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

Ming-Daw Tsai*

ABSTRACT: The stereochemical problem involving a pro-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 [^{16}O,^{17}O,^{18}O]thiophosphates (P_{si}). (*Rp*)- and (*Sp*)-[α-$^{18}O_1$]Adenosine 5′-thiophosphates (AMPS) were synthesized by a combined chemical and biochemical procedure. Hydrolysis of (*Rp*)- and (*Sp*)-[α-$^{18}O_1$]AMPS in $H_2{}^{17}O$ by 5′-nucleotidase gave two enantiomers of chiral P_{si} of unknown configuration. A ^{31}P NMR method based on the combination of the quadrupolar effect of ^{17}O [Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472] and the ^{18}O 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 P_{si}. The results indicate that hydrolysis of (*Rp*)- and (*Sp*)-[α-$^{18}O_1$]AMPS in $H_2{}^{17}O$ gave (*R*)- and (*S*)-[^{16}O,^{17}O,^{18}O]P_{si}, 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:

$ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_2OR''$ (prochiral) (A)

$ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_3$ (pro-prochiral) (B)

$ROPO_3$ (pro-prochiral) $\rightleftharpoons$ $R'OPO_3$ (pro-prochiral) (C)

$ROPO_3$ (pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral) (D)

PO_4 (pro-pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral) (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., P_i[1] $\rightleftarrows$ H_2O oxygen exchange) have not yet been solved. Thus, although the stereochemistry of nearly 30 enzymes catalyzing phosphoryl 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 $P_i \rightleftarrows H_2O$ oxygen exchange (reaction E) (Koshland & Springhorn, 1956). So far the existence of a phosphoryl-enzyme intermediate has not been evidenced (or ruled out). These 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[2] we report the first stereochemical study on a reaction involving a pro-pro-prochiral phosphorus center, the hydrolysis of AMP to adenosine and P_i 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 P_i chiral. Our approach involves synthesis of (*Rp*)- and (*Sp*)-[α-$^{18}O_1$]AMPS as analogues of AMP. Hydrolysis of these two substrates in $H_2{}^{17}O$ gave chiral [^{16}O,^{17}O,^{18}O]thiophosphates (P_{si}). The configurations of chiral P_{si} were then analyzed by combined use of the $^{31}P(^{17}O)$ (Tsai, 1979; Tsai et al., 1980) and the $^{31}P(^{18}O)$ (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% $H_2{}^{17}O$ (containing 52.8 atom % ^{17}O, 41.8 atom % ^{18}O, and 5.4 atom % ^{16}O) was obtained from Monsanto, whereas the 99.5% $H_2{}^{18}O$ was purchased from Norsk Hydro. 5′-Nucleotidase (*Crotalus atrox* venom, 250–500 units/mg), lactic dehydrogenase (pig heart, 500

† From the Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907. *Received April 21*, 1980. This work was supported by National Science Foundation Research Grant PCM 79-11478 and in part by National Institutes of Health Research Grant GM 26839. The high-field ^{31}P NMR spectra were measured at the Purdue University Biochemical Magnetic Resonance Laboratory which is supported by National Institutes of Health Grant RR 01077.

* Present address (after Jan 1, 1981): Department of Chemistry, Rutgers University, Newark, NJ 07102.

[1] Abbreviations used: P_i, inorganic phosphate; P_{si}, inorganic thiophosphate; PEP, phosphoenolpyruvate; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; TLC, thin-layer chromatography; PGK, phosphoglycerate kinase; PP_i, inorganic pyrophosphate; O, oxygen-16; Θ, oxygen-17; ●, oxygen-18; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-thiophosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); NAD, nicotinamide adenine dinucleotide; NADH, 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).

[2] A preliminary account of this work has been published as a communication (Tsai & Chang, 1980).

0006-2960/80/0419-5310$01.00/0

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, dithioerythritol, 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 ^{31}P NMR spectra were recorded at 32.2 MHz on a Varian FT-80 NMR spectrometer equipped with a multinuclear probe. High-resolution ^{31}P NMR spectra for measurements of ^{18}O isotope shifts were obtained at 145.7 MHz on a Nicolet NT-360 instrument. The field was locked on deuterium (D_2O) and all spectra were recorded at ambient temperature. All chemical shifts are expressed relative to 85% H_3PO_4 as the external reference.

Chromatography. A DEAE-Sephadex A-25 column (2.5 × 25 cm) was used for routine column chromatography to separate nucleotides, P_i, and P_{si}. 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 H_2O and 0.6 M ammonium bicarbonate. Nucleosides and nucleotides were located by UV absorption at 260 nm. Thin-layer chromatography was carried out on polyethylenimine–cellulose sheets (Brinkmann) in 0.75 M potassium phosphate buffer, pH 3.5.

Determination of P_i and P_{si}. P_i 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 H_2SO_4; solution C is 5 mL of A + 30 mL of B. Incubation of 0.05 μmol of P_i with 1 mL of solution C at 40 °C for 20 min gives a blue solution with $A_{810} \approx 1.2$. P_{si} does not give a positive color formation in the above test. However, when 0.05 μmol of P_{si} in 1 mL of solution C is heated at 100 °C for 10 min, an absorption of ~1.0 at 810 nm relative to blank solution C can be obtained. For accurate quantitation of P_i and P_{si}, a calibration curve is required in each set of measurements. P_{si} can also be determined by UV absorption directly: at pH 3.2, λ_{max} = 210 nm and ϵ = 4000; at pH 7, λ_{max} = 225 nm and ϵ = 4200. It is known that P_{si} could be hydrolyzed to P_i in certain pH ranges (Dittmer & Ramsay, 1963).

Synthesis of (Rp)- and (Sp)-[α-$^{18}O_1$]AMPS. [$^{18}O_2$]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 $PSCl_3$. After being stirred for 12 h at 0–4 °C, the resultant suspension was filtered and the precipitate was then hydrolyzed in 5 mL of $H_2{}^{18}O$ (99.5%). The resultant solution was brought to neutral pH with NaOH and purified by column chromatography to give 5.8 mmol of pure AMPS (**1**). ^{31}P NMR analysis indicated that **1** contained only $^{18}O_2$ species (shifted 0.067 ppm upfield) and no detectable $^{18}O_1$ or nonlabeled species. Since the signal/noise ratio of the NMR spectrum is ~20, the atom % ^{18}O enrichment of **1** is at least >95%.

Phosphorylation of **1** by the procedure of Eckstein & Goody (1976) gave a mixture of diastereomers, [α-$^{18}O_1$]ADPαS (A + B) (**2**), in 50% yield. Pyruvate kinase is known to be specific for the A isomer of ADPαS (Eckstein & Goody, 1976), but the stereospecificity may not be 100% (Jaffe & Cohn, 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 ATPαS (A) (**3**) as determined by ^{31}P NMR (Sheu & Frey, 1977) (no detectable P_α signal due to isomer B). The unreacted ADPαS contained 80% isomer B and 20% isomer A. Further incubation of this unreacted ADPαS with pyruvate kinase and PEP, followed by column chromatography, gave ATPαS (A + B) and >95% pure ADPαS (B) (**4**) (40% yield from **2**). ^{31}P NMR analysis indicated that one of the two ^{18}O atoms in **1** was retained in **3** and **4** in >95% enrichment.

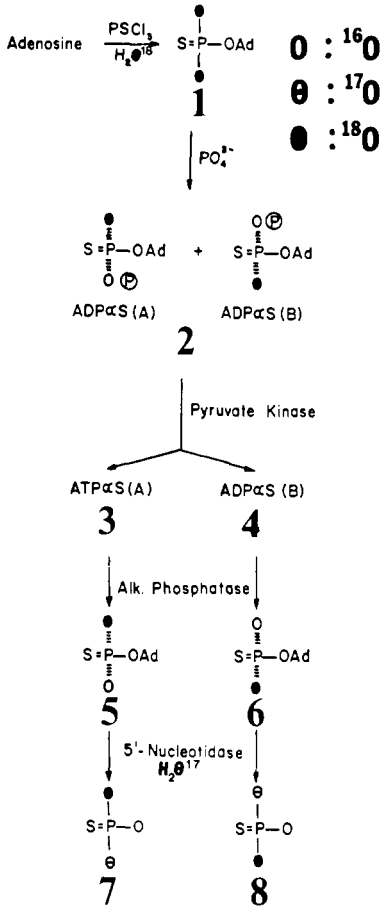
ATPαS (A) (**3**) and ADPαS (B) (**4**) were hydrolyzed to (*Sp*)-[α-$^{18}O_1$]AMPS (**5**) and (*Rp*)-[α-$^{18}O_1$]AMPS (**6**), respectively, by calf intestine alkaline phosphatase. The incubation mixture (pH 8.5) contained 0.1 M Tris, 0.01 M $MgCl_2$, 50 mM nucleotide, and 0.02 mg of enzyme/mL. 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_i by a DEAE-Sephadex column eluted with ammonium bicarbonate gradient, in a 90% yield. ^{31}P NMR analysis indicates >95% ^{18}O 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 $MgCl_2$, pH 8.5. The water was then removed by lyophilization and replaced by 0.6 mL of $H_2{}^{17}O$ (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 A_{265} due to deamination of the adenosine formed. After the reaction was complete, the product P_{si} (**7** and **8**, respectively) was converted into ATPγS 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 % ^{18}O enriched [$^{18}O_2$]-AMPS was hydrolyzed by 5′-nucleotidase in H_2O according to the same procedure. The product $PS^{18}O_2O^{3-}$ was isolated in a 60% yield by chromatography on a DEAE-Sephadex column eluted with ammonium bicarbonate gradient. No appreciable amount of P_i was found as a byproduct. The $P^{18}O_2O^{3-}$ was converted to its trimethyl ester by CH_2N_2 methylation and analyzed by GC–MS. The atom % ^{18}O enrichment found was 65%.

Conversion of P_{si} to ATPβS (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 50 mM Tris, 10 mM DTE, 10 mM $MgCl_2$, 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, dialyzed 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 ATPγS in 55% yield relative to AMPS. The ATPγS obtained was then dissolved in 5 mL of buffer (PH 7.5) containing 0.1 M Hepes, 50 mM KCl, 25 mM $MgCl_2$, 1 mM DTE, and 0.15 M AMP and incubated with 2000 units of myokinase at 25 °C. Formation of ADPβS was followed by TLC. The product ADPβS was isolated by column chromatography in 80% yield. For con-

Scheme I



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 $MgCl_2$, 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. ^{31}P NMR analysis indicates no detectable isomer A.

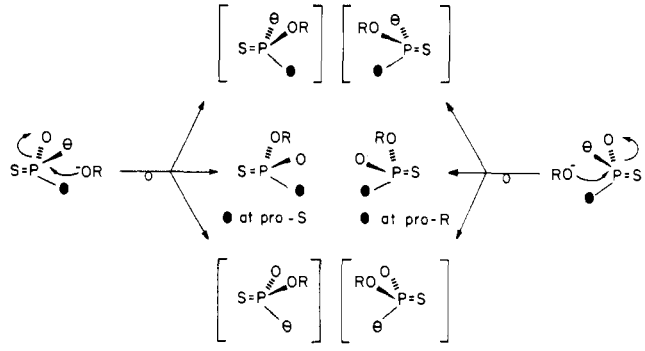
Results

Synthesis of (Sp)- and (Rp)-[α-$^{18}O_1$]AMPS. Scheme I summarizes the experimental procedure. Reaction of $PSCl_3$ with adenosine (Murray & Atkinson, 1968), followed by $H_2{}^{18}O$ (99.5%) hydrolysis, gave [$^{18}O_2$]AMPS (**1**) (>95% ^{18}O). Chemical phosphorylation (Eckstein & Goody, 1976) of **1** yielded [α-$^{18}O_1$]ADPαS (A + B) (**2**). Incubation of **2** with pyruvate kinase and phosphoenolpyruvate (Eckstein & Goody, 1976; Jaffe & Cohn, 1979) gave [α-$^{18}O_1$]ATPαS (A) (**3**) (>95% isomer A) from the first 30% reaction and [α-$^{18}O_1$]-ADPαS (B) (**4**) (>95% isomer B) from the last 40% unreacted ADPαS. Reaction of **3** and **4** with calf intestine alkaline phosphatase gave (*Sp*)-[$^{18}O_1$]AMPS (**5**) and (*Rp*)-[$^{18}O_1$]-AMPS (**6**), respectively. Recently different procedures for the synthesis of **5** and **6** have been developed (Jarvest & Lowe, 1979; Richard et al., 1979).

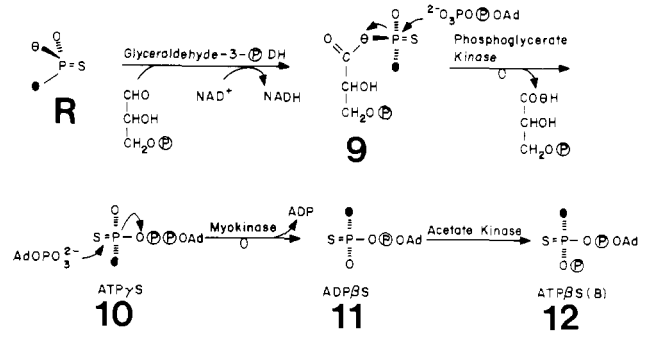
Hydrolysis of AMPS Catalyzed by 5'-Nucleotidase. The kinetic data for AMPS as a substrate of the venom 5'-nucleotidase have been obtained by Murray & Atkinson (1968): V_{max} = 0.026 μmol min^{-1} mg^{-1} and K_m = 0.02 mM. The corresponding values for AMP are V_{max} = 1.39 μmol min^{-1} mg^{-1} and K_m = 0.035 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 $H_2{}^{17}O$ (52.8% $H_2{}^{17}O$, 41.8% $H_2{}^{18}O$). The chiral P_{si} products (**7** and **8**, respectively) were converted into ATPγS (see later discussions) immediately, without being isolated, to minimize any possible racemization.

Scheme II



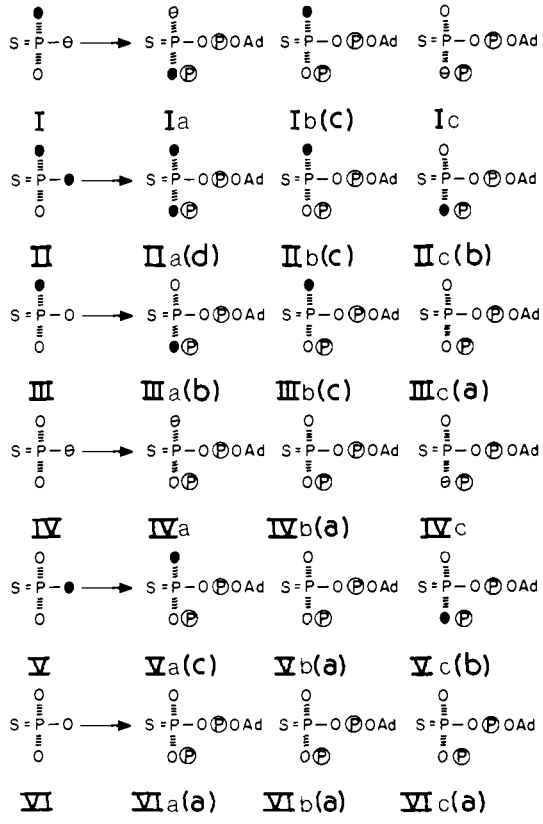
Scheme III



Rationale of Conformational Analysis by ^{31}P NMR. This is based on the combination of the $^{31}P(^{17}O)$ NMR method (Tsai, 1979; Tsai et al., 1980) and the $^{31}P(^{18}O)$ isotope shift method (Cohn & Hu, 1978). As illustrated by Scheme II, displacement of one of the three oxygen isotopes of (*S*)-[^{16}O,^{17}O,^{18}O]P_{si} by a nucleophile (RO^-) gives a mixture of three inseparable species. Among them, two (those in brackets) contain an ^{17}O isotope. According to the results of our recent work, ^{17}O causes the ^{31}P NMR signals of the ^{31}P nuclei directly bonded to ^{17}O to broaden in most biochemical phosphate derivatives ($\Delta P \approx$ 50–70 Hz for P–^{17}O–P; $\Delta P >$ 300 Hz for P–$^{17}O^-$) (Tsai et al., 1980). Thus, the two ^{17}O-containing species should give very broad ^{31}P signals which may not be observable. Only the species which contains only ^{16}O and ^{18}O (^{18}O at the *pro-S* position) should give a sharp, unquenched ^{31}P NMR signal. Analogously, the (*R*)-[^{16}O,^{17}O,^{18}O]P_{si} should give a corresponding non-^{17}O-containing species with ^{18}O at the *pro-R* position. The *pro-R* and *pro-S* ^{18}O can be distinguished by a stereospecific phosphorylation at one of the two positions. It is known that ^{18}O causes the ^{31}P NMR signal to shift upfield (~0.02–0.05 ppm) (Cohn & Hu, 1978). A nonbridge ^{18}O (P–$^{18}O^-$) should cause a larger shift of the ^{31}P signal than a bridge ^{18}O (P–^{18}O–P) does, due to a greater double bond character (Cohn & Hu, 1980; Lowe et al., 1979). The two main steps for configurational analysis by ^{31}P 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 thiophosphoryl group of ATPγS with P_{si}. We used this procedure to convert chiral P_{si} into ATPγS (**10**). As shown in Scheme III, *R*-chiral P_{si} was first incorporated into 3-phosphoglyceroyl thiophosphate (**9**) with intact configuration.

Scheme IV



Only the species with ^{17}O at the P–O–C bridge position is shown in the scheme since the other two species (P–^{16}O–C and P–^{18}O–C) will give species containing ^{17}O in the later steps. The thiophosphoryl group of **9** was then transferred to ADP by cleaving the P–OC bond to give ATPγS (**10**). Webb & Trentham (1980) have shown that the thiophosphoryl group transfer catalyzed by PGK proceeds with inversion of configuration at phosphorus. The thiophosphoryl group of ATPγS was then transferred to AMP by myokinase, with inversion of configuration (Richard & Frey, 1978), to give ADPβS (**11**). Stereospecific phosphorylation by acetate kinase (Richard et al., 1978) at the *pro-R* oxygen of ADPβS gave ATPβS (B) (**12**). On the basis of the stereochemistry involved (two inversions), (*R*)-[^{16}O,^{17}O,^{18}O]P_{si} should give [β-^{18}O]ATPβS (B) (^{18}O at the β-nonbridge position), whereas (*S*)-[^{16}O,^{17}O,^{18}O]P_{si} should yield [βγ-^{18}O]ATPβS (B) (^{18}O at the βγ-bridge position). Both enantiomers of the [^{16}O,^{17}O,^{18}O]P_{si} **7** and **8** obtained from hydrolysis catalyzed by 5′-nucleotidase were converted to ATPβS (B) according to Scheme III, with an overall yield of ~30%. The samples were then analyzed by ^{31}P NMR. In a separate experiment, [$^{18}O_3$]P_{si} was converted to [β,βγ-$^{18}O_2$]ATPβS (B) by the same procedure.

Theoretical Analysis. Although Scheme III shows that (*R*)-[^{16}O,^{17}O,^{18}O]P_{si} should give rise to [β-^{18}O]ATPβS (B) (**12**) which gives an observable P_β signal in ^{31}P NMR, it is technically impossible to obtain a chiral P_{si} 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_{si} sample and the three possible ATPβS (B) (e.g., Ia, Ib, and Ic) species which could result from each P_{si} species. Since the kinetic isotope effect due to oxygen isotopes should be very small, the three ATPβS (B) species from each P_{si} species should be in approximately equal amounts (e.g., Ia ≈ Ib ≈ Ic).

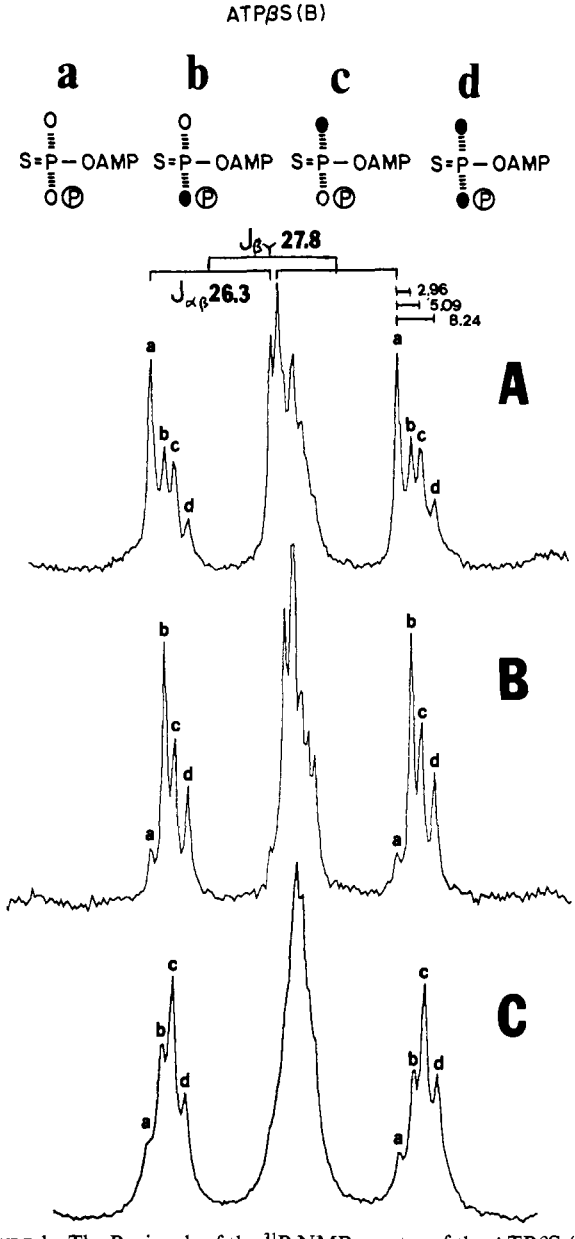


FIGURE 1: The P_β signals of the ^{31}P NMR spectra of the ATPβS (B) obtained from [$^{18}O_3$]P_{si} (A) and from the two chiral P_{si} **7** and **8** (B and C, respectively). The sample (30 μmol) was dissolved in 2.5 mL of D_2O containing 10 mM EDTA and the spectra were recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are expressed in hertz. The chemical shift of the P_β signal is −29.8 ppm from H_3PO_4. Number of transients = 2800. Pulse delay = 5 s.

Among the ATPβS (B) species in Scheme IV, Ia, Ic, IVa, and IVc contain an ^{17}O atom bonded to P_β. Each of the remaining species, as designated in parentheses, belongs to one of the four non-^{17}O-containing species: nonlabeled ATPβS (B) (**a**), [βγ-^{18}O]ATPβS (B) (**b**), [β-^{18}O]ATPβS (B) (**c**), and [β,βγ-$^{18}O_2$]ATPβS (B) (**d**). In the ^{31}P NMR spectrum of the ATPβS (B) obtained from (*R*)-[^{16}O,^{17}O,^{18}O]P_{si}, which may contain a mixture of species I–VI, only the species other than Ia, Ic, IVa, and IVc will give unquenched, sharp P_β signals. As will be shown later (Figure 1), the P_β signal of ATPβS (B) obtained from chiral P_{si} 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 I: ^{31}P NMR Analysis of the ATPβS (B) Derived from Chiral Thiophosphates

P_{si} samples	intensity[a] (%) a	b	c	d	F value	config
$PS^{18}O_3^{3-}$	41.3 ± 1.2	24.6 ± 0.1	22.1 ± 0.0	11.8 ± 1.2	1.11	
7	8.8 ± 0.5	42.8 ± 0.6	28.1 ± 0.5	20.3 ± 0.5	1.52	*S*
8	12.2 ± 0.5	26.5 ± 1.6	38.8 ± 0.1	22.4 ± 2.0	0.68	*R*
calcd[b]	5.0	50.0	25.0	20.0	2.0	*S*
optimal	5.0	25.0	50.0	20.0	0.5	*R*
calcd[c]	7.8	47.3	25.9	19.0	1.82	*S*
minimal	7.8	25.9	47.3	19.0	0.55	*R*

[a] Obtained from peak height measurements for the P_β signal of ATPβS. The errors represent deviations between the two nonoverlapping halves of the two doublets. [b] Calculated for chiral P_{si} of 50% purity and 100% chirality. [c] Calculated for chiral P_{si} of 47.5% purity and 90% chirality.

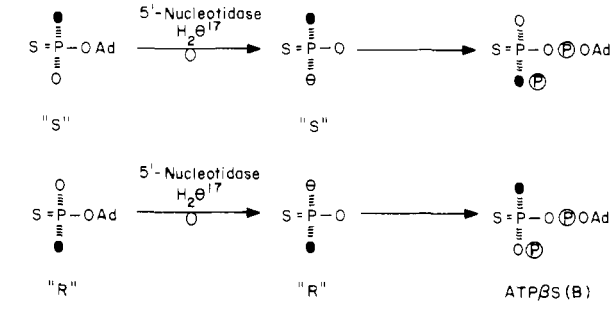
P_{si} sample should give $F < 1$. On the other hand, the species I of the opposite enantiomer, *S*-chiral P_{si}, should contribute to **b** instead of **c** and give $F > 1$.

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-$^{18}O_1$]AMPS (**5**) and (*Rp*)-[α-$^{18}O_1$]AMPS (**6**) are isotopically and isomerically pure based on NMR. The isotopic composition of $H_2{}^{17}O$, after being diluted by some additives, is 50% ^{17}O, 40% ^{18}O, and 10% ^{16}O. If **5** and **6** are 100% pure, the chiral P_{si} obtained from **5** or **6** should contain 50% I, 40% II, and 10% III, if the hydrolysis 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_{si}, 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 AMPS (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_{si} obtained may have a 47.5% "purity" and a 90% "chirality", if the hydrolysis is 100% stereospecific. The calculated compositions of ATPβS (B) obtained from such chiral P_{si} 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_β signals of ATPβS (B) obtained from [$^{18}O_3$]P_{si} and the two chiral P_{si} **7** and **8** (Figure 1, parts A, B, and C, respectively). The signal contains two overlapping doublets due to ^{31}P–^{31}P coupling ($J_{\alpha\beta}$ = 26.3 Hz, $J_{\beta\gamma}$ = 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,βγ-$^{18}O_2$]ATPβS (B) (**d**), respectively. Since a bridge ^{18}O is expected to cause a smaller magnitude of isotope shift due to its smaller double bond character compared to a nonbridge ^{18}O (Cohn & Hu, 1980; Lowe et al., 1979), peak b should come from [βγ-^{18}O]ATPβS (B) (**b**) whereas peak c can be assigned to [β-^{18}O]ATPβS (B) (**c**). The relative heights of peaks a–d are listed in Table I. As expected, the ATPβS (B) obtained from [$^{18}O_3$]P_{si} (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_{si} **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

Scheme V



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_{si} may occur in two steps. First, when a sample of [$^{18}O_2$]AMPS (**1**) with lower enrichment (85 atom % ^{18}O as determined by ^{31}P NMR at 145.7 MHz) was hydrolyzed by 5′-nucleotidase in H_2O with a prolonged incubation (1 additional h after the reaction was complete), the isolated product $PS^{18}O_2O^{3-}$ was only enriched with 65 atom % ^{18}O as determined by GC–MS for its trimethyl ester. The detailed kinetics and mechanism of this oxygen loss from P_{si}, enzyme-catalyzed or chemical, remain to be established by more detailed investigation. Second, when a $PS^{18}O_3^{3-}$ sample (75 atom % ^{18}O, obtained from hydrolysis of 50 μL of $PSCl_3$ in 200 μL of 99% $H_2{}^{18}O$ containing 120 mg of NaOH) was converted to ATPγS by a prolonged incubation (7 h), the [γ-$^{18}O_2$]ATPγS obtained was only enriched with 40 atom % ^{18}O by NMR analysis. This oxygen loss is apparently due to reversible conversions between P_{si} and ATPγS which may cause oxygen exchange between P_{si} and glyceraldehyde 3-phosphate, as can be seen from Scheme III.

In the work involving chiral P_{si} 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 avoidable and could cause partial racemization which accounts for the differences between the observed and the calculated *F* values.

P_γ Signal of ATPβS (B). The P_γ signals of ^{31}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 $^{31}P(^{16}O)$ and $^{31}P(^{18}O)$ (0.021 ppm upfield) species. The $^{18}O/^{16}O$ ratios are 1.21 in Figure 2A and 1.14 in Figure 2B. This ratio is not a measure of chirality since, as shown in Scheme IV, the chiral P_{si} species (I) contributes equally to Ia and Ib. The ratio, however, may be related to isotopic compositions. The calculated ratio for chiral P_{si} of 50% purity and 100% chirality is 1.27. The deviation in the observed values could be due to the additional $P_{si} \rightleftarrows H_2O$ oxygen exchange discussed above.

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 [^{16}O,^{17}O,^{18}O]thiophosphates. Possible

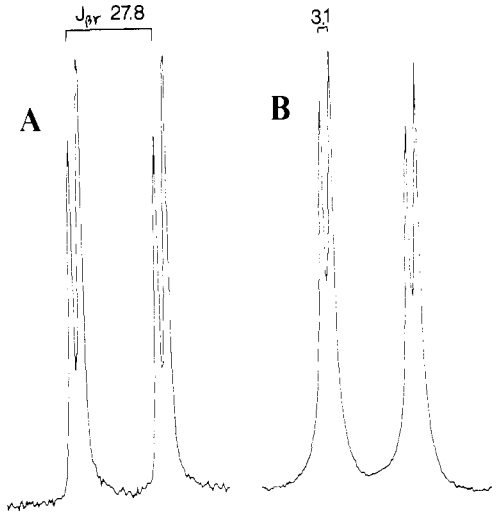


FIGURE 2: The P_γ signals the ^{31}P NMR spectra of the ATPβS (B) obtained from the two chiral P_{si} **7** and **8** (A and B, respectively). Experimental conditions are the same as in Figure 1. The coupling constants and isotope shifts are expressed in hertz. The chemical shift of the P_γ signal is 6.0 ppm from H_3PO_4.

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 Phosphomonoesterases. There are five important types of phosphomonoesterases: 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 $P_i \rightleftarrows H_2O$ 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 [^{16}O,^{17}O,^{18}O]phosphate monoesters catalyzed by *E. coli* alkaline phosphatase proceeds with net retention of configuration at phosphorus.

A phosphoryl-enzyme intermediate has been shown for the sarcoplasmic membrane ATPase (Hasselbach, 1974), which catalyzes both ATP hydrolysis and $P_i \rightleftarrows H_2O$ oxygen exchange (Kanazawa & Boyer, 1973). The yeast inorganic pyrophosphatase catalyzes extensive $P_i \rightleftarrows H_2O$ 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 [^{16}O,^{17}O,^{18}O]P_{si} 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 $P_i \rightleftarrows H_2O$ oxygen exchange (Koshland & Springhorn, 1956). On the basis of these and other mechanistic features, Koshland & 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_{si}. In the synthesis of **5** and **6** we have been able to optimize the ^{18}O isotopic purity (>95%) and the isomeric 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_{si} and glyceraldehyde 3-phosphate which might occur during the conversion of chiral P_{si} to ATPγS via reverse reactions. However, there are certain limitations in accurate quantitations. Of the three parameters, *F* values, isotopic compositions, and chirality, two have to be known in order to define the third. The *F* values, which are defined by the peak heights of ^{31}P NMR signals, may have an error of ±10%. Accurate determination of the isotopic compositions of chiral P_{si} is difficult due to the fact that (i) there are two labeled positions, ^{17}O and ^{18}O, (ii) the ^{17}O isotope used always contains some ^{18}O and ^{16}O, and (iii) sulfur also contains 4.2% of ^{34}S. In the present work the isotopic compositions of chiral P_{si} were obtained from that of the precursors **5** and **6** and the composition of $H_2{}^{17}O$ used for hydrolysis.

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

While this work was in progress, Webb & Trentham (1980) synthesized chiral P_{si} 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 ATPγS to ADP and P_{si} with inversion of configuration.

Acknowledgments

We are indebted to Dr. W. W. Cleland of the University of Wisconsin for a trial experiment on the CD spectrum of chiral P_{si}, to Drs. M. R. Webb and D. R. Trentham of the University of Pennsylvania for informing us of their work on chiral P_{si} prior to publication, to Drs. H. G. Floss (Purdue University) and J. R. Knowles (Harvard University) for useful discussions, to J. F. Kozlowski for obtaining some NMR spectra, and to T. T. Chang for excellent technical assistance.

References

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

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

Benkovic, S. J., & Schray, K. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 493–527, Plenum Press, New York.

Cohn, M., & Hu, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 200–203.

Cohn, M., & Hu, A. (1980) *J. Am. Chem. Soc. 102*, 913–916.

Dittmer, D. C., & Ramsay, O. B. (1963) *J. Org. Chem. 28*, 1268–1272.

Drummond, G. I., & Yamamoto, M. (1971) *Enzymes, 3rd Ed. 4*, 337–354.
Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160–166.
Eckstein, F. (1977) *Biochim. Biophys. Acta 483*, 1–5.
Eckstein, F. (1978) *Acc. Chem. Res. 12*, 204–210.
Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.
Hackney, D. D., & Boyer, P. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3133–3137.
Hasselbach, W. (1974) *Enzymes, 3rd Ed. 10*, 431–467.
Hollander, V. P. (1971) *Enzymes, 3rd Ed. 4*, 449–498.
Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839–10845.
Janson, C. A., Degani, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 3743–3749.
Jarvest, R. L., & Lowe, G (1979) *J. Chem. Soc., Chem. Commun.*, 364–366.
Jones, S. R., Kindman, L. A., & Knowles, J. R. (1978) *Nature (London) 275*, 564–565.
Kanazawa, T., & Boyer, P. D. (1973) *J. Biol. Chem. 248*, 3163–3172.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.
Koshland, D. E., Jr., & Springhorn, S. S. (1956) *J. Biol. Chem. 221*, 469–476.
Levine, D., Reid, T. W., & Wilson, I. B. (1969) *Biochemistry 8*, 2374–2380.
Lowe, G., Potter, B. V. L., Sproat, B. S., & Hull, W. E. (1979) *J. Chem. Soc., Chem. Commun.*, 733–735.
Morton, R. K. (1953) *Nature (London) 172*, 65.
Murray, W. A., & Atkinson, M. R. (1968) *Biochemistry 7*, 4023–4029.
Reid, T. W., & Wilson, I. B. (1971) *Enzymes, 3rd Ed. 4*, 373–415.
Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757–7758.
Richard, J. P., Ho, H.-T., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756–7757.
Richard, J. P., Douglas, C. P., Ives, D. H., & Frey, P. A. (1979) *J. Biol. Chem 254*, 4339–4341.
Sheu, K.-F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445–4448.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472.
Tsai, M.-D., & Chang, T. T. (1980) *J. Am. Chem. Soc. 102*, 5416–5418.
Tsai, M.-D., Huang, S. L., Kozlowski, J. F., & Chang, C. C. (1980) *Biochemistry 19*, 3531–3536.
Webb, M. R., & Trentham, D. R. (1980) *J. Biol. Chem. 255*, 1775–1779.

Nerve Growth Factor Zymogen. Stoichiometry of the Active-Site Serine and Role of Zinc(II) in Controlling Autocatalytic Self-Activation†

Michael Young* and Mary Jo Koroly

ABSTRACT: Mouse submandibular gland nerve growth factor (NGF) is a 116000 molecular weight protease with a high degree of specificity for certain lysyl and arginyl bonds. This protein can activate plasminogen and it is also a member of the general class of serine proteases [Orenstein, N. S., Dvorak, H. A., Blanchard, M. H., & Young, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497]. As isolated, NGF is an enzymically inactive zymogen. Upon dilution from high to very low protein concentrations or upon treatment with EDTA, the zymogen undergoes autocatalytic activation. Atomic absorption spectroscopy measurements reveal that NGF contains 1 g-atom of tightly bound Zn(II) per mol. Reaction of the fully autoactivated protease with [^{3}H]DFP yields 1 mol of labeled serine per mol of enzyme. All results indicate that as long as Zn(II) remains bound to the zymogen, autocatalytic activation is inhibited. Removal of this ion, by dilution of the protein or by chelation, initiates autoactivation. The physiologic purpose of this unusual reaction is not known but it may be that Zn(II) serves to act as a control ion which keeps the protein in an inactive form (the zymogen) until it recognizes its naturally occurring substrate.

Recent studies from this laboratory have shown that the predominant form of mouse submandibular gland nerve growth factor (NGF)[1] is a protein of molecular weight 116000 (Young et al., 1978) which is secreted at very high concentrations into mouse saliva (Murphy et al., 1977a,b). Other lower molecular weight forms of NGF are also present in both salivary gland extracts and saliva, and evidence has been presented which indicates that these smaller species are degradation products of the larger 116000 molecular weight form (Young et al., 1978; Murphy et al., 1977b).

The observation that the mouse submandibular gland contains a high molecular weight form of NGF is not a new one. In 1967, Varon et al. (1967) isolated a protein which they called 7S-NGF which was estimated to have a molecular weight of 140000. Evidence from several sources indicates that 7S-NGF is an unstable protein and that it dissociates into its subunits at relatively high protein concentrations (Varon et al., 1967; Greene et al., 1969; Smith et al., 1969; Baker, 1975; Pantazis et al., 1977). When the individual subunits of 7S-NGF are recombined, the resulting protein exhibits the

† From the Department of Biochemistry and Molecular Biology, University of Florida, College of Medicine, Gainesville, Florida 32610. *Received February 19, 1980.* This work was supported by National Institutes of Health Grants CA 17137 and NS 13674.

[1] Abbreviations used: NGF zymogen, 116000 molecular weight nerve growth factor zymogen prepared as described by Young et al. (1978); 7S-NGF, nerve growth factor prepared as described by Varon et al. (1967); EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphorofluoridate; Tame, N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester; TLME, N^{α}-*p*-toluenesulfonyl-L-lysine methyl ester; TLCK, N^{α}-*p*-toluenesulfonyl-L-lysine chloromethyl ketone; *p*NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/80/0419-5316$01.00/0