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Use of Phosphorus-31 Nuclear Magnetic Resonance to Distinguish Bridge and Nonbridge Oxygens of Oxygen-17-Enriched Nucleoside Triphosphates. Stereochemistry of Acetate Activation by Acetyl Coenzyme A Synthetase†

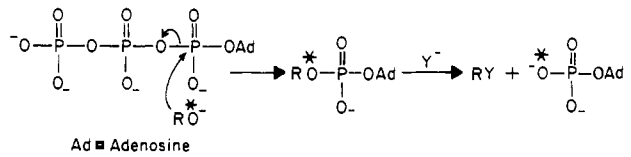
Ming-Daw Tsai

ABSTRACT: Adenosine 5′-(thiophosphate) (AMPS) contains a prochiral phosphorus center. Differentiation of the two diastereotopic oxygens would allow elucidation of the stereochemical course of biological adenylyl transfer reactions. A general method was developed to distinguish between the "pro-*R*" and "pro-*S*" oxygens. When we converted the AMPS to the isomer A of adenosine 5′-(1-thiotriphosphate) (ATPαS), which is known to have *S* configuration at P_α, the pro-*R* oxygen is incorporated into the bridge position, whereas the pro-*S* oxygen is located at the nonbridge position. The ^{31}P NMR spectra of the ^{17}O-enriched compounds were used to distinguish between the bridge and nonbridge oxygens based on the decrease in the peak intensity of ^{31}P NMR signals caused by the directly bound ^{17}O isotope. The method was used to elucidate the stereochemical course of acetate activation catalyzed by yeast acetyl coenzyme A (CoA) synthetase. The results indicate that yeast acetyl-CoA synthetase is specific for the isomer B of ATPαS and that the nucleophilic displacement proceeds with net inversion of configuration at P_α of ATPαS (B), supporting the "in-line" mechanism.

Adenylyl transfer reactions involve nucleophilic displacement of the pyrophosphoryl group of ATP by the second substrate (Scheme I). In most cases the functional group which undergoes nucleophilic displacement contains oxygen, e.g., carboxyl, hydroxyl, and imidol [HN=C(OH)NH–] groups, or sulfate. This type of reaction is involved in many classes of important biological processes (Stadtman, 1973), e.g., carboxyl group activation (fatty acyl-CoA synthetases, aminoacyl-tRNA synthetases, lipoate activating enzymes, biotin activating enzymes, etc.), biosynthesis of phosphodiester derivatives of adenosine (RNA polymerases, adenylation of amino glycoside antibiotics, etc.), synthesis of adenosine diphosphate derivatives (NAD, FAD, ADPglucose, etc.), sulfate activation, synthesis of imidol adenylate derivatives, and adenylylation of functional groups of proteins. In many cases a third substrate displaces the AMP moiety, resulting in the incorporation of an oxygen from the second substrate into the phosphoryl group of AMP.

Among the numerous enzymes catalyzing reactions involving chiral, prochiral, or proprochiral phosphorus centers, only a few have been analyzed unequivocally for their steric courses, and only two of them, e.g., DNA-dependent RNA polymerase (Eckstein et al., 1976) and tRNA nucleotidyltransferase (Eckstein et al., 1977), are adenylyl transfer reactions. Both enzymes specifically incorporate the isomer A of ATPαS.[1] In both cases, the configuration of the thiophosphate diester bond

Scheme I



was determined by correlation, through a combination of ribonuclease and chemical reactions, with the endo isomer of uridine 2′,3′-*O,O*-thiophosphate, the absolute configuration of which had been determined by X-ray crystallography (Eckstein, 1975). Following elucidation of the absolute configuration of ATPαS (A) (Burgers & Eckstein, 1978), it is now known that both enzymes catalyze the adenylyl transfer reactions with net inversion of configuration at P_α.

In this paper[2] a general method to elucidate the stereochemical course of adenylyl transfer reactions is presented. The method is based on the following rationale. If one of the two isomers of ATPαS is used as a substrate, the stereochemical course of the nucleophilic displacement can be elucidated by determining whether the labeled oxygen of the second substrate is incorporated into the pro-*R* or pro-*S* position of the product AMPS. By converting the AMPS to

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[1] Abbreviations used: AMPS, adenosine 5′-(thiophosphate); ADPαS, adenosine 5′-(1-thiodiphosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEP, phosphoenol pyruvate.

[2] An abstract of part of this work has been submitted for presentation at the 177th National Meeting of the American Chemical Society to be held in Honolulu, HI, April 1979.

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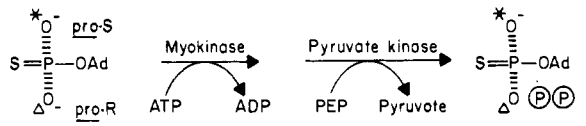
Scheme II



the isomer A of ATPαS (Sheu & Frey, 1977; Jaffe & Cohn, 1978), which is known to have *S* configuration at P_α (Burgers & Eckstein, 1978), we can locate the pro-*R* oxygen at the bridge position, whereas the pro-*S* oxygen is located at the nonbridge position, as shown in Scheme II.

A ^{31}P NMR method was developed to differentiate the bridge and nonbridge oxygens of nucleoside triphosphates based on the decrease in the peak intensity of the ^{31}P NMR signals caused by the directly bound ^{17}O isotope. Applicability of this method is demonstrated by elucidation of the stereochemical course of acetate activation catalyzed by the yeast acetyl-CoA synthetase which has been shown to incorporate the oxygen of acetate into the product AMP (Boyer et al., 1956).

Materials and Methods

Materials. Both 41% $H_2{}^{17}O$ (containing 41% ^{17}O and 28% ^{18}O) and 20% $H_2{}^{17}O$ (containing 20% ^{17}O and 20% ^{18}O) were purchased from Merck. Acetyl-CoA synthetase (yeast; 3 units/mg), myokinase (rabbit muscle; 360 units/mg), and pyruvate kinase (rabbit muscle; 200 units/mg) were purchased from Boehringer. Lactic dehydrogenase (pig heart; 500 units/mg), NADH, dithioerythritol, ATP, PEP, and adenosine were obtained from Sigma. DEAE-Sephadex was purchased from Pharmacia. Other chemicals used were of reagent grade or highest purity available commercially.

Preparation of Thiophosphate Analogues. AMPS was prepared according to the procedure of Murray & Atkinson (1968). ATPαS (A) was prepared enzymatically from AMPS (Sheu & Frey, 1977; Jaffe & Cohn, 1978). ATPαS (B) was prepared according to the procedure of Eckstein & Goody (1976).

Preparation of ^{17}O-Labeled Compounds. [^{17}O]Phosphate was prepared by addition of PCl_5 (0.15 mmol) to 50 μL of 41 atom % enriched $H_2{}^{17}O$. The 20% $H_2{}^{17}O$ was used for the preparation of all other ^{17}O compounds. [$^{17}O_2$]AMPS (**1**) was prepared according to the procedure of Murray & Atkinson (1968) by using $H_2{}^{17}O$ in the hydrolysis step. [α-^{17}O,αβ-^{17}O]ATPαS (A) (**3**) was prepared enzymatically (Sheu & Frey, 1977; Jaffe & Cohn, 1978) from [$^{17}O_2$]AMPS (**1**). [αβ,βγ-$^{17}O_2$,β-$^{17}O_2$]ATP (**2**) was synthesized by a modified procedure of Hoard & Ott (1965) as described below. AMP (0.5 mmol) was converted to the pyridinium form and then evaporated with 150 μL of tributylamine, followed by evaporation twice with 2 mL of dry pyridine. The sample was then dissolved in 5 mL of DMF and added to 100 mg of 1,1′-carbonyldiimidazole. After stirring at room temperature under an argon atmosphere for 23 h, we added tributylammonium [$^{17}O_4$]phosphate (prepared from 1 mmol of PCl_5 and 0.2 mL of 20% $H_2{}^{17}O$) to the activated AMP, and the reaction mixture was further stirred at room temperature for 16 h (some precipitate formed in 5 min). The reaction mixture was diluted with CH_3OH, evaporated to dryness, loaded to a column of DEAE-Sephadex A-25 (2.5 × 25 cm), and eluted with a gradient of 2 L each of 0.1 and 0.6 M triethylammonium bicarbonate (pH 7.5). The ADP isolated (2600 A_{260} units) was then converted to compound **2** by incubating with pyruvate kinase and phosphoenol pyruvate and purified chromatographically by using the same column described above.

[^{17}O]Acetate was prepared by incubation of 0.5 mmol of glacial acetic acid with 0.25 mL of $H_2{}^{17}O$ (20%) and 62.5 μL of 38% HCl at 100 °C for 24 h (Boyer et al., 1956). The atom enrichment of ^{17}O in the product was determined as 19% by conversion into acetanilide, followed by mass spectral analysis.

NMR Measurements. ^{31}P spectra were recorded at 32.2 MHz on a Varian FT-80 NMR spectrometer equipped with a multinuclear probe. The field was locked on deuterium, and all spectra were recorded with broad-band proton decoupling at ambient temperature. All samples were dissolved in 1.3–1.6 mL of 99% D_2O in 10-mm tubes. All chemical shifts are expressed relative to 85% H_3PO_4 as the external reference.

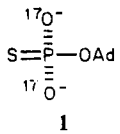
The following conditions were used in obtaining all spectra: 4000-Hz spectral width; 1.0-s acquisition time; 60° pulse width; and 10-s pulse delay. The integrations were obtained for each signal separately at the spectral width of 200 Hz (20-times expansion). Two spectra were obtained for each sample, and three integrations were obtained for each spectrum.

Assay of Acetyl-CoA Synthetase. For the measurement of the apparent K_m values and maximum velocities, the yeast acetyl-CoA synthetase was assayed by measuring the increase in the absorbance at 232 nm caused by the formation of acetyl-CoA. Each 0.5-mL reaction vial contained 100 mM Hepes, pH 8.0; 50 mM KCl; 25 mM $MgCl_2$; 1 mM dithioerythritol; 2 mM acetate; 2 mM coenzyme A; varying concentrations (0.1–4.0 mM) of ATPαS (B) or ATP; and acetyl-CoA synthetase [0.25 mg for ATPαS (B) and 0.025 mg for ATP]. The incubation mixtures were incubated at 25 °C for 2–20 min. The initial rates were obtained by measuring the increase in the absorbance at 232 nm as a function of time on a Cary-17 spectrometer in a cuvette of 1-mm path length. The assays in Figure 5 were carried out in a cuvette of 10-mm path length with lower concentration of substrates.

Stereochemical Study of Acetyl-CoA Synthetase. The incubation mixture (50 mL) contained 40 mM Hepes, pH 7.5; 20 mM KCl; 10 mM $MgCl_2$; 1 mM dithioerythritol; 70 μmol of [^{17}O]acetate; 30 μmol of ATPαS (B); 50 μmol of CoA; and 8 mg of acetyl-CoA synthetase. After incubation at room temperature for 16 h, the reaction mixture was chromatographed on a DEAE-Sephadex A-25 (HCO_3^-) column. The isolated [^{17}O]AMPS (20 μmol) was converted to ATPαS (A) by the coupled reactions of myokinase and pyruvate kinase as described above and analyzed by the ^{31}P NMR method.

Results

Effect of ^{17}O on the ^{31}P NMR Signals. The ^{17}O isotope has a spin number of 5/2 and a large electric quadrupole moment (-2.6×10^{-2} barns). When a quadrupolar nucleus and a dipolar nucleus X are spin–spin coupled, the multiplet of X is often broadened or collapsed due to the rapid relaxation rate caused by the quadrupolar nuclei (Witanowski & Webb, 1973). By use of inorganic phosphate labeled with 41% ^{17}O, it is shown (Figure 1) that ^{17}O causes the ^{31}P NMR signal to broaden substantially, to give a signal which was observable only when the signal to noise ratio was very high. For a compound with lower ^{17}O enrichment, e.g., [$^{17}O_2$]AMPS (**1**)



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in which each of the two oxygens of the thiophosphate group was enriched with 20% ^{17}O, the broad peak resulting from the ^{31}P (^{17}O) species could hardly be observed in the spectrum in which the signal to noise ratio was not especially high (Figure

Table I: Relative Peak Intensity of the ^{31}P NMR Signals of the ^{17}O-Labeled Compounds in Comparison with the Corresponding Nonlabeled Compounds[a]

compds	P_α			P_β			P_γ		
	% intensity	% original	calcd[b]	% intensity	% original	calcd[b]	% intensity	% original	calcd[b]
P_i[c]	195 ± 5								
[^{17}O]P_i[c]	52 ± 3	26 ± 2	12						
AMPS[d]	80 ± 3								
1[d]	46 ± 3	58 ± 4	64						
ATP[e]	340 ± 30			205 ± 9			223 ± 20		
2[e]	285 ± 20	84 ± 6	80	108 ± 10	53 ± 5	41	181 ± 15	81 ± 7	80
ATPαS (A)	138 ± 3			104 ± 4			100		
3	92 ± 1	67 ± 1	66	86 ± 4	83 ± 4	81	100		
ATPαS (A) from [^{17}O]acetate	111 ± 6	80 ± 4	81	85 ± 5	82 ± 5	81	100		

[a] Measured by spectral integration. The averaged values are obtained from two independent spectra, with three independent integrations for each spectrum. [b] Calculated percentage of the ^{31}P species free of ^{17}O according to the relationship $(a\,^{17}O + b\,^{16}O)^n$, where a = ^{17}O abundance per labeled oxygen, b = ^{16}O abundance per labeled oxygen, and n = number of position labeled. For example, in the [^{17}O]P_i the species present are derived from $(0.41\,^{17}O + 0.59\,^{16}O)^4$ where ^{18}O is considered as ^{16}O since ^{18}O has no effect on the peak intensity. The $P^{16}O_4$ species is therefore equal to $(0.59)^4$ of the total species, i.e., 12%. [c] Relative to the internal standard H_3PSO_3. [d] Relative to the external standard $H_4PO_4^+ClO_4^-$. [e] Relative to the internal standard P_i.

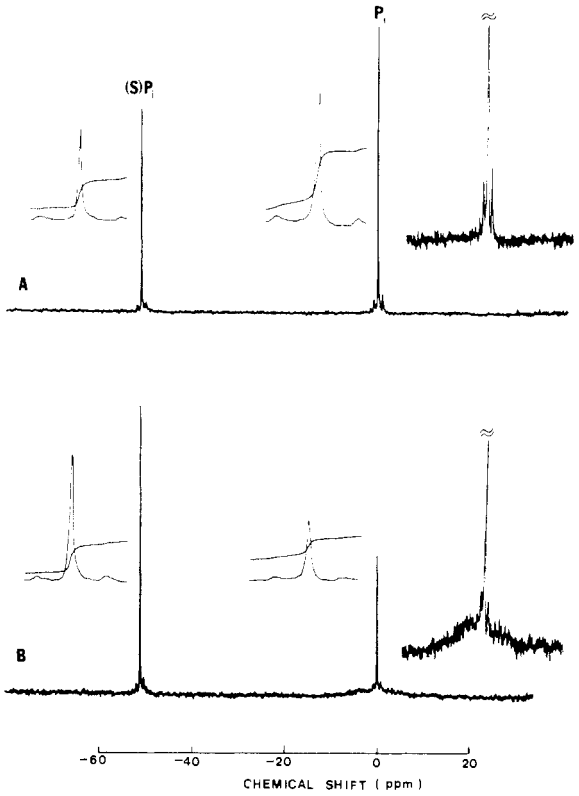


FIGURE 1: (A) Phosphate (100 mM), thiophosphate (90 mM), and EDTA (2 mM), pH 1.35; 2000 transients. (B) [^{17}O]Phosphate (100 mM), thiophosphate (90 mM), and EDTA (2 mM), pH 1.35; 3000 transients. The inset on the right-hand side represents the amplified origin of the phosphate signal. The other insets represent the integrations of the corresponding signals.

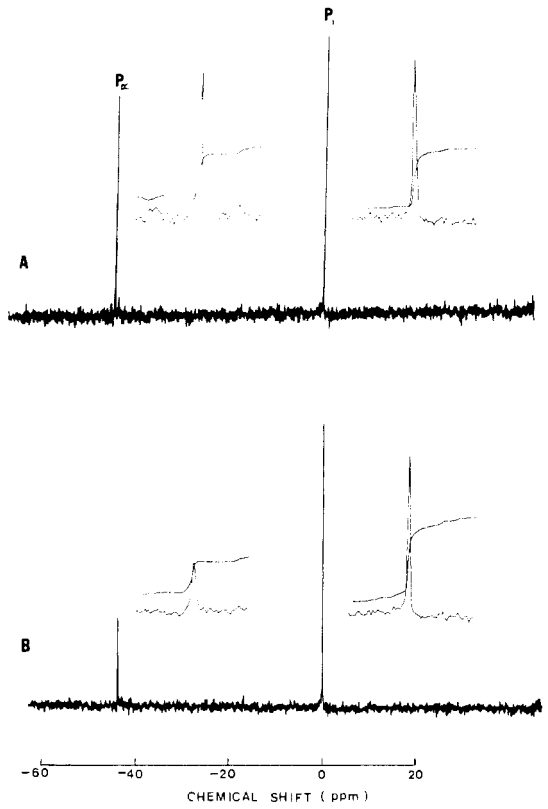
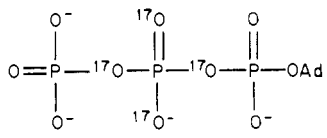


FIGURE 2: (A) AMPS (40 mM) and EDTA (2 mM), pH 5.7; 400 transients. (B) [$^{17}O_2$]AMPS (compound **1**) (40 mM), EDTA (2 mM), pH 5.7; 400 transients. $H_4PO_4^+ClO_4^-$ (0.2 M) in a 3-mm inner tube was used as the external standard. The insets represent the integrations of the corresponding signals.

2). When the integration was taken separately for each signal in the expanded spectra (200-Hz width), a flat base line could be obtained, as shown in Figure 1. The decrease in the integration of the labeled compounds relative to the corresponding nonlabeled compounds, as shown in Table I, correlates semiquantitatively with the theoretical values calculated based on the assumption that the intensity of the ^{31}P NMR signal of the ^{31}P nuclei labeled with one or more ^{17}O is negligible.

Differentiation of the Bridge and Nonbridge Oxygens. It was expected that ^{17}O at the bridge position of nucleoside triphosphates would cause a decrease in the integration of the ^{31}P signals of both of the directly bound phosphorus atoms, whereas ^{17}O at the nonbridge position would have such an effect only on the one ^{31}P nucleus directly bound to ^{17}O. To demonstrate this point, [$\alpha\beta,\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**2**) and [α-^{17}O,$\alpha\beta$-^{17}O]ATPαS (A) (**3**) were synthesized by using 20%



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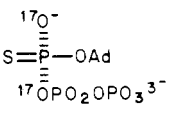

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atom enriched ^{17}O isotope. The ^{31}P NMR spectra of compound **2** and the corresponding nonlabeled ATP are shown in Figure 3, with inorganic phosphate as an internal reference. As revealed by the spectra and the integration data in Table

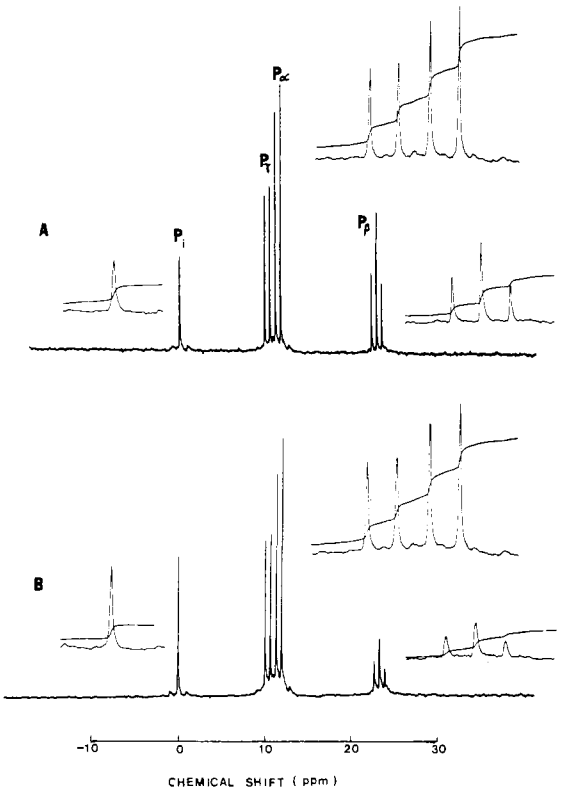


FIGURE 3: (A) ATP (70 mM), phosphate (56 mM), and EDTA (2 mM), pH 5.7; 1000 transients. (B) [$\alpha\beta,\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (compound 2) (70 mM), phosphate (56 mM), and EDTA (2 mM), pH 5.7; 2000 transients. The insets represent the integrations of the corresponding signals.

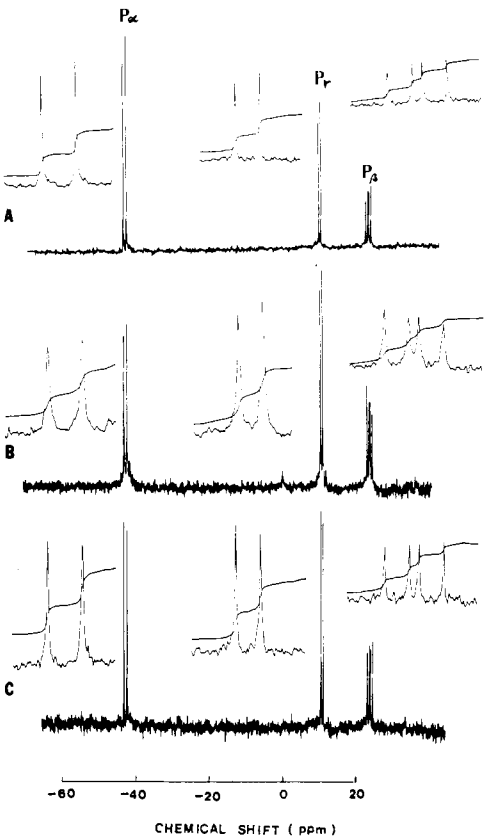


FIGURE 4: (A) ATPαS (A) (20 mM) and EDTA (2 mM), pH 5.7; 1800 transients. (B) [α-^{17}O,$\alpha\beta$-^{17}O]ATPαS (A) (compound 3) (60 mM) and EDTA (2 mM), pH 5.7; 700 transients. (C) ATPαS (A) from [^{17}O]acetate (5 mM), pH 5.7; 7600 transients. The insets represent the integrations of the corresponding signals.

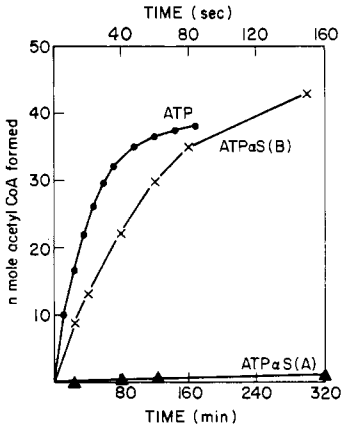
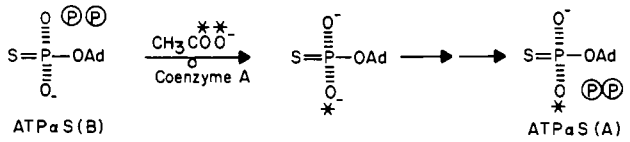


FIGURE 5: Substrate specificity of acetyl-CoA synthetase. The assay solution (1.0 mL) contained 40 mM Hepes, pH 7.5; 20 mM KCl; 10 mM $MgCl_2$; 1 mM dithioerythritol; 0.1 μmol of CoA; 0.5 μmol of acetate; 0.04 mg of acetyl-CoA synthetase; and 0.05 μmol of ATP [(●) time scale in seconds], ATPαS (A) [(▲) time scale in minutes], and ATPαS (B) [(×) time scale in minutes]. The solution was incubated at room temperature. The amount of acetyl-CoA formed was calculated from the increase in the absorbance at 232 nm in a cuvette of 10-mm path length.

Scheme III



I, the observed effect of ^{17}O on the ^{31}P NMR signals of compound **2** correlates well with the predicted values. Similar results were obtained for the ^{31}P NMR spectrum of compound **3** (Figure 4B) in comparison with the nonlabeled ATPαS (A) (Figure 4A).

Stereochemical Course of Acetate Activation Catalyzed by the Yeast Acetyl-CoA Synthetase. As shown in Figure 5, ATPαS (B) is a reasonable substrate for yeast acetyl-CoA synthetase, whereas ATPαS (A) is not a substrate. From Lineweaver–Burk plots the following data were obtained for ATPαS (B): apparent K_m = 1.7 mM and V_{max} = 32 (nmol/min)/mg. The corresponding values for ATP are apparent K_m = 1.0 mM and V_{max} = 4.0 (μmol/min)/mg. The AMPS obtained from the incubation of [^{17}O]acetate, ATPαS (B), and CoA with the yeast acetyl-CoA synthetase was converted to ATPαS (A) with myokinase and pyruvate kinase according to the procedure of Scheme II. The ^{31}P NMR spectrum of the ATPαS (A) obtained is shown in Figure 4C. From the integration and the data shown in Table I, it is clear that the peak intensity of both P_α and P_β signals decreased to approximately 82% of the corresponding signals of the nonlabeled ATPαS (A), indicating that the ^{17}O is located at the $\alpha\beta$-bridge position and that the activation of acetate catalyzed by the yeast acetyl-CoA synthetase proceeds with net inversion of configuration at P_α or ATPαS (B). The overall stereochemical course is shown in Scheme III. During the preparation of this manuscript a communication appeared (Midelfort & Sarton-Miller, 1978) reporting determination of the steric course of the yeast acetyl-CoA synthetase reaction, with the same result, by a more lengthy procedure.

Discussion

The results of this study establish a new NMR method for the analysis of the steric course of adenylyl transfer reactions,

based on the influence of the ^{17}O nucleus on the ^{31}P NMR signal. Presumably, the stereochemical course of adenylyl transfer reactions could also be elucidated by the ^{18}O isotope shift method (Cohn & Hu, 1978; Lowe & Sproat, 1978a) since the bridge ^{18}O would cause isotope shifts of both ^{31}P NMR signals, whereas the nonbridge ^{18}O would cause an isotope shift of only a single ^{31}P signal. This method, however, requires high magnetic field and high resolution. In the 18.8-kG magnetic field as used in this work, the expected ^{18}O isotope shift is only ≤ 0.7 Hz. The enzymes catalyzing adenylyl transfer reactions are normally not available on large quantity, and the signals of P_α and P_β of ATPαS (A) are separated 70 ppm apart. With a spectral width of 4000 Hz and a computer size of 8K data points, the magnitude of the ^{18}O isotope shift is within the limits of detection. Under these conditions, the ^{31}P (^{17}O) method has a clear advantage over the ^{31}P (^{18}O) method in that it does not require high magnetic field or high resolution, and thus is particularly useful for small amounts of sample.

Another type of problem which can potentially be studied by the ^{31}P (^{17}O) method is the enzyme-catalyzed randomization of oxygen between the bridge and nonbridge positions, which often has significant mechanistic implications. This problem has been studied by using mass spectroscopic methods, as in the case of the mechanism of oxygen exchange between water and the γ-PO_3 of ATP in the chloroplast phosphorylation system (Wimmer & Rose, 1977), and recently by the ^{31}P NMR method based on the ^{18}O isotope shift, as in the case of the mechanism of pyruvate kinase (Lowe & Sproat, 1978b).

Possible mechanisms for the phosphoryl transfer reactions have been discussed by Westheimer (1968) and Benkovic & Schray (1973). The finding that the yeast acetyl-CoA synthetase catalyzes displacement of the pyrophosphoryl group of ATPαS (B) by acetate with inversion of configuration at P_α is consistent with other phosphoryl-transferring enzymes of known stereochemistry, e.g., UDPglucose pyrophosphorylase (Sheu & Frey, 1978), ribonuclease A (Eckstein, 1975), ribonuclease T1 (Eckstein, 1975), DNA-dependent RNA polymerase (Eckstein et al., 1976; Burgers & Eckstein, 1978), and tRNA nucleotidyltransferase (Eckstein et al., 1977; Burgers & Eckstein, 1978). As suggested by the stereochemical results, all of these enzymes catalyze nucleophilic displacement at a chiral or prochiral phosphorus center by an in-line mechanism without pseudorotation. Alkaline phosphatase has been shown to catalyze phosphoryl transfer with net retention of configuration (Jones et al., 1978), which is not inconsistent with the in-line mechanism since the reaction proceeds via a double-displacement mechanism involving a covalent phosphoryl enzyme intermediate (Levine et al., 1969).

After this manuscript had been submitted for publication, two related communications have appeared. Richard et al. (1978) have developed a method to differentiate the pro-*R* and pro-*S* oxygens of ADPβS by enzymatic conversion of the ADPβS stereospecifically labeled with ^{18}O at P_β to ATPβS, followed by chemical degradation and mass spectral analysis of the phosphate derivatives to determine if ^{18}O is located at the bridge or nonbridge position. This method was then used to elucidate the stereochemical course of thiophosphoryl group transfer (inversion of configuration) catalyzed by adenylate kinase (Richard & Frey, 1978). The underlying principle in this work, which differentiates the two diastereotopic oxygens of ADPβS, is analogous to that of the present paper, which differentiates the two diastereotopic oxygens of AMPS. Both are based on stereospecific phosphorylation, followed by distinguishing between the bridge and nonbridge oxygens. The ^{31}P (^{17}O) NMR method has advantage over the mass spectral method in that it does not require degradation and derivatization of the nucleoside triphosphates.

Acknowledgments

The author is indebted to Professor H. G. Floss of this department for sharing some of his research facilities and for useful discussions and to J. Kozlowski for help in obtaining some of the NMR spectra.

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