tsai, 1979). Consequently only signals corresponding to ^{16}O- and ^{18}O-containing species appear in the ^{31}P NMR spectrum. The presence of oxygen-18 causes an upfield shift in the ^{31}P resonance, and the magnitude of the shift is dependent upon the bond order (Cohn & Hu, 1978; Lowe et al., 1978). Species containing P_β–O–P_γ oxygen-18 have their ^{31}P resonance shifted by about .021 ppm, and a compound containing $^{31}P{=}{}^{18}O$ has its resonance shifted by about 0.035 ppm to higher field compared to the unlabeled compound (Frey & Sammons, 1985). One set of ^{31}P NMR lines for P_β of (S_P)-[β-^{17}O,^{18}O]ATPβS is shown in Figure 2 to illustrate the effect. The ^{31}P NMR spectrum of the β-phosphorus in ATPβS is a triplet in which each peak of the triplet shows four distinct resonances due to the presence of ^{18}O. Some of the signals are due to the partial ^{18}O-isotopic enrichment at all ^{17}O positions in Scheme I (the ^{17}O-enrichment was 52.8%) and do not affect the stereochemical results.

Table I shows the predicted ^{16}O and ^{18}O distribution for the two possible stereochemical outcomes and the observed distribution. The experimental spectra show a 15% loss of ^{18}O and ^{17}O. The isotope washout is probably due to enzymatic hydrolysis of the intermediate glycerate 1-thiophosphate 3-phosphate during the incorporation of [^{17}O,^{18}O]inorganic thiophosphate into [^{17}O,^{18}O]ATPγS. The P–O bond cleavage results in loss of isotope and partial racemization (assuming the reaction proceeds with inversion) (Webb & Trentham, 1980). During the preparation of the [β-^{17}O,^{18}O]ATPβS samples for ^{31}P NMR analysis a considerable amount (30–40%) of the compound decomposed to [β-^{17}O,^{18}O]ADPβS. The resonances due to P_β of [β-^{17}O,^{18}O]ADPβS (doublet centered at 34.323 ppm) are well separated from those of P_β of [β-^{17}O,^{18}O]ATPβS (triplet centered at 29.257 and 29.437 ppm for the R_P and S_P isomers, respectively) and can be used to check the ^{18}O enrichment at P_β. Each peak of the doublet of ADPβS shows three distinct resonances due to the presence

Table I: Peak Distribution for the ^{31}P NMR Spectrum of the β-Phosphorus of [β-^{17}O,^{18}O]ATPβS

species[a]	theoretical values (%) retention	theoretical values (%) inversion	observed values[b] (%)	theoretical values (corrected for 15% washout)[c] retention	theoretical values (corrected for 15% washout)[c] inversion
(R_P)-[β-^{17}O,^{18}O]ATPβS					
$^{16}O_2$	19	19	33	29	29
b-^{18}O, nb-^{16}O	43	23	26	37	24
b-^{16}O, nb-^{18}O	23	43	33	24	37
$^{18}O_2$	15	15	8	10	10
(S_P)-[β-^{17}O,^{18}O]ATPβS					
$^{16}O_2$	19	19	30	29	29
b-^{18}O, nb-^{16}O	23	43	37	23	37
b-^{16}O, nb-^{18}O	43	23	22	37	23
$^{18}O_2$	15	15	11	10	10

[a] The ATPβS species are designated by their oxygen isotopes in the $\beta\gamma$-bridging (b) and the β-nonbridging (nb) positions. [b] Average values from the peak area of the extreme upfield and downfield peaks of the triplet corresponding to Pβ of [^{17}O,^{18}O]ATPβS. [c] On the basis of observed peak distribution of the β-phosphorus of [^{17}O,^{18}O]ADPβS.

Table II: Isotopic Distribution for the ^{31}P NMR Spectrum of the β-Phosphorus of [β-^{17}O,^{18}O]ADPβS

oxygen isotopes	theoretial values (%)	observed values[a] (%)	theoretical values corrected for 15% isotope washout[b]
$^{16}O_2$	19	29	29
$^{18}O_1$	66	61	61
$^{18}O_2$	14	10	10

[a] Average value from peak areas for P_β of [^{17}O,^{18}O]ADPβS formed from decomposition of (R_P)- and (S_P)-[^{17}O,^{18}O]ATPβS. [b] Isotope washout during incorporation of [^{17}O,^{18}O]thiophosphate to [^{17}O,^{18}O]-ATPβS. See Results and Discussion for explanation.

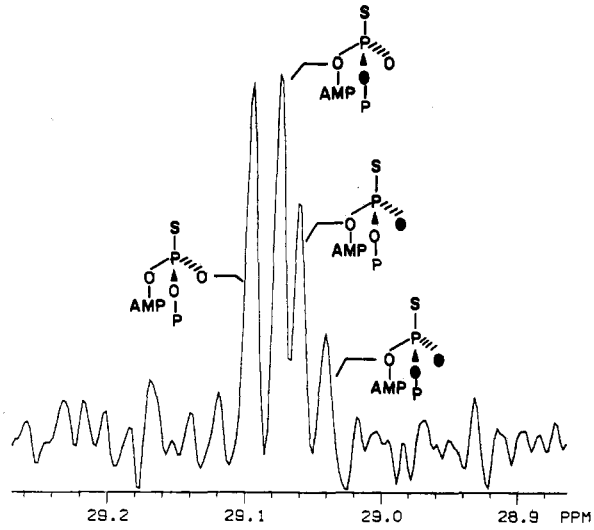
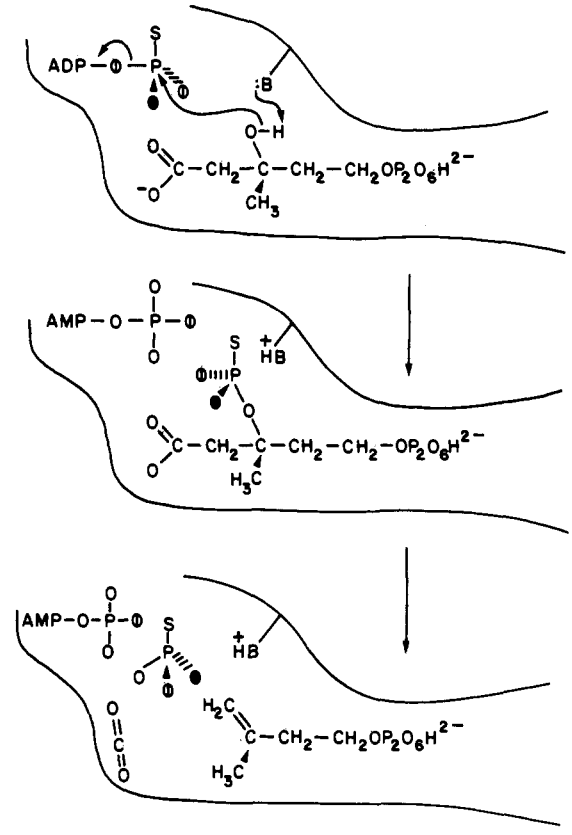


FIGURE 2: ^{31}P NMR spectra of the β-phosphorus of (S_P)-[^{17}O,^{18}O]ATPβS derived from the stereochemical analysis of [^{17}O,^{18}O]PSO_3^{2-}. The spectrum was obtained on a Nicolet 200 instrument at 80.1 MHz with a deuterium field lock and proton coupled: spectral width 400 Hz, aquisition time 2.56 s, and pulse width 6.0 μs. The signals for P_β are triplets. Shown are the extreme upfield spectral lines of the triplet corresponding to P_β of (S_P)-[^{17}O,^{18}O]-ATPβS. Chemical shift of the displayed resonances are $\delta_{P\beta}$ 29.096, 29.075, 29.061, 29.040. Other spectral parameters for signals not shown are $\delta_{P\beta}$ 29.781, 29.760, 29.746, 29.725 and 29.437, 29.415, 29.400, 29.379.

of oxygen-18. Table II gives the observed and calculated isotopic distribution. The experimental spectra for [β-^{17}O,^{18}O]ADPβS also show a 15% loss of isotope, probably due to the reason discussed earlier. When the theoretical values for the peak distribution of the β-phosphorus of [β-^{17}O,^{18}O]ATPβS are corrected for this 15% isotopic washout (Table I), the ^{31}P NMR spectra results are conclusive; chicken liver MVADP decarboxylase catalyzes the formation of [^{17}O,^{18}O]thiophosphate from (S_P)-[γ-$^{17}O_2$,^{18}O]ATPγS with

Scheme II



overall inversion of configuration at phosphorus.

Our observations are inconsistent with a double-displacement reaction mechanism in which the thiophosphoryl group is first transferred to an enzymic nucleophilic and in a subsequent step to the 3-OH group of MVADP. It has been established by several investigators that thiophosphoryl and phosphoryl transfer by such a mechanism proceeds with overall retention of configuration at phosphorus (Frey, 1982; Webb, 1982). The stereochemical results we have obtained, thus, suggests that ATPγS and MVADP are simultaneously bound to the enzyme at adjacent sites and the thiophosphoryl group is transferred directly from ATPγS to the 3-hydroxy group of MVADP. Such a mechanism is outlined in Scheme II for a two-step reaction pathway involving the initial abstraction of a proton from the 3-hydroxyl group of MVADP by a basic group in the enzyme in concert with thiophosphorylation of MVADP. In the second step decarboxylation and departure of thiophosphate generate the product isopentenyl diphosphate. A mechanism in which the thiophosphorylation and the de-

carboxylation are concerted cannot be excluded by our results but is less favored for lack of chemical precedent. The postulated initial enzymatic deprotonation of the tertiary hydroxyl group of MVADP is analogous to similar processes in the reaction mechanism of kinases (Dunaway-Mariano & Cleland, 1980). pH studies carried out in the University of Santiago (A. M. Jabalquinto and E. Cardemil, unpublished results) suggest a $pK < 4$ for such a group. The binding of the substrates to the active site of MVADP decarboxylase is probably strengthened by the presence of at least one arginyl residue in the region (Jabalquinto et al., 1983). We have not included a role for the divalent metal ions required for the reaction, although they most likely play a crucial role in the mechanism of phosphoryl group transfer and perhaps also in the decarboxylation step. This point is currently under investigation and will be the subject of a forthcoming publication.

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Registry No. MVADP, 103025-21-4; ATP, 56-65-5; (S_P)-[α-$^{17}O_2$,^{18}O]ATPγS, 88454-60-8; (R_P)-[β-^{17}O,^{18}O]ATPβS, 103025-22-5; (S_P)-[β-^{17}O,^{18}O]ATPβS, 103067-95-4; [5′-^{18}O]adenosine, 28080-29-7; [α-$^{17}O_2$,5′-^{18}O]AMPαS, 103025-23-6; (S_P)-[α-$^{17}O_2$,5′-^{18}O]ATPαS, 103025-24-7; (S_P)-[α-$^{17}O_2$,5′-^{18}O]ADPαS, 103025-25-8; 2′,3′-methoxymethylidene-AMP, 68973-49-9; 2′,3′-isopropylideneadenosine, 362-75-4; (S_P)-P^1-[5′-^{18}O]adenosine-5′ P^3-2′,3′-methoxymethylideneadenosine-5′ 1-thio[1-$^{17}O_2$]triphosphate, 103025-26-9; mevalonate-5-diphosphate decarboxylase, 9024-66-2.

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