Stereochemistry of the Hydrolysis of Adenosine 5'-Thiophosphate Catalyzed by Venom 5'-Nucleotidase†

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ABSTRACT: The stereochemical problem involving a pro-prochiral phosphorus center, the hydrolysis of adenosine 5'-monophosphate to adenosine and inorganic phosphate catalyzed by the venom 5'-nucleotidase, has been studied by use of chiral [18O,P,18O,P,18O,P]thiophosphates (P₄). (R₄)- and (S₄)-[18O,P,18O,P]Adenosine 5'-thiophosphates (AMPS) were synthesized by a combined chemical and biochemical procedure. Hydrolysis of (R₄)- and (S₄)-[18O,P,18O,P]AMPS in H₂¹⁸O by 5'-nucleotidase gave two enantiomers of chiral P₄, of unknown configuration. A 3¹P NMR method based on the combination of the quadrupolar effect of ¹³O [Tsai, M.-D. (1979) Biochemistry 18, 1468-1472] and the ¹⁸O isotope shift [Cohn, M., & Hu, A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 200-203] has been developed to analyze the configuration of chiral P₄. The results indicate that hydrolysis of (R₄)- and (S₄)-[18O,P,18O,P]AMPS in H₂¹⁸O gave (R₄)- and (S₄)-[¹³O,P,¹³O,P]P₄, respectively. Therefore the hydrolysis of AMPS catalyzed by the venom 5'-nucleotidase must proceed with inversion of configuration at phosphorus, which suggests that the reaction is most likely an "in line" single displacement without involving a phosphoryl-enzyme intermediate and without pseudorotation.

The enzyme-catalyzed reactions involving a P-O bond cleavage can be categorized into the following types based on the stereochemistry involved:

ROPO₂OR' (prochiral) ⇄ ROPO₂OR" (prochiral) (A)

ROPO₃OR' (prochiral) ⇄ ROPO₃OR (pro-chiral) (B)

ROPO₃ (pro-chiral) ⇄ ROPO₃ (pro-chiral) (C)

ROPO₄ (pro-chiral) ⇄ PO₄ (pro-chiral) (D)

PO₄ (pro-chiral) ⇄ PO₄ (pro-chiral) (E)

During the past few years sophisticated methods have been developed to analyze the stereochemical courses of the reactions belonging to types A, B, and C (Eckstein, 1975, 1978; Knowles, 1980). However, the stereochemical problems of reaction D (i.e., hydrolysis of phosphate monoesters) and reaction E (i.e., P₄ = H₂O oxygen exchange) have not yet been solved. Thus, although the stereochemistry of nearly 30 enzymes catalyzing phosphor transfer reactions has been solved (Knowles, 1980), only one of them (Escherichia coli alkaline phosphatase) is a phosphomonoesterase. Since the alkaline phosphatase also catalyzes transphosphorylation, its stereochemistry has been elucidated by the method developed for kinases (Jones et al., 1978).

The venom 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) (Drummond & Yamamoto, 1971) catalyzes hydrolysis of 5'-mononucleotides (reaction D) but does not catalyze transphosphorylation (reaction C) (Morton, 1953) or P₄ = H₂O oxygen exchange (reaction E) (Koshland & Springhorn, 1956). So far the existence of a phosphor-enzyme intermediate has not been evidenced (or ruled out). Those features suggest that 5'-nucleotidase is mechanistically different from another class of phosphomonoesterases, the alkaline phosphatase, which involves a phosphoryl-enzyme intermediate in catalysis (Levine et al., 1969). Koshland & Springhorn (1956) have proposed that the "single displacement" pathway is the more likely mechanism for 5'-nucleotidase. The most direct evidence to this mechanism would be to show that the reaction proceeds with "inversion" of configuration at phosphorus.

In this paper we report the first stereochemical study on a reaction involving a pro-prochiral phosphorus center, the hydrolysis of AMP to adenosine and P₄ catalyzed by the venom 5'-nucleotidase. Since there are only three oxygen isotopes available, it is necessary to use a different atom, e.g., sulfur, in order to make the P₄ chiral. Our approach involves synthesis of (R₄)- and (S₄)-[18O,P,18O,P]AMPS as analogues of AMP. Hydrolysis of these two substrates in H₂¹⁸O gave chiral [¹³O,P,¹³O,P]thiophosphates (P₄). The configurations of chiral P₄ were then analyzed by combined use of the 3¹P(¹³O) (Tsai, 1979; Tsai et al., 1980) and the 3¹P(¹⁸O) (Cohn & Hu, 1978) NMR methods. The results indicate that the hydrolysis of AMPS by 5'-nucleotidase proceeds with inversion of configuration, which suggests that the reaction catalyzed by 5'-nucleotidase is most likely an "in line" single displacement without involving a phosphoryl-enzyme intermediate and without pseudorotation.

Materials and Methods

Materials. The 52.8% H₂¹⁸O (containing 52.8% atom % ¹⁸O, 41.8 atom % ¹⁰O, and 5.4 atom % ¹²O) was obtained from Monsanto, whereas the 99.9% H₂¹⁰O was purchased from Norsk Hydro. 5'-Nucleotidase (Crotalus atrox venom, 250-500 units/mg, lactic dehydrogenase (pig heart, 500

† Abbreviations used: P₄, inorganic phosphate; P₈, inorganic thiophosphate; PEP, phosphoenolpyruvate; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; TLC, thin-layer chromatography; PGK, phosphoglycerate kinase; PP, inorganic pyrophosphate; O, oxygen-16; O, oxygen-18; AMP, adenosine 5'-monophosphate; AMPS, adenosine 5'-thiophosphate; ADP, adenosine 5'-diphosphate; ADP-S, adenosine 5'-diphosphate; ATPS, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD, nicotinamide adenine dinucleotide; NAD[N], reduced NAD; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; GC, gas chromatography; MS, mass spectroscopy; the diastereomers A and B are designated on the basis of their enzymatic activity (Eckstein & Goody, 1976).

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units/mg), alkaline phosphatase (calf intestine, 1000 units/mg), 3-phosphoglycerate kinase (yeast, 2000 units/mg), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 40-80 units/mg), pyruvate, phosphoenolpyruvate, dihydroxyacetone, EDTA, and glyceraldehyde 3-phosphate were obtained from Sigma Chemical Co. Myokinase (rabbit muscle, 360 units/mg), pyruvate kinase (rabbit muscle, 200 units/mg), adenosine deaminase (calf intestine, 200 units/mg), acetate kinase (E. coli, 200 units/mg), AMP, ADP, ATP, adenosine, NAD, and NADH were purchased from Boehringer. DEAE-Sephadex A-25 was obtained from Pharmacia. Other chemicals used were of reagent grade or highest purity available commercially.

**Instrumental Methods.** Mass spectra were measured on a Du Pont 21-492 GC–MS by using chemical ionization (isobutane) and an OV-17 gas chromatographic column. Routine 31P NMR spectra were recorded at 32.2 MHz on a Varian FT-80 NMR spectrometer equipped with a multinuclear clear probe. High-resolution 31P NMR spectra for measurements of 1H isotope shifts were obtained at 145.7 MHz on a Nicolet NT-360 instrument. The field was locked on deuterium (D2O) and all spectra were recorded at ambient temperature. All chemical shifts are expressed relative to 85% H3PO4 as the external reference.

**Chromatography.** A DEAE-Sephadex A-25 column (2.5 x 25 cm) was used for routine column chromatography to separate undesirable, P and P0. Two gradient systems have been used: 2 L each of 0.1 and 0.6 M triethylammonium bicarbonate (pH 7.5) and 2 L each of H2O and 0.6 M ammonium bicarbonate. Nucleosides and nucleotides were located by UV absorption at 260 nm. Thin-layer chromatography was carried out on polyethyleneimine-cellulose sheets (Brinkmann) in 0.75 M potassium phosphate buffer, pH 3.5.

**Determination of P** and **P0.** P was determined by a modified Fiske–Subbarow procedure (Ames, 1966). Solution A is 10% ascorbic acid; solution B is 0.42% ammonium molybdate tetrahydrate in 1 N H2SO4; solution C is 5 mL of A + 30 mL of B. Incubation of 0.05 μmol of P, with 1 mL of solution C at 40 °C for 20 min gives a blue solution with an absorption at approximately 1200 nm. A solution of P0 does not give a positive color from the solution C at 40 °C for 20 min. According to quantitation of P and P0 in a calibration curve is required in each set of measurements. P0 can also be measured by UV absorption directly: at pH 3.2, λmax = 210 nm and ε = 4000; at pH 7, λmax = 225 nm and ε = 4200. It is known that P0 could be hydrolyzed to P in certain pH ranges (Dittmer & Ramsey, 1963).

**Synthesis of (R)- and (S)-[1-18O]AMPS.** [1-18O]AMPS (1) was prepared by a modified procedure of Murray & Atkinson (1968). Adenosine (20 mmol) was suspended in triethyl phosphate (50 mL) at 100 °C; the solution was then cooled to 0 °C and mixed with 6 mL of PSCl3. After being stirred for 12 h at 0-4 °C, the resultant suspension was filtered and purified by column chromatography to give 5.8 mmol of pure AMPS (1). 31P NMR analysis indicated that 1 contained only 18O species (shifted 0.067 ppm upfield) and no detectable 16O or nonlabeled species. Since the signal/noise ratio of the NMR spectrum is 20, the atom % 18O enrichment of 1 is at least >95%.

Phosphorylation of 1 by the procedure of Eckstein & Goody (1976) gave a mixture of diastereomers, [1-18O]ADP0S (A + B) (2), in 50% yield. Pyruvate kinase is known to be specific for the A isomer of ADP0S (Eckstein & Goody, 1976), but the stereospecificity may not be 100% (Jaffe & Cohen, 1979). We have found that, when 2 was incubated with pyruvate kinase and phosphoenolpyruvate, the first 30% product isolated by column chromatography was >95% pure ATP0S (A) (3) as determined by 31P NMR (Sheu & Frey, 1977) (no detectable Ps signal due to isomer B). The unreacted ADP0S contained 80% isomer B and 20% isomer A. Further incubation of this unreacted ADP0S with pyruvate kinase and PEP, followed by column chromatography, gave ATP0S (A + B) and >95% pure ADP0S (4) (40% yield from 2). 31P NMR analysis indicated that one of the two 18O atoms in 1 was retained in 3 and 4 in >95% enrichment.

ATP0S (A) (3) and ADP0S (B) (4) were hydrolyzed to (S)-[1-18O]AMPS (5) and (R)-[1-18O]AMPS (6), respectively, by calf intestine alkaline phosphatase. The incubation mixture (pH 8.5) contained 0.1 M Tris, 0.01 M MgCl2, 50 mM nucleotide, and 0.02 mg of enzyme/mL. The reaction at 25 °C was followed by TLC and was stopped as soon as the hydrolysis was complete. The product AMPS was separated from P, by a DEAE-Sephadex column eluted with ammonium bicarbonate gradient, in a 90% yield. 31P NMR analysis indicates >95% 18O enrichment in both 5 and 6.

**Hydrolysis of AMPS by 5’-Nucleotidase.** Compounds 5 and 6 (200 μmol) were dissolved in 1 mL of Tris buffer containing 0.01 M MgCl2, pH 8.5. The water was then removed by lyophilization and replaced by 0.6 mL of H218O (52.8%). The hydrolysis was started by addition of the venom 5’-nucleotidase (500 units) and 10 μL of adenosine deaminase (4 units) and was followed by the decrease in A265 due to deamination of the adenosine formed. After the reaction was complete, the product P (7 and 8, respectively) was converted into ATP6S directly without being isolated, as will be described later. The commercial 5’-nucleotidase was used without further purification. The observed enzyme activity cannot be due to alkaline phosphatase since we have shown that alkaline phosphatase catalyzes only very slow hydrolysis of AMPS.

In a separate experiment, 85 atom % 18O enriched [1-18O]AMPS was hydrolyzed by 5’-nucleotidase in H2O according to the same procedure. The product PS18O6S2- was isolated in a 60% yield by chromatography on a DEAE-Sephadex column eluted with ammonium bicarbonate gradient. No appreciable amount of P, was found as a byproduct. The PS18O6S2- was converted to its trimethyl ester by CH3N2 methylation and analyzed by GC–MS. The atom % 18O enrichment found was 65%.

**Conversion of** P **to** ATP6S (B) (Scheme III). The reaction mixture for the hydrolysis of 5 and 6 by 5’-nucleotidase was directly mixed with 40 mL of a solution (pH 8.0) containing 25 mM Tris, 10 mM DTE, 10 mM MgCl2, 1 mM EDTA, 15 mM ADP, 0.5 mM NAD+, 40 mM pyruvate, 20 mM glyceraldehyde-3-phosphate, lactic dehydrogenase (200 units), glyceraldehyde-3-phosphate dehydrogenase (2000 units, diazyed against buffer before use), and phosphoglycerate kinase (2000 units). After incubation at 30 °C for 12 min, the reaction mixture was quickly cooled to 0-2 °C and loaded to the DEAE-Sephadex column. Elution with the triethylammonium bicarbonate gradient gave ATP6S in 55% yield relative to AMPS. The ATP6S obtained was then dissolved in 5 mL of buffer (pH 7.5) containing 0.1 M Hepes, 50 mM KCl, 25 mM MgCl2, 1 mM DTE, and 0.15 M AMP and incubated with 2000 units of myokinase at 25 °C. Formation of ADP0S was followed by TLC. The product ADP0S was isolated by column chromatography in 80% yield. For con-
version of ADPβS to ATPβS (B) (Richard et al., 1978), the reaction mixture (pH 7.5) contained 10 mM ADPβS, 100 mM acetyl phosphate, 0.1 M Hepes, 50 mM KCl, 25 mM MgCl2, and 1 mM DTE. Incubation with acetate kinase (0.1 mg/mL) at 25 °C followed by column chromatography gave ATPβS (B) in 70% yield. 31P NMR analysis indicates no detectable isomer A.

Results

Synthesis of (Sp)- and (Rp)-[α-18O]AMPS. Scheme I summarizes the experimental procedure. Reaction of PSCI with adenosine (Murray & Atkinson, 1968), followed by H218O (99.5%) hydrolysis, gave [18O]2AMPs (I) (>95% 18O). Chemical phosphorylation (Eckstein & Goody, 1976) of I yielded [α-18O]2ADPβS (A + B) (2). Incubation of 2 with pyruvate kinase and phosphoenolpyruvate (Eckstein & Goody, 1976; Jaffe & Cohn, 1979) gave [α-18O]3ATPβS (A) (3) (95% isomer A) from the first 30% reaction and [α-18O]2-ADPβS (B) (4) (>95% isomer B) from the last 40% unreacted ADPβS. Reaction of 3 and 4 with calf intestine alkaline phosphatase gave (Sp)-[18O]2AMPS (5) and (Rp)-[18O]2-AMPS (6), respectively. Recently different forms of the synthesis of 5 and 6 have been developed (Jarvest & Lowe, 1979; Richard et al., 1979).

Hydrolysis of AMPS Catalyzed by 5'-Nucleotidase. The kinetic data for AMPS as a substrate of the venom 5'-nucleotidase have been obtained by Murray & Atkinson (1968): $V_{max} = 0.026 \text{ umol min}^{-1} \text{ mg}^{-1}$ and $K_m = 0.02 \text{ mM}$. The corresponding values for AMP are $V_{max} = 1.39 \text{ umol min}^{-1} \text{ mg}^{-1}$ and $K_m = 0.035 \text{ mM}$. Thus, AMPS is a reasonably good substrate for the venom 5'-nucleotidase.

To determine the stereochemical course of the hydrolysis catalyzed by 5'-nucleotidase, we hydrolyzed 5 and 6 in H218O (52.8% H218O, 41.8% H216O). The chiral Pn products (7 and 8, respectively) were converted into ATPγS (see later discussions) immediately, without being isolated, to minimize any possible racemization.

Rationale of Conformational Analysis by 31P NMR. This is based on the combination of the 31P(18O) NMR method (Tsai, 1979; Tsai et al., 1980) and the 31P(18O) isotope shift method (Cohn & Hu, 1978). As illustrated by Scheme II, displacement of one of the three oxygen isotopes of (S)-[18O]218O18OPn by a nucleophile (R0) gives a mixture of three inseparable species. Among them, two (those in brackets) contain an 18O isotope. According to the results of our recent work, 18O causes the 31P NMR signals of the 31P nuclei directly bonded to 18O to broaden in most biochemical phosphate derivatives (∆P ≈ 50–70 Hz for P-18O-P; ∆P > 300 Hz for P-18O-P) (Tsai et al., 1980). Thus, the two 18O-containing species should give very broad 31P signals which may not be observable. Only the species which contains only 16O and 18O (18O at the pro-S position) should give a sharp, unquenched 31P NMR signal. Analogously, the (R)-[18O]218O18OPn should give a corresponding non-18O-containing species with 18O at the pro-R position. The pro-R and pro-S 18O can be distinguished by a stereospecific phosphorylation at one of the two positions. It is known that 18O causes the 31P NMR signal to shift upfield (∆P ≈ 0.02–0.05 ppm) (Cohn & Hu, 1978). A nonbridge 18O (P-18O) should cause a larger shift of the 31P signal than a bridge 18O (P-18O-P) does, due to a greater double bond character (Cohn & Hu, 1980; Lowe et al., 1979). The two main steps for configurational analysis by 31P NMR are therefore the nucleophilic displacement and the stereospecific phosphorylation.

Procedure of Configurational Analysis. Eckstein (1977) has shown that glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (PGK) together catalyze exchange of the phosphohydryl group of ATPγS with Pn. We used this procedure to convert chiral Pn into ATPγS (10). As shown in Scheme III, R-chiral Pn was first incorporated into 3-phosphoglyceroyl thionophosphate (9) with intact configuration.
Only the species with $^{17}$O at the P-O-C bridge position is shown in the scheme since the other two species (P-$^{18}$O-C and P-$^{18}$O-C) will give species containing $^{18}$O in the later steps. The thionophosphor group of $^{18}$O was then transferred to ADP by cleaving the P-OC bond to give ATP+$^{18}$S (10). Webb & Trentham (1980) have shown that the thionophosphor group transfer catalyzed by PGK proceeds with inversion of configuration at phosphorus. The thionophosphor group of ATP+$^{18}$S was then transferred to AMP by myokinase, with inversion of configuration (Richard & Frey, 1978), to give ADP+$^{18}$S (11). Stereospecific phosphorylation by acetate kinase (Richard et al., 1978) at the pro-OH oxygen of ADP+$^{18}$S gave ATP+$^{18}$S (B) (12). On the basis of the stereodynamics involved (two inversions), (R)-$^{17}$O-$^{18}$O-$^{17}$O-$^{18}$O$^+_p$ should give $[^{18}$O$]_{P_a}$ATO+P$^+$S (B) ($^{18}$O at the nonbridge position), whereas (S)$^{17}$O-$^{18}$O-$^{17}$O-$^{18}$O$^+_p$ should yield $[^{18}$O-Pb]ATO+P$^+$S (B) ($^{18}$O at the $^{17}$O-bridge position). Both enantiomers of the [^{18}O,^{17}O]P$_a$ 7 and 8 obtained from hydrolysis catalyzed by S'-nucleotidase were converted to ATP+$^{18}$S (B) according to Scheme III, with an overall yield of ~30%. The samples were then analyzed by $^{31}$P NMR. In a separate experiment, [^{18}O]$^+_p$ was converted to $[^{18}$O-ATP]S (B) by the same procedure.

**Theoretical Analysis.** Although Scheme III shows that (R)-$^{17}$O-$^{18}$O-$^{17}$O-$^{18}$O$^+_p$ should give rise to $[^{18}$O$]_{P_a}$ATO+P$^+$S (B) (12) which gives an observable P$_b$ signal in $^{31}$P NMR, it is technically impossible to obtain a chiral P$_b$ sample with 100% purity. In practice, the position enriched with $^{18}$O may also contain some $^{16}$O, whereas the position enriched with $^{17}$O may also contain some $^{18}$O and some $^{16}$O since the $^{17}$O-enriched water also contains $^{18}$O. Scheme IV shows the six possible species (I-VI) in a real chiral P$_b$ sample and the three possible ATPS (B) (e.g., Ia, Ib, and Ic) species which could result from each P$_b$ species. Since the kinetic isotope effect due to oxygen isotopes should be very small, the three ATPS (B) species from each P$_b$ species should be in approximately equal amounts (e.g., Ia $\approx$ Ib $\approx$ Ic).

**FIGURE 1:** The $P_b$ signals of the $^{31}$P NMR spectra of the ATPS (B) obtained from $[^{18}$O$_2$]P$_a$ (A) and from the two chiral P$_b$ 7 and 8 (B and C, respectively). The sample (30 pmol) was dissolved in 2.5 mL of D$_2$O containing 10 mM EDTA and the spectra were recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are expressed in hertz. The chemical shift of the $P_b$ signal is -29.8 ppm from H$_3$PO$_4$. Number of transients = 2800. Pulse delay = 5 s.

Among the ATPS (B) species in Scheme IV, Ia, Ib, IC, IVa, and IVc contain an $^{18}$O atom bonded to P$_b$. Each of the remaining species, as designated in parentheses, belongs to one of the four non-$^{17}$O-containing species: nonlabeled ATPS (B) (a), $[^{18}$O$]_{P_b}$ATO+P$^+$S (B) (b), $[^{18}$O$]_{P_a}$ATO+P$^+$S (B) (c), and $[^{18}$O$]_{P_b}$ATO+P$^+$S (B) (d). In the $^{31}$P NMR spectrum of the ATPS (B) obtained from (R)-$^{17}$O-$^{18}$O-$^{17}$O$^+_p$, which may contain a mixture of species I-VI, only the species other than Ia, Ib, and IVa, and IVc will give unquenched, sharp P$_b$ signals. As will be shown later (Figure 1), the P$_b$ signal of ATPS (B) obtained from chiral P$_b$ contains four peaks a, b, c, and d due to the species a, b, c, and d, respectively. As shown in Scheme IV, the species I, which is chirally labeled, contributes to c but not to b, whereas each of the other nonchirally labeled species (II-VI) contributes equally to both b and c. We define the ratio of the peak heights b/c as the "F value." An R-chiral
Table 1: $^3$P NMR Analysis of the ATPβS (B) Derived from Chiral Thiophosphates

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<th>c</th>
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$^a$ Obtained from peak height measurements for the P$_2$ signal of ATPβS. The errors represent deviations between the two non-overlapping halves of the two doublets. 
$^b$ Calculated for chiral P$_2$ at 50% purity and 100% chirality. 
$^c$ Calculated for chiral P$_2$ at 47.5% purity and 90% chirality.

P$_2$ sample should give F < 1. On the other hand, the species I of the opposite enantiomer, S-chiral P$_2$, should contribute to b instead of c and give F > 1.

To avoid possible confusion, we define the term “purity” as the percentage of the chirally labeled species (i.e., the M + 3 species) and the term “chirality” as the optical purity of the chirally labeled species. In the present experiments, both (Sp)-[α-$^{15}$O]AMP and (Rp)-[α-$^{15}$O]AMP are isotopically and isomerically pure based on the NMR. The isotopic composition of H$_3^3$PO$_4$, after being diluted by some additives, is 50% 15O, 40% 16O, and 10% 18O. If 5 and 6 are 100% pure, the chiral P$_2$ obtained from 5 or 6 should contain 50% 15O, 40% 16O, and 10% 18O, if the hydrogen is 100% stereospecific. Thus the optimal “purity” of 7 or 8 is 50%, whereas the optimal “chirality” of 7 or 8 could be 100%. The optimal F values calculated on the basis of these data are 2.0 and 0.5 for “S” and “R” P$_2$, respectively, as shown in Table I (calculated, optimal).

Since the NMR method may not detect signals of <5%, the two samples 5 and 6 may actually contain 5% of nonlabeled AMP (95% isotopic purity) and 5% of the opposite isomer (which may cause a 10% decrease in chirality). If this is the case, the chiral P$_2$ obtained may have a 47.5% “purity” and a 90% “chirality”, if the hydrogen is 100% stereospecific. The calculated compositions of ATPβS (B) obtained from such chiral P$_2$ samples and the corresponding F values (1.82 and 0.55) are also listed in Table I (calculated, minimal).

**Observed Results.** Figure 1 shows the P$_2$ signals of ATPβS (B) obtained from [15O]P$_2$ and the two chiral P$_2$ 7 and 8 (Figure 1, parts A, B, and C, respectively). The signal contains two overlapping doublets due to 31P–31P coupling ($J_{31P} = 26.3$ Hz, $J_{31P} = 27.8$ Hz). Each half of a doublet contains four lines, a, b, c, and d. The upfield shifts of peaks b, c, and d from peak a are 0.0203, 0.0349, and 0.0565 ppm, respectively. Peaks a and d should be due to nonlabeled ATPβS (B) (a) and [β,γ-15O]ATPβS (B) (d), respectively. Since a bridge 15O is expected to cause a smaller magnitude of isotope shift due to its smaller double bond character compared to a non-bridge 15O (Cohn & Hu, 1980; Lowe et al., 1979), peak b should come from [β,γ-15O]ATPβS (B) (b) whereas peak c can be assigned to [β,γ-15O]ATPβS (B) (c). The relative heights of peaks a–d are listed in Table I. As expected, the ATPβS (B) obtained from [15O]P$_2$ (Figure 1A) has an F value of 1.11 which indicates b = c within experimental error (the reproducibility of peak heights is ±10%). The chiral P$_2$ 7 gave ATPβS (B) with b > c (F = 1.52), whereas the opposite enantiomer 8 gave ATPβS (B) with b < c (F = 0.68). These results indicate that the absolute configurations of 7 and 8 are “S” and “R”, respectively, and that hydrolysis of AMPS by 5’-nucleotidase must proceed with inversion of configuration at phosphorus. Scheme V shows these stereochemical results.

**Possible Causes of Racemization.** As shown in Table I, the observed F values somewhat deviate from the calculated values. In order to find the possible causes for this deviation, we have shown that oxygen exchange of P$_2$ may occur in two steps. First, when a sample of [15O]AMP (I) with lower enrichment (85 atom % 15O as determined by $^3$P NMR at 145.7 MHz) was hydrolyzed by 5’-nucleotidase in H$_2$O with a prolonged incubation (1 additional h after the reaction was complete), the isolated product [PS$_{30}$O$_2$] was only enriched with 65 atom % 15O as determined by GC–MS for its trimethyl ester. The detailed kinetics and mechanism of this oxygen loss from P$_2$ enzyme-catalyzed or chemical, remain to be established by more detailed investigation. Second, when a PS$_{30}$O$_2$ sample (75 atom % 15O, obtained from hydrolysis of 50 mL of PSC$_3$ in 200 mL of 99% H$_2$O containing 120 mg of NaOH) was converted to ATPβS by a prolonged incubation (7 h), the [γ-15O]ATPβS obtained was only enriched with 40 atom % 15O by NMR analysis. This oxygen loss is apparently due to reversible conversions between P$_2$ and ATPβS which may cause oxygen exchange between P$_2$ and glycerol-aldehyde 3-phosphate, as can be seen from Scheme III.

In the work involving chiral P$_2$ we have tried to minimize possible oxygen exchanges in the above two steps by stopping the reaction as soon as it is complete. However, such exchanges may not be completely avoided and could cause partial racemization which accounts for the differences between the observed and the calculated F values.

**P$_2$, Signal of ATPβS (B).** The P$_2$ signals of $^3$P NMR spectra of ATPβS (B) obtained from 7 and 8 are shown in Figure 2, parts A and B, respectively. Each signal contains two doublets due to 31P–31P coupling ($J_{31P} = 26.3$ Hz, $J_{31P} = 27.8$ Hz). Each half of a doublet contains four lines, a, b, c, and d. The upfield shifts of peaks b, c, and d from peak a are 0.0203, 0.0349, and 0.0565 ppm, respectively. Peaks a and d should be due to nonlabeled ATPβS (B) (a) and [β,γ-15O]ATPβS (B) (d), respectively. Since a bridge 15O is expected to cause a smaller magnitude of isotope shift due to its smaller double bond character compared to a non-bridge 15O (Cohn & Hu, 1980; Lowe et al., 1979), peak b should come from [β,γ-15O]ATPβS (B) (b) whereas peak c can be assigned to [β-15O]ATPβS (B) (c). The relative heights of peaks a–d are listed in Table I. As expected, the ATPβS (B) obtained from [15O]P$_2$ (Figure 1A) has an F value of 1.11 which indicates b = c within experimental error (the reproducibility of peak heights is ±10%). The chiral P$_2$ 7 gave ATPβS (B) with b > c (F = 1.52), whereas the opposite enantiomer 8 gave ATPβS (B) with b < c (F = 0.68). These results indicate that the absolute configurations of 7 and 8 are “S” and “R”, respectively, and that hydrolysis of AMPS by 5’-nucleotidase must proceed with inversion of configuration at phosphorus. Scheme V shows these stereochemical results.

**Discussion**

**Mechanism Suggested by Stereochemical Results.** Our results establish that hydrolysis of AMPS catalyzed by 5’-nucleotidase proceeds with inversion of configuration at phosphorus. This is the first stereochemical course elucidated for a problem involving a pro-pro-prochiral phosphorus center. Since 5’-nucleotidase does not catalyze transphosphorylation (Morton, 1953), its stereochemical course can only be studied by use of chiral [15O,15O,18O]thiophosphates. Possible
mechanisms for the phosphoryl transfer reactions have been discussed by Benkovic & Schray (1973, 1978). The relationship between the stereochemical outcomes and the possible mechanisms has recently been discussed by Knowles (1980). The "inversion" of configuration suggests that the hydrolysis catalyzed by 5'-nucleotidase proceeds by the "in-line" associative pathway without pseudorotation, although the dissociative pathway involving a metaphosphate intermediate cannot be exclusively ruled out. Our stereochemical results also suggest that 5'-nucleotidase catalyzes hydrolysis by an "odd" number of displacements which is most likely a single displacement without involving a phosphoryl-enzyme intermediate.

Comparison with Other Phosphomonoesters. There are five important types of phosphomonoesters: alkaline phosphatase, acid phosphatase, ATPase, inorganic pyrophosphatase, and 3'- or 5'-nucleotidases. Whether the reaction proceeds by a double displacement mechanism involving a phosphoryl-enzyme intermediate or by a single displacement mechanism has been studied extensively in recent years by kinetic methods and by attempts to isolate the phosphoryl-enzyme intermediate. The work related to alkaline phosphatases (Reid & Wilson, 1971) and acid phosphatases (Hollander, 1971), which generally catalyze transphosphorylation and P_i \rightleftharpoons H_2O oxygen exchange in addition to phosphomonoester hydrolysis, has been reviewed recently (Knowles, 1980). It appears that both alkaline and acid phosphatases involve phosphoryl-enzyme intermediates. Consistent with this, Knowles and co-workers (Jones et al., 1978) have shown that the transphosphorylation of chiral [18O,17O,16O]phosphate monoesters catalyzed by E. coli alkaline phosphatase proceeds with net retention of configuration at phosphorus.

A phosphoryl-enzyme intermediate is known for the sarcoplasmic membrane ATPase (Hasselbach, 1974), which catalyzes both ATP hydrolysis and P_i \rightleftharpoons H_2O oxygen exchange (Kanazawa & Boyer, 1973). The yeast inorganic pyrophosphatase catalyzes extensive P_i \rightleftharpoons H_2O oxygen exchange in addition to PP_i hydrolysis (Hackney & Boyer, 1978). On the basis of kinetic studies, Boyer and co-workers (Janson et al., 1979) proposed that the intermediate for P_i exchange is an enzyme-bound PP_i rather than a phosphoryl-enzyme intermediate. Stereochemical studies by use of chiral [16O,17O,18O]P_i may support or refute the proposed mechanisms for these two enzymes.

The venom 5'-nucleotidase is unique in that it does not catalyze transphosphorylation or P_i \rightleftharpoons H_2O oxygen exchange (Kosshland & Springhorn, 1956). On the basis of these and other mechanistic features, Kosshland & Springhorn (1956) have proposed the single displacement pathway as the preferred mechanism over the double displacement pathway. Our stereochemical results apparently have supported this proposal and excluded the existence of a phosphoryl-enzyme intermediate.

Configurational Analysis of Chiral P_n. In the synthesis of 5 and 6 we have been able to optimize the 18O isotopic purity (>95%) and the isomic purity (>95%). The deviation between the observed F values and the calculated F values can be qualitatively explained by the partial oxygen exchange during hydrolysis and by the oxygen exchange between P_n and glyceraldehyde 3-phosphate which might occur during the conversion of chiral P_n to ATP\_iS via reverse reactions. However, there are certain limitations in accurate quantitations. The peak heights of 31P NMR signals, may have an error of ±10%. Accurate determination of the isotopic compositions of chiral P_n is difficult due to the fact that (i) there are two labeled positions, 17O and 18O, (ii) the 17O isotope usually always contains some 17O and 18O, and (iii) sulfur also contains 4.2% of 34S. In the present work the isotopic compositions of chiral P_n were obtained from that of the precursors 5 and 6 and the composition of H_218O used for hydrolysis.

Would there be a more accurate and straightforward way of measuring the chirality of chiral P_n? The circular dichroic method may not be a good candidate. The highest Δε/ε we have ever detected, if it is at all real, is only 1.5 × 10^4 (corrected for a 100% "pure" chiral P_n) at 225 nm.

While this work was in progress, Webb & Trentham (1980) synthesized chiral P_n of known configuration and developed a similar NMR procedure for configurational analysis. Added in Proof

Trentham and Webb have also shown that myosin catalyzes hydrolysis of ATP\_iS to ADP and P_n with inversion of configuration.

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References

Benkovic, S. J., & Schray, K. J. (1973) Enzymes, 3rd Ed. 8, 201–238.