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Phosphoenolpyruvate Synthetase and Pyruvate, Orthophosphate Dikinase: Stereochemical Consequences at both the β-Phospho and γ-Phospho Groups of ATP†

Arthur G. Cook and Jeremy R. Knowles*

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received June 15, 1984

ABSTRACT: [(*R*)-^{16}O,^{17}O,^{18}O]Phosphoenolpyruvate and adenosine 5′-*O*-[(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) have been synthesized and used to determine the stereochemical course of the several displacements at phosphorus catalyzed by phosphoenolpyruvate synthetase and by pyruvate, orthophosphate dikinase, two enzymes that catalyze the formation of phosphoenolpyruvate from pyruvate and ATP. The catalytic mechanisms for each of these enzymes are believed to involve both phospho- and pyrophospho-enzyme intermediates. The stereochemical results are entirely in accord with these pathways: the β-phospho group of ATP suffers overall retention of configuration that is presumably the consequence of two displacements with inversion, and the γ-phospho group of ATPγS suffers inversion of configuration that is most probably the consequence of a single displacement at this center.

The energetically favorable synthesis of phosphoenolpyruvate (PEP)[1] from pyruvate and ATP in extracts of *Escherichia coli* was first reported by Cooper & Kornberg (1965). The reaction yielded AMP and P_i as well as PEP, and the enzyme

$$\text{ATP} + \text{pyruvate} + H_2O \rightleftarrows \text{AMP} + \text{PEP} + P_i \quad (1)$$

involved has been named phosphoenolpyruvate synthetase (EC 2.7.9.2). In 1968, three groups (Hatch & Slack, 1968; Reeves, 1968; Reeves et al., 1968; Evans & Wood, 1968) reported a reaction that occurs in various microorganisms and several

$$\text{ATP} + \text{pyruvate} + P_i \rightleftarrows \text{AMP} + \text{PEP} + PP_i \quad (2)$$

plants that yields PEP, AMP, and PP_i. The enzyme catalyzing this reaction was named pyruvate, orthophosphate dikinase (EC 2.7.9.1) (Wood et al., 1977).

The initial proposals for the reaction sequence of PEP synthetase from *E. coli* and *Salmonella typhimurium* (Cooper & Kornberg, 1967a-c) and of pyruvate, orthophosphate dikinase from *Propionibacteria shermanii* (Evans & Wood, 1968) suggested three partial reactions for each of these two enzymes: eq 3, 4a, and 5 and eq 3, 4b, and 5, respectively.

$$\text{ATP} + \text{enzyme} \rightleftarrows \text{AMP} + \text{enzyme}\text{–}P_\beta P_\gamma \quad (3)$$

$$H_2O + \text{enzyme}\text{–}P_\beta P_\gamma \rightleftarrows P_{\gamma i} + \text{enzyme}\text{–}P_\beta \quad (4a)$$

$$P_i + \text{enzyme}\text{–}P_\beta P_\gamma \rightleftarrows P_\gamma P_i + \text{enzyme}\text{–}P_\beta \quad (4b)$$

$$\text{pyruvate} + \text{enzyme}\text{–}P_\beta \rightleftarrows \text{PEP} + \text{enzyme} \quad (5)$$

These mechanistic schemes were proposed primarily on the basis of investigations of the following exchange reactions: [^{14}C]AMP/ATP; $H_2{}^{18}O/P_i$; [^{32}P]P_i/PP_i; [^{32}P]PEP/ATP; [^{32}P]P_i/ATP; [^{32}P]PP_i/ATP; [^{14}C]pyruvate/PEP (Cooper &

† This work was supported by grants from the National Institutes of Health (to J.R.K.) and the National Science Foundation (to the Department of Chemistry) toward the purchase of the Bruker WM-300 and Bruker AM-300 spectrometers.

[1] Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$; PEP, phosphoenolpyruvate; P_i, orthophosphate; PP_i, pyrophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-0051$01.50/0

Kornberg, 1967a,b; Berman et al., 1967; Evans & Wood, 1968; Milner & Wood, 1972).

Further studies on pyruvate, orthophosphate dikinase from *Bacteroides symbiosus* by Reeves et al. (1968) support the above mechanistic path. It has been shown that the phospho group of the phospho-enzyme intermediate is bound to the enzyme through an acid-labile phosphoramidate linkage to the 3′-nitrogen of a histidine residue (Spronk et al., 1976; Milner et al., 1978; Goss et al., 1980). A pyrophospho-enzyme intermediate, also bound through an acid-labile linkage, has been detected by Milner et al. (1978); however, the instability of this species has prevented its isolation and characterization. Initial velocity and exchange kinetics (Milner & Wood, 1976) are in accord with a tri-uni-uni ping-pong mechanism, as required by the partial reactions detailed above.

Additional work on PEP synthetase from *E. coli* has also demonstrated the existence of a phospho-enzyme intermediate, again bound through a phosphoramidate linkage to the 3′-nitrogen of a histidine residue (Narindorasorak & Bridger, 1971). However, no pyrophospho-enzyme form has been detected for this system. Kinetic studies (Berman & Cohn, 1970b) have shown that AMP is required to make the P_i/H_2O exchange rate (measured by using $H_2{}^{18}O$) as fast as the P_i/ATP exchange rate (measured by using $[^{32}P]P_i$). These data suggest that a free pyrophospho-enzyme intermediate, as indicated by reactions 3 and 4a, is improbable. Indeed, Berman & Cohn (1970b) have suggested a mechanism that excludes such a free pyrophospho-enzyme species (eq 6 and 7). It is

$$\text{ATP} + \text{H}_2\text{O} + \text{enzyme} \rightleftarrows \text{AMP} + \text{P}_{\gamma\text{i}} + \text{enzyme–P}_\beta \quad (6)$$

$$\text{pyruvate} + \text{enzyme–P}_\beta \rightleftarrows \text{PEP} + \text{enzyme} \quad (7)$$

interesting to note that the pyruvate, orthophosphate dikinases isolated from tropical grass, sugar cane, maize leaves, and sorghum also appear to avoid a free pyrophospho-enzyme intermediate (Andrews & Hatch, 1969).

The mechanistic pathways apparently followed by these two PEP-synthesizing enzymes are bizarre when compared to the relative simplicity of the reactions catalyzed by the majority of phosphokinases. Moreover, since the putative pyrophospho-enzyme has never been fully characterized for either system, we decided to test the validity of each of the proposed schemes by evaluating the overall stereochemical consequence at each of the two phosphorus centers that suffer nucleophilic displacement during the enzymatic reactions.

To establish the stereochemical consequence of phospho- and pyrophospho-transfer reactions for PEP synthetase and for pyruvate, orthophosphate dikinase, we have determined the stereochemical course of the reactions at phosphorus. The stereochemical fate of the β-phosphorus of ATP was established by using [(*R*)-^{16}O,^{17}O,^{18}O]phosphoenolpyruvate, generated in situ from synthetic 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate, as the substrate and then determining the absolute configuration of the derived adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate using the stereoanalytical method described by Jarvest et al. (1981). The stereochemical fate of the γ-phosphorus of ATP was established by using synthetic adenosine 5′-*O*-[(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) and determining the absolute configuration of the derived inorganic [^{16}O,^{17}O,^{18}O]thiophosphate using the stereoanalytical method developed independently by Tsai (1979) and by Webb & Trentham (1980a).

Experimental Procedures

Materials. The following materials were obtained from Sigma Chemical Co.: aldolase (rabbit muscle, ammonium sulfate suspension, 15 units/mg), enolase (yeast, lyophilized powder, 68 units/mg), D-glucose-6-phosphate dehydrogenase (yeast, ammonium sulfate suspension, 330 units/mg), D-glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, ammonium sulfate suspension, 80 units/mg), glycerokinase (*E. coli*, lyophilized powder, 79 units/mg), α-glycerophosphate dehydrogenase (rabbit muscle, ammonium sulfate suspension, 119 units/mg), hexokinase (yeast, ammonium sulfate suspension, 320 units/mg), lactate dehydrogenase (bovine heart, ammonium sulfate suspension, 545 units/mg), malic dehydrogenase (pigeon muscle, ammonium sulfate suspension, 5300 units/mg), myokinase (rabbit muscle, ammonium sulfate suspension, 1160 units/mg), 3-phospho-D-glycerate phosphokinase (yeast, ammonium sulfate suspension, 1800 units/mg), pyruvate kinase (rabbit muscle, ammonium sulfate suspension, 355 units/mg), triosephosphate isomerase (rabbit muscle, ammonium sulfate suspension, 4500 units/mg), AMP sodium salt, ADP sodium salt, D-fructose 1,6-bisphosphate trisodium salt, 2′,3′-isopropylideneadenosine, NAD^+, NADH disodium salt, $NADP^+$ sodium salt, and phosphoenolpyruvate trisodium salt. Phosphoenolpyruvate carboxylase (yeast, ammonium sulfate suspension, 5 units/mg) and inorganic pyrophosphatase (yeast, ammonium sulfate suspension, 250 units/mg) were obtained from Boehringer Mannheim Biochemicals. All enzymes obtained as ammonium sulfate suspensions were dialyzed for 2 h before use against 50 mM Tris-HCl buffer, pH 6.7 or 8.0, containing dithioerythritol (10 mM) and EDTA (1 mM). Enzymes obtained as lyophilized powders were dissolved immediately before use in 50 mM Tris-HCl buffer, pH 6.7 or 8.0. Sodium pyrophosphate was obtained from Fisher Chemical Co. All other chemicals were of the highest grade available commercially. [^{17}O]Water (containing 9.9% ^{16}O, 51.1% ^{17}O, and 39.0% ^{18}O) and [^{18}O]water (containing 1.6% ^{16}O, 0.3% ^{17}O, and 98.1% ^{18}O) were obtained from Monsanto Research Corp. DEAE-cellulose (DE-52) was obtained from Whatman, Inc. Dowex-1 (200–400 mesh, 8% cross-linked), Dowex-50 (100–200 mesh, 8% cross-linked), and Sephadex G-25 (50–150 μm) were obtained from Sigma Chemical Co. AG 1-X8 (200–400 mesh) was obtained from Bio-Rad Laboratories. Ultrafiltration membrane cones were obtained from Amicon Corp. Polygram CEL PEI/UV254 thin-layer chromatography plates were obtained from Brinkman Instruments, Inc.

Clostridium symbiosus (ATCC 14940, formerly *Bacteroides symbiosus*) and *E. coli*, strain B, were obtained from the American Type Culture Collection.

2-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate was prepared as described by Blättler & Knowles (1980). Adenosine 5′-*O*-[(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) was prepared as described by Webb (1982).

Phosphoenolpyruvate synthetase was isolated according to the method described by Berman & Cohn (1970a) and had a specific activity of 6.03 units/mg. Pyruvate, orthophosphate dikinase was partially purified according to the method of Milner et al. (1975) and had a specific activity of 8.70 units/mg.

Methods. ^{1}H NMR spectra were recorded on a Varian FT-80, Bruker WM-300, or Bruker AM-300 instrument. Routine ^{31}P NMR spectra were recorded on a Varian XL-100 instrument. ^{13}C NMR spectra were recorded on a Bruker WM-300 or Bruker AM-300 machine. The high-resolution ^{31}P NMR spectra for stereochemical analysis were recorded on a Bruker WM-300 instrument. Chemical shifts are reported relative to external 85% phosphoric acid. Downfield shifts are positive. Mass spectra were recorded on an AEI

MS-9 or Kratos MS-50L double-focusing instrument.

Production of Adenosine [β-^{16}O,^{17}O,^{18}O]Diphosphate by Phosphoenolpyruvate Synthetase. 2-[(*R*)-^{16}O,^{17}O,^{18}O]-Phospho-D-glycerate ammonium salt (1 mmol) was dissolved in 105 mM potassium phosphate buffer, pH 6.8 (10 mL), containing AMP (1.1 mmol), $NADP^+$ (1.1 mmol), $MgCl_2$ (5 mM), D-glucose (660 mM), enolase (45 units), hexokinase (50 units), and D-glucose-6-phosphate dehydrogenase (60 units). The synthetase reaction was initiated by the addition of PEP synthetase (48 units). The reaction catalyzed by PEP synthetase was coupled to that catalyzed by hexokinase; thus, all adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate formed in the synthetase reaction was converted to adenosine [β-^{16}O,^{17}O,^{18}O]diphosphate. Coupling of the hexokinase reaction to that of the dehydrogenase also allowed monitoring of the PEP synthetase reaction from the change in absorbance at 340 nm corresponding to the appearance of NADPH. After 2 h at room temperature the enzymes were removed by ultrafiltration. The ultrafiltrate was applied to a column (2 L) of DEAE-cellulose equilibrated with 50 mM triethylammonium bicarbonate buffer, pH 7.0. The column was eluted with a linear gradient (4 L plus 4 L) of 50–500 mM triethylammonium bicarbonate, pH 7.0. Fractions containing adenosine [β-^{16}O,^{17}O,^{18}O]diphosphate [identified by thin-layer chromatography on poly(ethylene imine) plates eluted with 0.75 M KH_2PO_4 adjusted to pH 3.4 with concentrated HCl] were pooled, and the solvent was removed by evaporation under reduced pressure. Buffer salts were removed by three successive evaporations of added 2-propanol. The proton-decoupled ^{31}P NMR spectrum (D_2O, pD 7.0) showed two doublets, at δ −10.46 (d, J = 21.0 Hz, P_β) and −11.33 (d, J = 21.0 Hz, P_α).

Production of Inorganic [^{16}O,^{17}O,^{18}O]Thiophosphate by Phosphoenolpyruvate Synthetase. Adenosine 5′-*O*-[(γ*S*)-β,γ-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) triethylammonium salt (30 μmol) was dissolved in 50 mM Tris-HCl buffer, pH 8.0 (10 mL), containing dithioerythritol (10 mM), EDTA (1 mM), $MgCl_2$ (10 mM), sodium pyruvate (5 mM), $KHCO_3$ (5 mM), NADH (100 μmol), phosphoenolpyruvate carboxylase (50 units), and malic dehydrogenase (50 units). The synthetase reaction was initiated by the addition of PEP synthetase (48 units). The reaction was monitored by recording the change in absorbance at 340 nm, corresponding to the disappearance of NADH. After 24 h at room temperature the enzymes were removed by ultrafiltration. The proton decoupled ^{31}P NMR spectrum (D_2O) indicated all of the [(γ*S*)-β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS had been consumed and inorganic [^{16}O,^{17}O,^{18}O]thiophosphate had been produced. The ultrafiltrate was applied to a column (2 L) of DEAE-cellulose equilibrated with 75 mM triethylammonium bicarbonate buffer, pH 7.0. The column was eluted with a linear gradient (4 L plus 4 L) of 75–400 mM triethylammonium bicarbonate buffer, pH 7.0. Fractions that contained thiophosphate [identified by detecting the thiol with 5,5′-dithiobis(2-nitrobenzoic acid)] were pooled, and the solvent was removed by evaporation under reduced pressure. Buffer salts were removed by successive evaporation of three additions of 2-propanol. The proton-decoupled ^{31}P NMR spectrum (D_2O, pD 7.0) had a singlet at δ 35.220 ppm.

Production of Adenosine [β-^{16}O,^{17}O,^{18}O]Diphosphate by Pyruvate, Orthophosphate Dikinase. 2-[(*R*)-^{16}O,^{17}O,^{18}O]-Phospho-D-glycerate ammonium salt (1 mmol) was dissolved in 50 mM imidazole hydrochloride buffer, pH 6.7 (10 mL), containing AMP (1.1 mmol), PP_i (1.1 mmol), $NADP^+$ (1.1 mmol), $MgCl_2$ (20 mM), NH_4Cl (25 mM), D-glucose (105 mM), enolase (10 units), hexokinase (17 units), and D-glucose-6-phosphate dehydrogenase (18 units). The dikinase reaction was initiated by the addition of pyruvate, orthophosphate dikinase (15 units). The reaction catalyzed by pyruvate, orthophosphate dikinase was coupled to that catalyzed by hexokinase, as described previously for PEP synthetase. The product, adenosine [β-^{16}O,^{17}O,^{18}O]diphosphate, was isolated and purified as described for PEP synthetase. The ^{31}P NMR spectrum (D_2O, pD 7.0) showed two doublets at δ −10.47 (d, J = 20.5 Hz, P_β) and −11.33 (d, J = 20.5 Hz, P_α).

Production of Inorganic [α-S,α-^{17}O,^{18}O]Thiopyrophosphate by Pyruvate, Orthophosphate Dikinase. Adenosine 5′-*O*-[(γ*S*)-β,γ-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) triethylammonium salt (30 μmol) was dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing dithioerythritol (10 mM), EDTA (1 mM), $MgCl_2$ (10 mM), NH_4Cl (10 mM), sodium pyruvate (5 mM), potassium phosphate (5 mM), $KHCO_3$ (5 mM), NADH (100 μmol), phosphoenolpyruvate carboxylase (50 units), and malic dehydrogenase (50 units). The dikinase reaction was initiated by the addition of pyruvate, orthophosphate dikinase (45 units). The reaction was monitored as described for PEP synthetase. After 24 h at room temperature the enzymes were removed by ultrafiltration. The proton-decoupled ^{31}P NMR spectrum (D_2O) indicated all of the [(γ*S*)-β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS had been consumed and [α-S,α-^{17}O,^{18}O]thiopyrophosphate had been produced. The ultrafiltrate was applied to a column (2 L) of DEAE-cellulose equilibrated with 75 mM triethylammonium bicarbonate buffer, pH 7.0. The column was eluted with a linear gradient (4 L plus 4 L) of 75–400 mM triethylammonium bicarbonate buffer, pH 7.0. Fractions that contained thiopyrophosphate [identified by detecting the thiol with 5,5′-dithiobis(2-nitrobenzoic acid)] were pooled, and solvent was removed by evaporation under reduced pressure. Buffer salts were removed by successive evaporation of three additions of 2-propanol.

[α-S,α-^{17}O,^{18}O]Thiopyrophosphate was converted to inorganic [^{16}O,^{17}O,^{18}O]thiophosphate by inorganic pyrophosphatase. [α-S,α-^{17}O,^{18}O]Thiopyrophosphate triethylammonium salt (30 μmol) was dissolved in 50 mM Tris-HCl buffer, pH 8.0 (5 mL), containing dithioerythritol (10 mM), $MgCl_2$ (10 mM), and inorganic pyrophosphatase (1000 units). After 1 h at room temperature the proton-decoupled ^{31}P NMR spectrum (D_2O) indicated all of the [α-S,α-^{17}O,^{18}O]thiopyrophosphate had been consumed and inorganic [^{16}O,^{17}O,^{18}O]thiophosphate had been produced. The enzyme was removed by ultrafiltration and the ultrafiltrate applied to a DEAE-cellulose column as previously described for PEP synthetase. Inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was isolated and purified as described for PEP synthetase. The proton-decoupled ^{31}P NMR spectrum (D_2O,pD 7.0) appeared as a singlet at δ 35.175.

Stereochemical Analysis of Adenosine [β-^{16}O,^{17}O,^{18}O]Diphosphate. Adenosine [β-^{16}O,^{17}O,^{18}O]diphosphate triethylammonium salt was dissolved in 70 mM triethanolamine hydrochloride buffer, pH 7.6 (10 mL), containing $MgCl_2$ (10 mM) and D-glucose (105 mM). Myokinase (60 units) and hexokinase (60 units) were added to initiate the formation of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate. After 30 min at room temperature the enzymes were removed by ultrafiltration. The ultrafiltrate was applied to a column (60 mL) of AG 1-X8 (HCO_3^- form) equilibrated with 25 mM triethylammonium bicarbonate buffer, pH 7.0. The column was eluted with a linear gradient (300 mL plus 300 mL) of 25–250 mM triethylammonium bicarbonate buffer, pH 7.0. Fractions con-

taining D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate were pooled, and the solvent was removed by evaporation under reduced pressure. Buffer salts were removed by successive evaporation of three additions of 2-propanol. The D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate triethylammonium salt was converted to the bis(monocyclohexylammonium) salt by passage through a column (2 mL) of Dowex-50 (H^+ form) into dioxane containing excess monocyclohexylamine. D-Glucose 6-[^{16}O,^{17}O,^{18}O]phosphate bis(monocyclohexylammonium) salt was isolated by evaporation of the solvent under reduced pressure.

The yield of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate from the PEP synthetase reaction, as determined by enzymatic assay (Bergmeyer, 1974), was 0.81 mmol (81% from 2-phosphoglycerate). The proton-decoupled ^{31}P NMR spectrum (D_2O) showed a singlet at δ 4.101, which, when undecoupled, split into a triplet [J(PH) = 6.30 Hz].

The yield of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate from the pyruvate, orthophosphate dikinase reaction, as determined by enzymatic assay (Bergmeyer, 1974), was 0.90 mmol (90% from 2-phosphoglycerate). The proton-decoupled ^{31}P NMR spectrum (D_2O) showed a singlet at δ 4.088, which, when undecoupled, split into a triplet [J(PH) = 6.31 Hz].

D-Glucose 6-[^{16}O,^{17}O,^{18}O]phosphate was prepared for stereochemical analysis by modification of the procedure described by Begley et al. (1982). The bis(monocyclohexylammonium) salt of the product D-glucose 6-[^{16}O,^{17}O,^{18}O]-phosphate was converted to the mono(tri-*n*-butylammonium) mono(tri-*n*-octylammonium) salt by passage through a column (10 mL) of Dowex-50 (H^+ form) into dioxane (5 mL) containing equimolar amounts of tri-*n*-butylamine and tri-*n*-octylamine. The resulting solution was evaporated to dryness under reduced pressure 4 times from added dry dioxane and then freeze-dried once from dry dioxane. Dioxane–dimethylformamide (1:1, v/v) (1 mL) was added followed by 10 molecular sieves (4 Å). The solution was stirred for 3 h, and freshly distilled diphenyl phosphorochloridate (0.9 equiv) was then added. After the solution was stirred for 20 min, a solution of freshly sublimed potassium *tert*-butoxide (10 equiv) in dry dimethylformamide (5 mL) was added, and the golden solution was stirred for an additional 10 min. The reaction was then quenched by pouring onto Dowex-50 (pyridinium form) which had been dried for 24 h at 105 °C. The mixture was filtered and the filtrate evaporated to dryness. The cyclic diester product was purified by ion-exchange chromatography on a column (20 mL) of AG 1-X8 (HCO_3^- form) equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 7.0. The column was eluted with a linear gradient (200 mL plus 200 mL) of 10–100 mM triethylammonium bicarbonate buffer, pH 7.0. Fractions containing the diester were pooled, and the solvent was removed by evaporation under reduced pressure. Buffer salts were removed by successive evaporation of three additions of 2-propanol. The purified diester was then converted into the potassium salt by passage down a column (10 mL) of Dowex-50 (K^+ form). The eluate was evaporated to dryness.

The diester resulting from the PEP synthetase reaction showed a singlet in the proton-decoupled ^{31}P NMR spectrum (D_2O) at δ −2.525. When proton undecoupled, this signal appeared as a doublet [J(PH) = 22.0 Hz]. The diester resulting from the pyruvate, orthophosphate dikinase reaction showed a singlet in the proton-decoupled ^{31}P NMR spectrum at δ −2.614. When undecoupled this appeared as a doublet [J(PH) = 22.0 Hz].

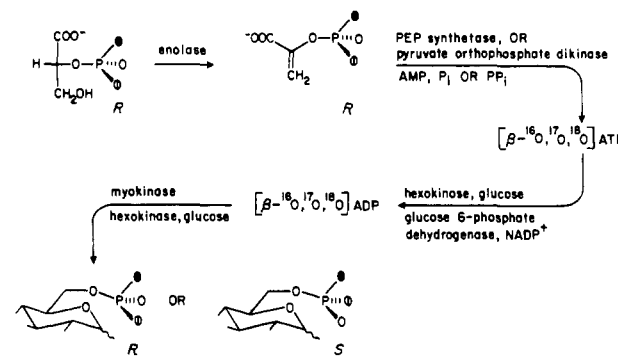

FIGURE 1: Scheme for the investigation of the stereochemical course at P_β, in the reactions catalyzed by PEP synthetase and by pyruvate, orthophosphate dikinase.

The cyclic diester was dissolved in H_2O (3 mL), and the cyclic polyether 18-crown-6 (25 mg) was added. The solution was then evaporated to dryness 4 times from added dry dioxane. Dry dimethyl-d_6 sulfoxide (1 mL) was added followed by methyl iodide (100 μL), and the mixture was stirred for 20 h. Dry methanol was added and the solution transferred to a dry 10-mm NMR tube for high-resolution ^{31}P NMR analysis.

Stereochemical Analysis of Inorganic [^{16}O,^{17}O,^{18}O]*Thiophosphate.* Inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was converted to isotopically labeled ATPβS according to the method of Webb (1982) as modified by Senter et al. (1983), which is shown in Figure 5.

Results and Discussion

Stereochemical Fate of the β-Phosphorus of ATP. [(*R*)-^{16}O,^{17}O,^{18}O]Phosphoenolpyruvate, generated in situ from 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate in the presence of enolase, has been used as substrate for PEP synthetase and for pyruvate, orthophosphate dikinase (Figure 1), and the absolute configuration at P_β of the product, adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate, has been determined. Control experiments demonstrated that no ATP was produced when PEP was omitted from the PEP synthetase reaction mixture or when PP_i was omitted from the pyruvate, orthophosphate dikinase reaction mixture.

Analysis of the product, adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate, involved enzymatic transfer of the β-phospho group of ATP to D-glucose to form D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate, in reactions catalyzed by hexokinase and myokinase, as illustrated in Figure 1. The net result of these two transfers, each of which is known from earlier work (Blättler & Knowles, 1979; Richard & Frey, 1978) to proceed with inversion of the configuration at phosphorus, is overall retention of the configuration at the β-phosphorus of adenosine [β-^{16}O,^{17}O,^{18}O]-diphosphate when this β-phospho group is transferred to D-glucose to yield D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate.

The configuration at phosphorus in these derived samples of D-glucose 6-phosphate was now determined. The stereoanalytical method depends upon the different perturbations of the NMR signal of a ^{31}P nucleus produced by the oxygen isotopes that are bonded to it. The torsional equivalence of the three peripheral phospho group oxygens of glucose 6-phosphate is first removed by ring closure to the glucose cyclic 4,6-phosphate diester by using a reaction of known in-line stereochemistry (Jarvest et al., 1981), as shown in Figure 2. Any of the three isotopes may be lost in this reaction (primary kinetic isotope effects are negligible), and there are three cyclic diesters formed in a 1:1:1 ratio from each anomer of D-glucose 6-phosphate. To accentuate the difference between the two

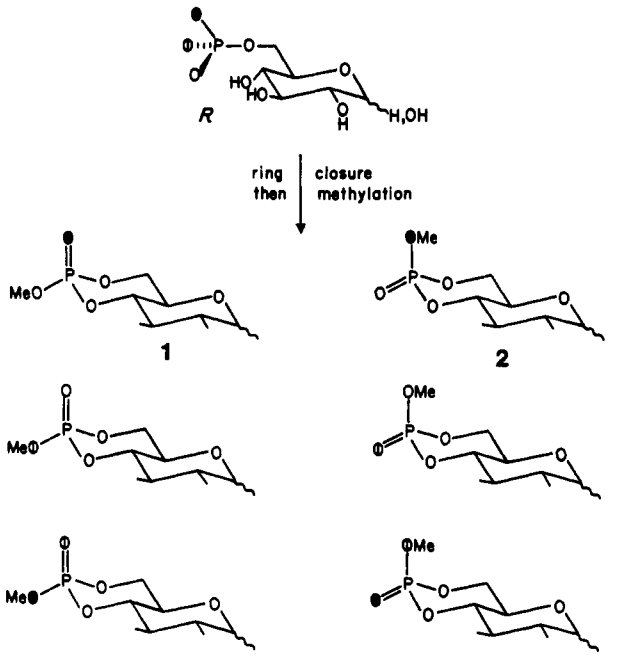

FIGURE 2: Stereochemical analysis of the absolute configuration at phosphorus of glucose 6-[^{16}O,^{17}O,^{18}O]phosphate. Only the products from the (*R*)-phospho compound are shown. Had the configuration of the original phospho group been *S*, the axial and equatorial exocyclic phospho group oxygens would have been in the opposite positions from those illustrated.

remaining oxygen atoms, this mixture of six cyclic diesters is methylated to yield three axial and three equatorial triesters for each anomer. The ^{31}P NMR signals for the axial triesters appear at higher field than the equatorial triesters by about 2 ppm (Jarvest et al., 1981). Now, the electrical quadrupole moment of ^{17}O so broadens the ^{31}P resonances of those ^{31}P nuclei involved in a ^{31}P–^{17}O bond that their signals are not observed in the ^{31} P NMR spectrum. Only species **1** and **2** (Figure 2) are seen. Had the configuration at phosphorus in the glucose 6-phosphate been *S*, the spectrum would have been dominated by the species corresponding to **1** and **2** with the exocyclic oxygens switched (i.e., the ^{18}O label would have been equatorial). Stereochemical assignment is now possible because these two sets give rise to different ^{31}P NMR spectra. When ^{18}O replaces ^{16}O in a phosphoric ester, the ^{31}P NMR resonance is shifted by an amount proportional to the ^{31}P–^{18}O bond order: a compound containing ^{31}P–^{18}O has its ^{31}P resonance shifted by about 0.02 ppm and a compound containing ^{31}P=^{18}O has its ^{31}P resonance shifted by about 0.04 ppm, to higher field compared to the unlabeled compound. In practice, the ^{17}O position is contaminated with 9.9% ^{16}O and 39.0% ^{18}O (deriving from the isotopic content of the $H_2{}^{17}O$ used synthetically), leading to additional resonances in the observed ^{31}P NMR spectrum. These additional resonances do not affect the stereochemical analysis.

The high field axial resonances in the ^{31}P NMR spectrum of the cyclic triesters derived from the sample of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained from the adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate produced in the PEP synthetase reaction are shown in Figure 3. The spectrum consists of a set of four lines from each anomer of the triesters and indicates that the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate, and thus the adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate from which it is derived, is of the *R* configuration. The anomer resonances of the equatorial triesters, while of lower intensity, were consistent with this conclusion. Calculation of the quality index (Buchwald et al., 1982) yields values of Q_{max} = 0.49 (from

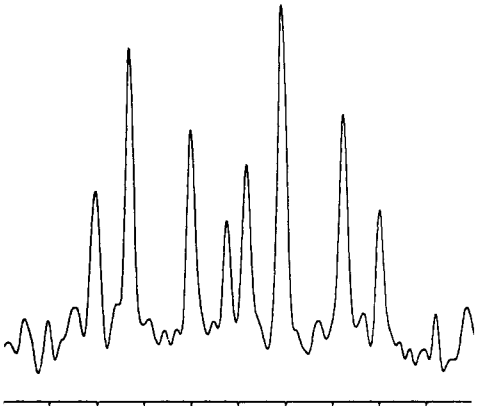

FIGURE 3: ^{31}P NMR spectrum of the axial triesters derived from D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained in the stereochemical analysis of adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate from the PEP synthetase reaction. The sample was prepared in dimethyl-d_6 sulfoxide–methanol (1:1 v/v). The spectrum was obtained on a Bruker WM-300 instrument at 121.5 MHz with a deuterium field lock and broad-band decoupling: spectral width 600 Hz; acquisition time 13.6 s; pulse width 24.5 μs; number of transients 1368; Gaussian multiplication with Gaussian broadening 0.05 Hz; line broadening −0.30 Hz. The chemical shifts for the eight resonances are δ −4.8117, −4.8264, −4.8524, −4.8678, −4.8762, −4.8913, −4.9175, and −4.9329, upfield from external 85% phosphoric acid. Scale: 0.02 ppm/division.

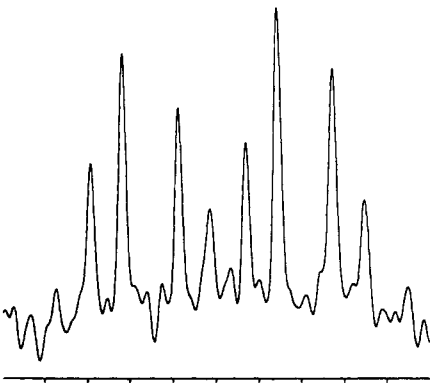

FIGURE 4: ^{31}P NMR spectrum of the axial triesters derived from D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained in the stereochemical analysis of adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate from the pyruvate, orthophosphate dikinase reaction. The sample was prepared in dimethyl-d_6 sulfoxide–methanol (6:4 v/v). The spectrum was obtained on a Bruker WM-300 instrument at 121.5 MHz with a deuterium field lock and broad-band decoupling: spectral width 600 Hz; acquisition time 13.6 s; pulse width 24.5 μs; number of transients 4543; Gaussian multiplication with Gaussian broadening 0.05 Hz; line broadening −0.40 Hz. The chemical shifts for the eight resonances are δ −4.7529, −4.7682, −4.7941, −4.8090, −4.8254, −4.8405, −4.8665, and −4.8814, upfield from external 85% phosphoric acid. Scale: 0.02 ppm/division.

mass spectral data) and Q_{obsd} = 0.44. The enantiomeric excess at phosphorus is about 90%. PEP synthetase therefore transfers the phospho group from PEP to the β-position of ATP with overall stereochemical *retention* of the configuration at phosphorus.

The high field axial resonances in the ^{31}P NMR spectrum of the cyclic triesters deriving from the sample of D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate obtained from the adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate produced in the pyruvate, orthophosphate dikinase reaction are shown in Figure 4. This spectrum indicates the D-glucose 6-[^{16}O,^{17}O,^{18}O]phosphate, and thus the adenosine [β-^{16}O,^{17}O,^{18}O]triphosphate from which it is derived, is also of the *R* configuration. The anomer resonances of the equatorial triesters were consistent with this conclusion. Calculation of the quality index yields values of

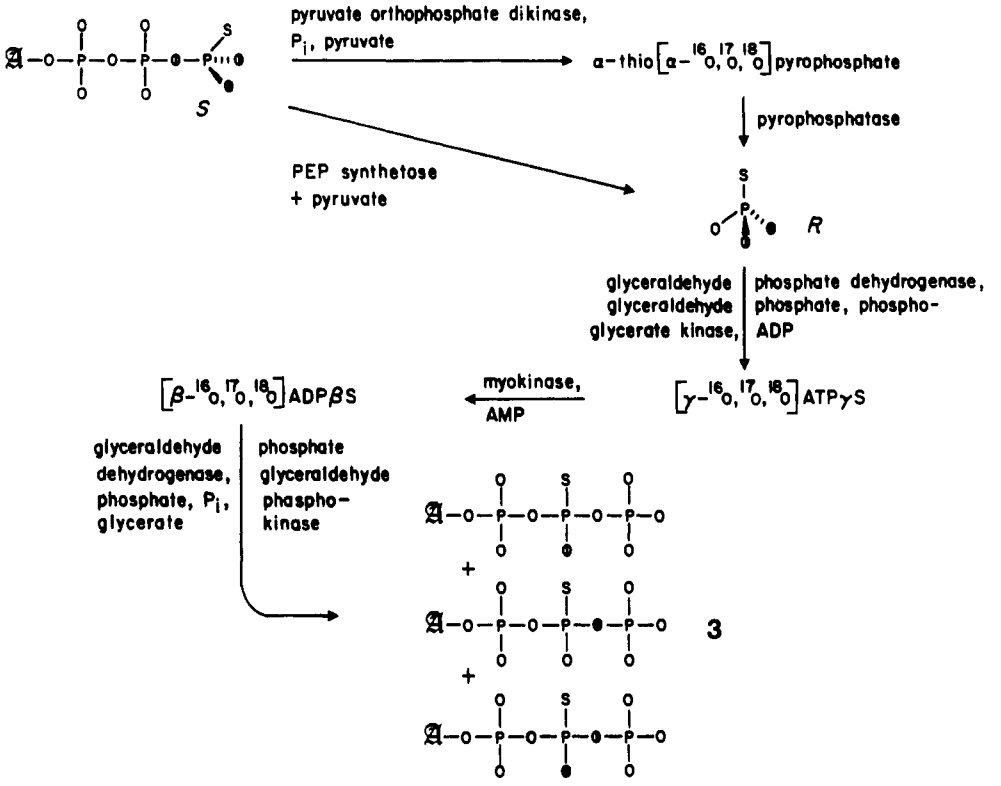

FIGURE 5: Scheme for the investigation of the stereochemical course at P_γ, in the reactions catalyzed by PEP synthetase and by pyruvate, orthophosphate dikinase. Only the products from *R*-[^{16}O,^{17}O,^{18}O]thiophosphate are illustrated. Had the thiophosphate been *S*, the ^{18}O label in **3** would have been in the *β-non*bridging position.

Q_{max} = 0.49 and Q_{obsd} = 0.41. The enantiomeric excess at phosphorus is about 84%. Pyruvate, orthophosphate dikinase therefore also transfers the phospho group from PEP to the β-position of ATP with overall stereochemical *retention* of the configuration at phosphorus.

We have argued earlier that the stereochemical behavior of the more than 40 phosphotransferases that have been investigated is consistent with the view that each act of enzyme-catalyzed phospho group transfer results in inversion of the configuration at phosphorus (Knowles, 1982). This view is based upon studies of simple phosphokinases (where the bond between P_γ and the β,γ-bridge oxygen of ATP is cleaved), of nucleotidyltransferases (where the bond between P_α and the α,β-bridge oxygen of the nucleoside triphosphate is cleaved), and of one pyrophosphoryltransferase (where the bond between P_β and the α,β-bridge oxygen of the triphosphate is cleaved; Li et al., 1978). This last reaction provides, of course, the closest analogy to the *pyro*phosphoryl-transfer reaction (eq 3) proposed for the enzymes under scrutiny here. Accordingly, the simplest interpretation of overall retention is that *the phospho group has suffered two transfers*, each of which results in inversion. This conclusion is entirely in accord with the earlier mechanistic proposals for PEP synthetase and pyruvate, orthophosphate dikinase. An enzyme nucleophile first attacks the β-phosphorus of ATP displacing AMP and yielding a pyrophospho-enzyme. After cleavage of the pyrophospho group either by water (PEP synthetase) or by P_i (pyruvate, orthophosphate dikinase) a phospho-enzyme results. This phospho group, which was originally the β-phospho group of ATP, is now transferred to pyruvate in a second displacement, to yield PEP having the same configuration at phosphorus as the original ATP. These stereochemical results thus strongly support mechanistic formulations involving pyrophospho-enzymes, even though these species have proved elusive, at best, in classical attempts to isolate them.

Stereochemical Fate of the γ-Phosphorus of ATPγS. Adenosine 5′-*O*-[(γ*S*)-βγ-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) has been used as a substrate for PEP synthetase and for pyruvate, orthophosphate dikinase (Figure 5), and the absolute configuration of the products, [^{16}O,^{17}O,^{18}O]thiophosphate (from PEP synthetase) and [α-S,α-^{17}O,^{18}O]thiopyrophosphate (from pyruvate, orthophosphate dikinase), has been determined. The product of the pyruvate, orthophosphate dikinase reaction, [α-S,α-^{17}O,^{18}O]thiopyrophosphate, was hydrolyzed to [^{16}O,^{17}O,^{18}O]thiophosphate by using inorganic pyrophosphatase, which proceeds exclusively via H_2O attack on the β-phosphorus (Webb & Trentham, 1980a), leaving the stereochemistry at the α-center (the thiophospho group) unperturbed. Control experiments demonstrated that no thiophosphate was produced from the hydrolysis of ATPγS in the absence of enzyme, nor was PEP produced when pyruvate was omitted from the enzyme reaction mixtures.

Analysis of the product [^{16}O,^{17}O,^{18}O]thiophosphate followed that developed by Tsai (1979) and Webb & Trentham (1980a,b) by incorporating the inorganic thiophosphate stereospecifically into the β-position of ATP as shown in Figure 5. The reactions that affect the stereochemistry of the thiophospho group are those catalyzed by 3-phospho-D-glycerate phosphokinase to yield ATPγS, and by myokinase to yield ADPβS, each of which proceeds with inversion of the configuration (Richard & Frey, 1978; Webb & Trentham, 1980a). The net result is overall retention of the stereochemistry of the thiophospho center from [^{16}O,^{17}O,^{18}O]thiophosphate to ATPβS.

The stereoanalytical method depends upon the same principles as described earlier. The electrical quadrupole moment of ^{17}O so broadens the ^{31}P resonances of the ^{31}P atom to which it is attached such that no species containing a ^{31}P–^{17}O bond are observed in the ^{31}P NMR spectrum. Consequently, only species **3** (Figure 5) will be seen. (If the thiophosphate had

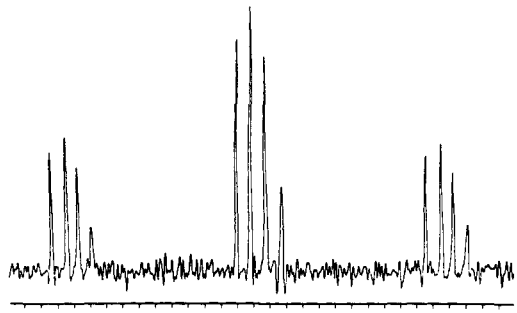

FIGURE 6: ^{31}P NMR spectrum of the β-phosphorus of the ATPβS derived from the stereochemical analysis of inorganic [^{16}O,^{17}O,^{18}O]thiophosphate obtained in the PEP synthetase reaction. The spectrum was obtained on a Bruker WM-300 instrument at 121.5 MHz with a deuterium field lock and broad-band decoupling: spectral width 500 Hz; acquistion time 16.4 s; pulse width 24.5 μs; number of transients 5365; shifted sine-bell multiplication $\pi/4$. The chemical shifts of the 12 resonances are δ 29.8291, 29.8089, 29.7938, 29.7735, 29.6010, 29.5806, 29.5656, 29.5453, 29.3732, 29.3528, 29.3377, and 29.3173, downfield from external 85% phosphoric acid. Scale: 0.02 ppm/division.

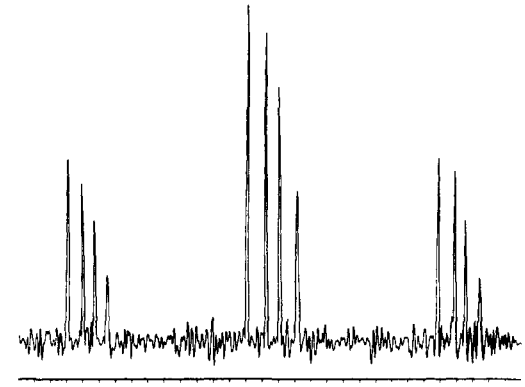

FIGURE 7: ^{31}P NMR spectrum of the β-phosphorus of the ATPβS derived from the stereochemical analysis of inorganic [α-S,α-^{17}O,^{18}O]thiopyrophosphate obtained in the pyruvate, orthophosphate dikinase reaction. The spectrum was obtained on a Bruker WM-300 instrument at 121.5 MHz with a deuterium field lock and broad-band decoupling: spectral width 500 Hz; acquisition time 16.4 s; pulse width 24.5 μs; number of transients 4181; shifted sine-bell multiplication $\pi/4$. The chemical shifts of the 12 resonances are δ 29.8414, 29.8208, 29.8058, 29.7855, 29.6132, 29.5927, 29.5776, 29.5572, 29.3855, 29.3652, 29.3501, and 29.3294, downfield from external 85% phosphoric acid. Scale: 0.02 ppm/division.

been *S*, the ^{18}O label would have been in the *β-non*bridging position.) Stereochemical assignment is now possible as before due to the fact that the bond order in a bridging P–O bond is near one, whereas in a *non*bridging P–O bond it is between one and two. An ^{18}O label in the *β-non*bridging position therefore shifts the ^{31}P NMR resonance of the β–phosphorus further upfield than if the label is in the β,γ bridge. The contamination of the ^{17}O position with ^{16}O and ^{18}O, as well as some label loss during conversion of the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate into ATPβS (Webb & Trentham, 1980a), leads to additional resonances in the observed ^{31}P NMR spectrum. These additional resonances do not, however, affect the stereochemical analysis.

The NMR spectrum of the β-phosphorus of the ATPβS obtained from the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate produced in the PEP synthetase reaction is shown in Figure 6. The spectrum indicates that the product [^{16}O,^{17}O,^{18}O]thiophosphate is of the *R* configuration [see Webb (1982)]. Calculation of the quality index yields values of $Q_{max} = 0.45$ and $Q_{obsd} = 0.39$. The enantiomeric excess at phosphorus is about 87%. PEP synthetase therefore transfers the thiophospho group from [(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O]ATPγS to water with stereochemical *inversion* of the configuration at phosphorus.

The spectrum of the β-phosphorus of the ATPβS obtained from the inorganic [α-S,α-^{17}O,^{18}O]thiopyrophosphate produced in the pyruvate, orthophosphate dikinase reaction is shown in Figure 7. This spectrum indicates that the product [^{16}O,^{17}O,^{18}O]thiopyrophosphate is of the *R* configuration. (It may be noted that the larger peak heights of the resonances from isotopically unlabeled species simply reflect a somewhat higher level of isotopic washout that derives from the larger number of manipulations required of this material compared with that illustrated in Figure 6.) Calculation of the quality index yields values of $Q_{max} = 0.45$ and $Q_{obsd} = 0.37$. The enantiomeric excess at phosphorus is about 82%. Pyruvate, orthophosphate dikinase therefore also transfers the thiophosphoryl group from [(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O]ATPγS to inorganic phosphate with stereochemical *inversion* of the configuration at phosphorus.

The stereochemical results obtained at both P_β and P_γ, for both the synthetase and the dikinase, are in accord with the mechanisms earlier proposed for these enzymes. We may ask, however, if the observed stereochemical behavior is consistent with any alternative pathways. One such possibility is that only a phospho-enzyme is formed, for both the synthetase and the dikinase: eq 8a, 9, and 10 and eq 8b, 9, and 10, respectively. This pathway would be predicted to give rise to the observed stereochemical results. However, for PEP synthetase, such a mechanism is inconsistent with the observed exchange reactions (Cooper & Kornberg, 1967a,b). For pyruvate, orthophosphate dikinase, this pathway is inconsistent with the exchange reactions (Evans & Wood, 1968; Milner & Wood, 1972), with the initial velocity and exchange kinetics (Milner & Wood, 1976), and with the detection of a pyrophosphoenzyme intermediate (Milner et al., 1978). In the absence of other data, therefore, we favor the mechanistic proposals summarized in eq 3–7.

$$\text{ATP} + \text{H}_2\text{O} + \text{enzyme} \rightleftarrows \text{ADP} + \text{P}_{\gamma i} + \text{enzyme} \quad (8a)$$

$$\text{ATP} + \text{P}_i + \text{enzyme} \rightleftarrows \text{ADP} + \text{P}_\gamma\text{P}_i + \text{enzyme} \quad (8b)$$

$$\text{ADP} + \text{enzyme} \rightleftarrows \text{AMP} + \text{enzyme–P}_\beta \quad (9)$$

$$\text{PEP} + \text{enzyme–P}_\beta \rightleftarrows \text{PEP} + \text{enzyme} \quad (10)$$

While the molecular details of the rather curious sequence of phospho group transfers mediated by these two enzymes remain obscure, the overall pathway, and the intermediacy of both a phospho-enzyme *and* a pyrophospho-enzyme, rests on firmer ground.

ACKNOWLEDGMENTS

We are grateful to David E. Hansen for assistance and advice.

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Thrombin Binds to a High-Affinity ~900 000-Dalton Site on Human Platelets†

Joan T. Harmon* and G. A. Jamieson

American Red Cross Blood Services, Bethesda, Maryland 20814
Received June 8, 1984

ABSTRACT: The functional sizes of the binding sites for thrombin on human platelets and isolated membranes have been determined by the technique of radiation inactivation: similar results were obtained. Independent studies using different radiation doses (0, 3, and 48 Mrad) and different thrombin concentrations (10^{-10}, 10^{-8}, and 10^{-6} M) confirmed the presence of three binding sites with functional sizes of 900 000, 30 000, and 4000 daltons. The binding site of lowest apparent size (4000 daltons) probably corresponds to what has been termed nonspecific binding since its dissociation constant (2900 nM) is well outside the physiological range. The site of intermediate size (30 000 daltons) is also probably not involved in platelet activation since its dissociation constant (11 nM) is also beyond the concentration range required for activation, although it may be involved in other aspects of platelet–thrombin interaction. The sites with the largest functional size are probably important in platelet function since their dissociation constant (0.3 nM) is in the range required for platelet activation. The functional size of these sites (900 000 daltons) suggests that the high-affinity site for thrombin binding to platelets may involve a multimolecular complex of membrane components.

Thrombin (α-thrombin) is one of the most potent physiological activators of platelet function and can induce platelet aggregation and secretion at concentrations below 1 nM. The mechanism of its action on platelets is not understood but appears to embody aspects both of an enzyme-catalyzed reaction and of agonist–receptor equilibrium (Martin et al., 1975).

Thrombin binds to intact platelets (Detwiler & Feinman, 1973; Tollefson et al., 1974; Ganguly, 1974) and to isolated membranes (Tam & Detwiler, 1978). The nature of these binding sites is not known: glycoprotein I (GPI; M_r 185 000) has been suggested as being a binding site on the basis of the ability of its proteolytic product glycocalicin (Okumura et al., 1978) and GPI itself (Ganguly & Gould, 1979) to inhibit the binding of thrombin to platelets. The possible role for GPI in thrombin binding and activation has been supported by the correlation between decreased GPI and the reduction of thrombin reactivity in Bernard–Soulier syndrome (Jamieson & Okumura, 1978), in myeloproliferative disorders (Bolin et al., 1977a; Ganguly et al., 1978), and in other pathological

† This is Contribution No. 585 from American Red Cross Blood Services, Bethesda, MD 20814. This work was supported, in part, by U.S. Public Health Service Grants RR05737 and HL14697.

0006-2960/85/0424-0058$01.50/0