Enzyme-Catalyzed Positional Isotope Exchange by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy Using Either ^{18}O- or ^{17}O-β,γ-Bridge-Labeled Adenosine 5′-Triphosphate

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Abstract: Tetrasodium pyrophosphate with ^{18}O enrichment in the bridge position has been prepared from $H_2{}^{18}O$ in an overall yield of 32%. ^{31}P NMR analysis at 97.3 MHz has given the percentages of unlabeled, ^{18}O-nonbridge, and ^{18}O-bridge species as 13%, 11%, and 76%, respectively. Resonances corresponding to ^{18}O-nonbridge and ^{18}O-bridge pyrophosphate are shifted upfield by 0.011 and 0.019 ppm, respectively, from the resonance corresponding to the unlabeled species. This material has, in turn, been used to synthesize ^{18}O-labeled ATP with unlabeled, ^{18}O-nonbridge, and β,γ-^{18}O-bridge species at the β-phosphorus position of 18%, 6%, and 76%, respectively. The β-phosphorus resonances corresponding to ^{18}O-nonbridge and ^{18}O-bridge label appear 0.026 and 0.016 ppm upfield from those corresponding to unlabeled material. ^{31}P NMR analysis at 97.3 MHz has shown that carbamoyl-phosphate synthetase from *Escherichia coli* catalyzes the bicarbonate-dependent β,γ-bridge to β-nonbridge positional oxygen exchange in β,γ-^{18}O-bridge ATP. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was 1.2 ± 0.1 in the presence of bicarbonate and the absence of glutamine and ornithine. By the same procedures, pyrophosphate and ATP with ^{17}O enrichments in the bridge and β,γ-bridge were prepared from $H_2{}^{17}O$ (containing 33.6% ^{16}O, 39.1% ^{17}O, and 27.3% ^{18}O). The presence of ^{18}O in this sample of ATP enabled the determination of positional oxygen exchange with carbamoyl-phosphate synthetase by ^{31}P NMR at 97.3 MHz as described above. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was found in this case to be 1.3 ± 0.1. In addition, positional oxygen exchange could be observed by ^{31}P NMR at 40.5 MHz as an increase in the intensity of the P_γ doublet due to movement of ^{17}O from the β,γ-bridge to β-nonbridge position. The significance of this observation lies in the ability to observe positional oxygen exchange of this type on virtually any lower frequency NMR instrument capable of observing phosphorus.

The positional isotope exchange (PIX) technique of Midelfort and Rose[1] was developed to detect either a phosphoenzyme intermediate for phosphotransfer reactions or a phosphorylated substrate intermediate in synthetase reactions in cases where ATP ⇌ ADP exchange reactions cannot otherwise be observed. That is, the ADP is generated at the active site of the enzyme and is not released during the phosphotransfer step. Briefly, this method follows the positional exchange of ^{18}O in ATP from a bridge (β, γ) to a nonbridge (β) position during the re-formation of ATP from enzyme-bound ADP and the proposed phosphoenzyme or phosphorylated substrate (Scheme I). If free rotation of the β-phosphoryl group of enzyme-bound ADP is assumed, there is a 67% probability that the labeled oxygen will be found in one of the β-nonbridge positions in the back-reaction. Detection of this exchange was originally done using mass spectrometry, which required a series of enzymatic and derivatization steps to prepare the sample for analysis.

Recently, it has been shown[2-4] that the ^{31}P NMR resonances in ATP are shifted upfield by about 0.02 ppm when an ^{16}O atom is replaced by ^{18}O. The magnitude of this shift is slightly greater when the ^{18}O atom is in a nonbridging position.[5-7] Applying these principles, Raushel and Villafranca[8] have used ^{31}P NMR to follow the PIX reaction of [γ-$^{18}O_4$]ATP with carbamoyl-phosphate synthetase from *Escherichia coli* and have obtained results similar to those derived from the more laborious mass spectral analysis.[9] An important advantage of ^{31}P NMR as applied to PIX experiments is that it provides a nondestructive means of detection while the reaction is in progress. In the practical application of this method, however, a high-frequency NMR instrument is desirable to resolve the individual peaks.

Independently, Tsai and co-workers have shown that the line-broadening effect of an ^{17}O nucleus directly bonded to phosphorus causes a quantitative decrease in the ^{31}P NMR signal in direct proportion to the amount of ^{17}O enrichment.[10,11] This effect has been demonstrated for two ^{17}O-labeled ATP analogues: [$\alpha\beta,\beta\gamma$-$^{17}O_2,\beta$-$^{17}O_2$]-[10] and [γ-$^{17}O_3$]ATP.[11] We were particularly interested in Tsai's proposal that the effect of ^{17}O on the ^{31}P NMR signal could be utilized in performing PIX experiments.[10] It was apparent from this work that if an ATP analogue could be made with an exclusive ^{17}O label in the β,γ-bridge position, then the positional isotope exchange of this label to a β-nonbridge position could be observed by an increase in the intensity of the $^{31}P_\gamma$ resonance on a lower frequency NMR instrument, which Tsai calls the $^{31}P(^{17}O)$ NMR method. It was also felt that the corresponding analogues with an ^{18}O label would be useful in the more traditional, higher field $^{31}P(^{18}O)$ method.

To date, no synthesis of ATP with an exclusive ^{17}O or ^{18}O label in the β,γ-bridge has been available. Thus, in the PIX experiments reported by Midelfort and Rose,[1] Wimmer et al.,[9] and Raushel and Villafranca,[8] a multiply labeled ATP analogue, [γ-$^{18}O_4$]ATP, was used. Alternatively, PIX experiments have been performed with an ATP analogue possessing multiple ^{18}O labels in the α,-β-bridge and in the two β-nonbridge positions, as was used, for example, by Lowe and Sproat in a study of pyruvate kinase.[12]

We now wish to report a synthetic method for preparing specific ^{17}O- and ^{18}O-labeled ATP analogues in sufficient enrichment at the β,γ-bridge for PIX to be readily observed using either the $^{31}P(^{18}O)$ or $^{31}P(^{17}O)$ NMR methods. In this paper we describe their use in prototype PIX experiments with carbamoyl-phosphate

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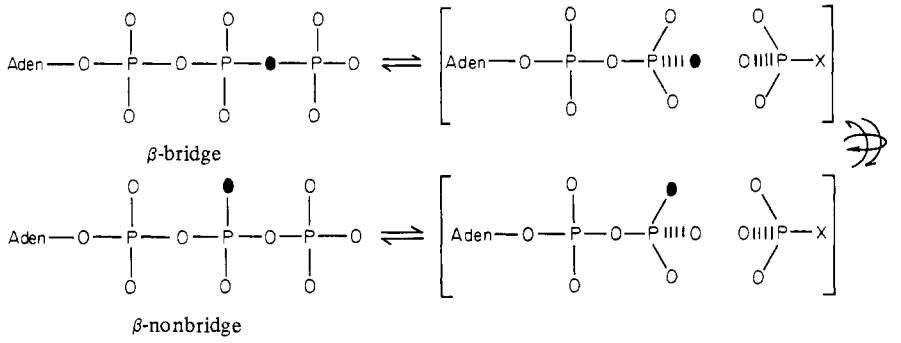
Scheme I. General Scheme for Positional Isotope Exchange[a]



[a] "X" is meant to indicate a phosphoryl acceptor leading to the proposed phosphoenzyme or phosphorylated substrate intermediate. In the reaction catalyzed by carbamoyl-phosphate synthetase discussed in this paper, X = HCO_3^-.

synthetase from *E. coli* and bicarbonate, the substrate that is phosphorylated.

Experimental Section

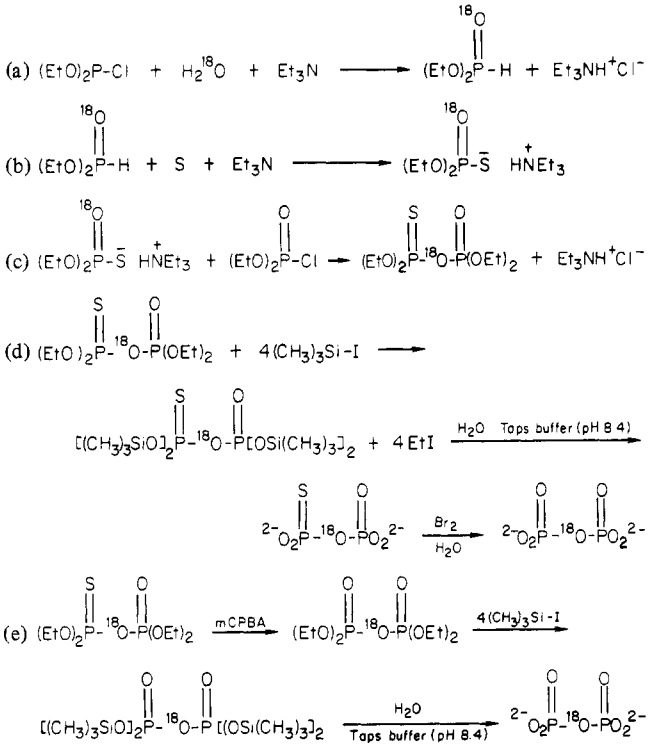
Materials. $H_2{}^{18}O$ (98 atom %) and $H_2{}^{17}O$ (containing 33.6% ^{16}O, 39.1% ^{17}O, and 27.3% ^{18}O) were purchased from Cambridge Isotopes. DEAE (diethylaminoethyl)-Sephadex A-25 was purchased from Pharmacia. Iodotrimethylsilane was from Aldrich, and adenosine 5'-monophosphoric acid was from Sigma. Finely powdered elemental sulfur was stored in a vacuum desiccator over sodium hydroxide pellets prior to use. Diethyl chlorophosphite, diethyl chlorophosphate, and triethylamine were distilled under nitrogen prior to use. Pyridine, dimethylformamide, and dichloromethane were heated at reflux over calcium hydride and freshly distilled. Diethyl ether was heated at reflux over sodium filings in a nitrogen atmosphere, distilled, and used immediately. Carbamoyl-phosphate synthetase from *E. coli* was purified as described earlier[8] and was a generous gift from Dr. Joseph J. Villafranca. Other reagents were of the finest quality available. All reactions were performed under nitrogen unless stated otherwise.

(1) Diethyl [^{18}O]phosphite was prepared according to Scheme IIa. A mixture of dry diethyl ether (100 mL), triethylamine (7.0 mL, 50 mmol), and $H_2{}^{18}O$ (1.0 g, 50 mmol) was cooled in an ice bath to 0 °C. Diethyl chlorophosphite (7.0 mL, 49 mmol) was added dropwise with vigorous stirring over the course of ca. 15 min. After complete addition, the mixture was stirred at room temperature overnight. Triethylammonium chloride was then removed by filtration and the solvent was removed. The product was purified by vacuum distillation [bp 73 °C (14 mmHg)], 5.83 g (85%).

(2) ^{18}O-bridged tetraethyl thiopyrophosphate was prepared by using a modification of the procedure of Arbuzov[13] (Scheme IIb,c). Powdered sulfur (1.31 g, 40.7 mmol) was added slowly to a stirred mixture of diethyl [^{18}O]phosphite (5.70 g, 40.7 mmol) and triethylamine (4.12 g, 40.7 mmol). After addition was complete, the mixture was heated at 45 °C until all of the sulfur had dissolved (ca. 30 min). The pale yellow syrup was dissolved in dry diethyl ether (100 mL). Next, diethyl chlorophosphate (6.94 g, 40.7 mmol) was added dropwise with stirring. Stirring was continued overnight, and the reaction was completed by heating at reflux for 2 h. The cooled solution was filtered to remove triethylammonium chloride, washed with water (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed, and the resulting pale yellow oil was degassed under high vacuum, yielding 11.6 g. The ^{31}P NMR spectrum (proton decoupled, $CDCl_3$ solvent) showed the crude product to be greater than 95% pure (88% yield): δ 53.7 (d, diethyl phosphorothioyl group) and δ 14.3 (d, diethyl phosphoryl group), J_{P-P} = 21.6 Hz, in agreement with literature values.[14]

(3) ^{18}O-bridged pyrophosphate tetrasodium salt was prepared from ^{18}O-bridged tetraethyl thiopyrophosphate by two methods (Scheme IId,e). In both methods, dealkylation was adapted from the procedure of Chojnowski et al.[15] The use of *m*-chloroperoxybenzoic acid in oxidation of organophosphorus compounds has been described by Bellet and Casida.[16] The use of bromine to replace sulfur with oxygen in thiopyrophosphate was adapted from the procedure that Lowe et al. used in the replacement of sulfur with ^{17}O in the synthesis of adenosine 5'-[(*R*)-α-^{17}O]triphosphate.[17]

Scheme II[a]



[a] Et = C_2H_5; mCPBA = *m*-chloroperoxybenzoic acid.

(4) Method A. Iodotrimethylsilane (3.52 g, 17.6 mmol) was added dropwise to a stirred solution of ^{18}O-bridged tetraethyl thiopyrophosphate (1.23 g, 4 mmol) in dry dichloromethane (3 mL) at −40°C (dry ice/acetone bath). After 1 h the reaction mixture was left at room temperature for 2 days, followed by 2 h of heating at reflux. Next, the mixture was added directly to 50 mL of 1 M Taps buffer [3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid], pH 8.4. Bromine was added dropwise to the stirring mixture until a brick red color persisted. The pH was maintained between 7–8 by dropwise addition of concentrated NaOH solution. After 5 min remaining bromine was eliminated by addition of $NaHSO_3$. The resulting aqueous solution was extracted with CH_2Cl_2 and then diluted with water to a final volume of 700 mL. This solution was applied to a DEAE-Sephadex A-25 (HCO_3^-) column (40 cm × 2.5 cm). The column was eluted with a 3-L linear gradient of 0.05–0.60 M triethylammonium bicarbonate buffer (pH 7.8) at 2–4 °C. Fractions were assayed for acid-labile phosphate according to the procedure of Ames.[18] Pyrophosphate is eluted from the column by 0.4 M triethylammonium bicarbonate under these conditions. The pooled fractions were concentrated to a syrup under vacuum using a bath temperature below 25 °C. Residual water and triethylammonium bicarbonate were removed by evaporation of several 25-mL aliquots of

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absolute CH_3OH under vacuum. The resulting syrup was transferred to a 30-mL glass centrifuge tube with three 2-mL rinses of CH_3OH. The sodium salt of ^{18}O-bridged pyrophosphate was precipitated by addition of 10–12 equiv of 1 M NaI in acetone and recovered by centrifugation. After the precipitate was washed twice with cold acetone, it was dissolved in about 20 mL of cold water. The resulting solution was adjusted to pH 11.5 with concentrated NaOH, and the product was precipitated by addition of ethanol to give white needles (0.76 g, 42.6% yield). ^{31}P NMR analysis of this material gave a single peak identical with that for authentic pyrophosphate at two pH values (6 and 10).

(5) Method B. *m*-Chloroperoxybenzoic acid (2.59 g, 12 mmol based on 80% assay) in dry CH_2Cl_2 (35 mL) was added slowly dropwise to a stirred solution of ^{18}O-bridged tetraethyl thiopyrophosphate (1.23 g, 4 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C. The reaction was continued at 0 °C for 90 min. Precipitated *m*-chlorobenzoic acid was removed by filtration. The resulting solution was washed with 20 mL of 20% $NaHSO_3$, three 10-mL aliquots of 10% $NaHCO_3$, and 10 mL of saturated NaCl solution, in that order. The organic layer was then dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure to give an oil (0.86 g). ^{31}P NMR (proton decoupled, $CDCl_3$ solvent) showed this material to contain approximately 75% tetraethyl pyrophosphate: δ 13.5, in agreement with the literature value.[19] This oil was dissolved in dry CH_2Cl_2 (2 mL) and cooled to −40 °C with a dry ice/acetone bath. Iodotrimethylsilane (2.80 g, 14.0 mmol) was added dropwise with stirring. Following complete addition, the reaction was continued at −20 °C for 2 h. After being warmed to room temperature, the solution was added to 30 mL of 1 M Taps buffer (pH 8.4). The pH was readjusted to 7.8 after 15 min, and the aqueous layer was partitioned with CH_2Cl_2 (20 mL) and then diluted to a final volume of 500 mL with water. This solution was applied to the DEAE-Sephadex A-25 column. The product, ^{18}O-bridged tetrasodium pyrophosphate, was eluted, recovered, and recrystallized as described under method A, giving 0.39 g (22% yield).

(6) β,γ-^{18}O-ATP was prepared according to the procedure of Hoard and Ott[20] from AMP using 3 equiv 1,1′-carbonyldiimidazole and 2 equiv of ^{18}O-labeled pyrophosphate. Of the pyrophosphate used, 1.01 equiv precipitated as the imidazolium salt, was recovered from the reaction mixture by centrifugation, and was reconverted to the sodium salt by passage through a column of DOWEX 50 WX8 (Na^+ form). The product was purified on the DEAE-Sephadex A-25 column described above by using a linear gradient (1.5 L + 1.5 L, 0.1–0.7 M) of triethylammonium bicarbonate buffer (pH 7.8). Fractions containing ATP were pooled, and the solvent and buffer were removed as described above. Precipitation of the sodium salt of [β,γ-^{18}O]ATP was achieved as described for ^{18}O-bridged pyrophosphate, and the solution was washed 3 times with cold acetone and dried in a vacuum desiccator. The yield was 38% based on unrecovered ^{18}O-labeled pyrophosphate. This sample had an identical R_f value (0.26) when compared to authentic unlabeled ATP by using poly(ethyleneimine)/cellulose thin-layer chromatography with 1.2 N LiCl as the eluent.[21]

(7) Diethyl[^{17}O]phosphite and ^{17}O-bridged tetraethyl thiopyrophosphate were prepared from $H_2{}^{17}O$ by using the same procedures described above for the ^{18}O analogues.

(8) ^{17}O-bridged pyrophosphate tetrasodium salt was prepared as described for ^{18}O-bridged pyrophosphate (Method A).

(9) [β,γ-^{17}O]ATP was prepared as described for [β,γ-^{18}O]ATP.

Methods. (1) Positional Isotope Exchange in [β,γ-^{18}O]ATP and [β,γ-^{17}O]ATP. Conditions were similar to those described by Raushel and Villafranca.[8] Carbamoyl-phosphate synthetase (5 mg) was incubated with 67 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 133 mM KCl, 13% D_2O, 17 mM HCO_3^-, and either 12.5 mM [β,γ-^{18}O]ATP (125 μmol) or 17.5 mM [β,γ-^{17}O]ATP (175 μmol) at 30 °C in a total volume of 10 mL. The bicarbonate-dependent ATPase reaction was followed by ^{31}P NMR at 40.5 MHz. After the reaction had proceeded to about 50% completion (3–4 h), the solutions were cooled in ice and applied immediately to the DEAE-Sephadex A-25 column, and the ATP samples were reisolated as their sodium salts according to the procedure given above. Controls without enzyme were incubated at 30 °C for the same lengths of time, and the resulting ATP samples were recovered in the same manner.

(2) Sample Preparation. The ^{18}O-bridged and ^{17}O-bridged pyrophosphate samples (55 μmol) and the PIX and control [^{18}O]- and [^{17}O]ATP samples were percolated through columns of Chelex 100 (Na^+ form) and lyophilized. These samples were then dissolved in 0.4 mL of 50% D_2O containing 0.5 mM EGTA. The 5-mm NMR tubes were made metal free by soaking overnight in 1/1 concentrated HNO_3/concentrated H_2SO_4 and rinsing thoroughly with distilled deionized water.

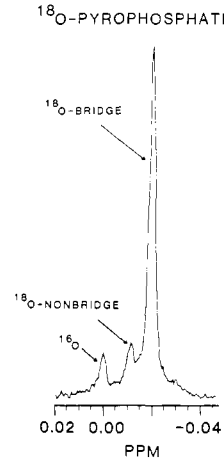

Figure 1. ^{31}P NMR spectrum of ^{18}O-labeled pyrophosphate taken at 97.3 MHz. The peaks corresponding to ^{18}O-nonbridge and ^{18}O-bridge pyrophosphate occur 0.011 and 0.019 ppm, respectively, upfield from unlabeled pyrophosphate. Details of these assignments are described in the text.

(3) NMR Measurements. ^{31}P NMR spectra of the tetraethyl esters of thiopyrophosphate and pyrophosphate were taken at 40.5 MHz in 12-mm NMR tubes with a Varian XL-100 spectrometer with $CDCl_3$ as the solvent. A spectral width of 4000 Hz and 8192 data points were used to acquire the free induction decay; a 90° pulse with a time delay between pulses of 5 s was employed. All spectra were taken with broad-band ^1H decoupling. Chemical shifts were measured relative to 85% H_3PO_4 as an external standard.

The course of the ATP → ADP reaction in the PIX assays was monitored by using ^{31}P NMR at 40.5 MHz on the Varian XL-100 spectrometer in 12-mm tubes by using a spectral width of 1000 Hz, 4096 data points, and 1000 data acquisitions.

High-resolution ^{31}P NMR spectra of the labeled pyrophosphate and ATP samples were taken in 5-mm NMR tubes (metal free) at 97.3 MHz by using the UCSF wide-bore 240-MHz spectrometer or at 202.5-MHz by using a Bruker 500-MHz spectrometer located in the Southern California Regional NMR Facility at the California Institute of Technology. For these experiments 8192 data points were used with a spectral width of 400 Hz (at 97.3 MHz) or 1000 Hz (at 202.5 MHz) to acquire the free induction decays, which after processing gave digital resolutions of 0.05 and 0.03 Hz/data point, respectively. Typically, 128 data acquisitions were collected for the ATP samples, and 32 acquisitions were taken for the pyrophosphate samples.

^{17}O NMR spectra at 36.6 MHz were recorded on a Bruker WH-270 spectrometer at Yale University as previously described.[22] Chemical shifts were measured relative to ^{17}O-enriched water.

Results and Discussion

A ^{31}P NMR spectrum taken at 97.3 MHz of a sample of ^{18}O-labeled pyrophosphate prepared by method A is given in Figure 1. The presence of small amounts of unlabeled and ^{18}O-nonbridge pyrophosphate is indicated (13% and 11%, respectively) in addition to the desired ^{18}O-bridge material. The resonance corresponding to ^{18}O-bridge pyrophosphate appears 0.019 ppm upfield from that corresponding to unlabeled pyrophosphate, in reasonable agreement with the magnitude of an upfield chemical shift associated with an ^{18}O-bridging oxygen in ATP.[5]

At first glance it was unexpected that a peak corresponding to ^{18}O-nonbridge pyrophosphate does not appear at higher field from the peak for the ^{18}O-bridge species. What must be taken into consideration is that for ^{18}O-nonbridge pyrophosphate the phosphorus atoms are nonequivalent and are expected to be strongly coupled (approximately 20 Hz based on the coupling constants observed in ATP). Thus, they will give rise to an AB splitting pattern. At 97.3 MHz, the peaks associated with this splitting pattern are collapsed into an apparent singlet at 0.011 ppm upfield from the resonance for unlabeled pyrophosphate,

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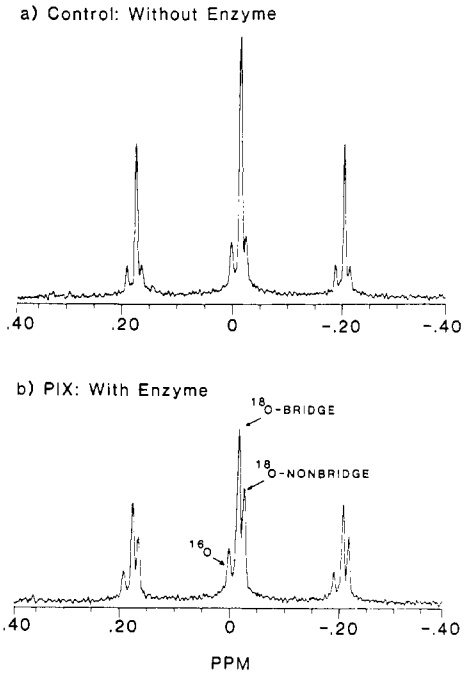

Figure 2. ^{31}P NMR spectra taken at 97.3 MHz of the P_β resonances in ^{18}O-labeled ATP after incubation at 30 °C without enzyme (a) and in the presence of enzyme (b) as described in the text. The peaks corresponding to ^{18}O-nonbridge label at P_β appear at higher magnetic field than those corresponding to ^{18}O-bridge label as has been described previously.[5-8]

which is the calculated average of two isolated ^{16}O- and ^{18}O-nonbridge phosphoryl resonances separated by 0.022 ppm. As expected for such an AB splitting pattern, at 202.5 MHz the two innermost peaks of this splitting pattern were resolved, giving rise to an apparent doublet with a separation of about 0.5 Hz. A more detailed description of this unusual observation will be the subject of a separate publication from this laboratory.

Samples of ^{18}O-labeled pyrophosphate prepared by either method A or method B were shown to have the same relative amounts of unlabeled, ^{18}O-nonbridge, and ^{18}O-bridge species, provided that the same sample of ^{18}O-labeled tetraethyl thiopyrophosphate (TETPP) was used. Different preparations of ^{18}O-labeled TETPP yielded [^{18}O]pyrophosphate with percentages of unlabeled and ^{18}O-nonbridge species ranging from 13% and 11% to 22% and 20%, respectively. In each case, the relative amounts of unlabeled and ^{18}O-nonbridge species were similar in magnitude, suggesting that both of these minor amounts of undesired side products may have originated by way of a common scrambling mechanism in the synthesis of ^{18}O-labeled TETPP. The same results were obtained when ^{18}O-labeled TETPP was prepared from purified triethylammonium diethyl [^{18}O]thiophosphate and diethyl chlorophosphate, suggesting that unreacted sulfur is not responsible for the observed scrambling. A detailed explanation for the appearance of unlabeled and ^{18}O-nonbridge pyrophosphate in these syntheses must await further study.

When ^{18}O-nonbridge pyrophosphate is coupled with AMP to make ATP, the undesired ^{18}O-nonbridge atom is found at either the P_β or P_γ position with equal probability. Thus, the percentage of ^{18}O-nonbridge label found at the P_β or P_γ position of ATP is thus (fortunately) only half that found in the pyrophosphate starting material. Also, the [^{16}O]ATP that results serves as a convenient internal standard.

The ^{18}O-labeled ATP synthesized was shown by high-resolution ^{31}P NMR to consist of unlabeled, ^{18}O-nonbridge, and β,γ-^{18}O-bridge species at the β-phosphorus position of 18%, 6%, and 76%, respectively. The β-phosphorus resonances corresponding to ^{18}O-bridge and ^{18}O-nonbridge label appear 0.016 and 0.026 ppm upfield from those corresponding to unlabeled material, in agreement with published literature values.[5] It is significant that the relative amount of ^{18}O-nonbridge label indicated is consistent

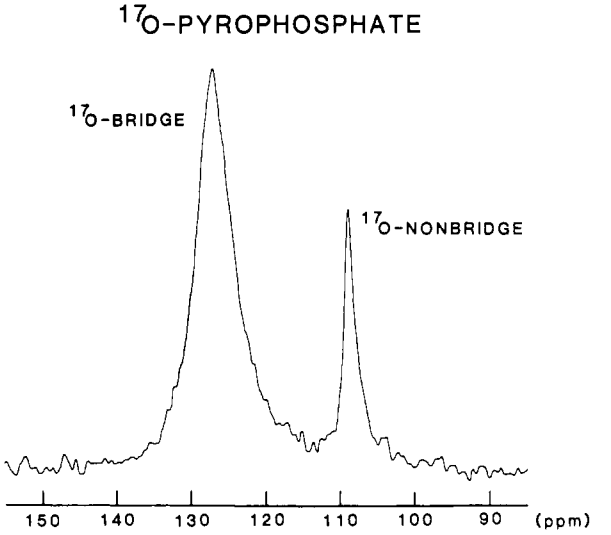

Figure 3. ^{17}O NMR spectrum taken at 36.6 MHz of a sample of pyrophosphate enriched with ^{17}O in the bridging position. Peak assignments were originally reported by Gerlt et al.[23]

Table I. Positional Isotope Exchange of [β,γ-^{18}O]ATP Catalyzed by Carbamoyl-Phosphate Synthetase in the Presence of Bicarbonate[a]

ATP analogue	method	fraction of chemical reaction[b]	fraction of exchange reaction[c]	μmol of ATP exch./ μmol of ADP prod.[d]
(i) ^{18}O ATP	$^{31}P(^{18}O)$ NMR	0.48 ± 0.02	0.55 ± 0.02	1.2 ± 0.1
(ii) ^{17}O ATP	$^{31}P(^{18}O)$ NMR	0.67 ± 0.01	0.76 ± 0.03	1.3 ± 0.1
(iii) ^{17}O ATP	$^{31}P(^{17}O)$ NMR	0.67 ± 0.01	0.63 ± 0.16	0.89 ± 0.43

[a] Incubation conditions were described in the text. [b] The fraction of chemical reaction, X, is defined as $X = (ADP)_t/(ATP)_{initial}$ where t = time at which the incubation was terminated. [c] In the $^{31}P(^{18}O)$ NMR method, the fraction of the exchange reaction, F, is defined as $F = (P_t - P_0)/(P_\infty - P_0)$ where P refers to the percentage of ^{18}O-nonbridge label at P_β at times t and zero and at equilibrium. In the $^{31}P(^{17}O)$ NMR method, $F = (A_t - A_0)/(A_\infty - A_0)$, where A refers to the summed areas of the peaks in the P_γ doublet, normalized with respect to the P_α peak, at times t and zero, and at equilibrium (∞). [d] As derived by Litwin and Wimmer.,[24] micromoles of ATP exchanged = $X/[\ln(1-X)](ATP)_{initial} \ln(1-F)$, where X = fraction of ATP lost and F = fraction of equilibrium attained in the final ATP pool during positional isotope exchange.

with our prediction based on the peak assignment for ^{18}O-nonbridge pyrophosphate. The percentage of these three species remained unchanged when this sample was used as a control under the conditions (minus enzyme) used in the PIX experiment (Figure 2a).

In the synthesis of ^{17}O-labeled pyrophosphate, the $H_2{}^{17}O$ used was not isotopically pure (it contained 33.6% ^{16}O and 27.3% ^{18}O), which led to concomitant formation of substantial amounts of ^{16}O- and ^{18}O-labeled material. Therefore, it was possible to detect the presence of nonbridge labeled pyrophosphate by high-resolution ^{31}P NMR. The ratio of peak areas corresponding to ^{18}O-nonbridge pyrophosphate in the ^{31}P NMR spectrum taken at 97.3 MHz gave a value of 21% for the percentage of nonbridge label out of total label for either given oxygen isotope (^{17}O or ^{18}O). In the ^{17}O NMR spectrum of this sample at pH 10.5 (Figure 3) the broad ^{17}O-bridge resonance appears at 126.6 ppm and the narrower ^{17}O-nonbridge resonance appears at 108.5 ppm. These peak assignments were previously reported by Gerlt et al.[23]

Figure 2b shows the ^{31}P NMR spectrum of ^{18}O-labeled ATP when treated in the presence of bicarbonate and carbamoyl-

(23) Gerlt, J. A.; Reynolds, M. A.; Demou, P. C.; Kenyon, G. L. *J. Am. Chem. Soc.* **1983**, in press.
(24) Litwin, S., Wimmer, M. J. *J. Biol. Chem.* **1979**, *254*, 1859.

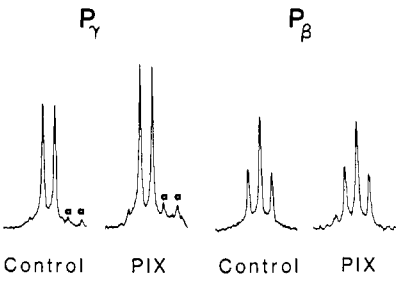

Figure 4. ^{31}P NMR spectra taken at 40.5 MHz of the P_γ and P_β resonances of ^{17}O-labeled ATP samples that were reisolated after incubation at 30 °C in the presence of enzyme (PIX) or absence of enzyme (control). Spectra were normalized with respect to the P_α resonances. The increase in the intensity of the P_γ peaks represents a decrease in the relative amount of ^{17}O bound at this group due to β,γ-bridge to β-nonbridge positional isotope exchange. Peaks labeled "a" correspond to small amounts of ADP that were formed after the ATP samples had been prepared for NMR analysis.

phosphate synthetase under the conditions described above. A marked increase in the intensity of the triplet corresponding to ^{18}O-nonbridge ATP is observed, along with a decrease in the intensity of the triplet corresponding to ^{18}O-bridge ATP, indicating that positional isotope exchange (bridge to nonbridge) has occurred. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was found to be 1.2 ± 0.1 [Table I (i)]. Wimmer et al.[9] have determined this ratio to be 1.4–1.7 at 37 °C using mass spectral analysis, whereas Raushel and Villafranca have obtained a value of 0.42 under the same conditions using ^{31}P NMR analysis.

Positional isotope exchange upon incubation of the ^{17}O-labeled ATP sample (containing also substantial ^{18}O label) in the presence of carbamoyl-phosphate synthetase and bicarbonate was determined by using both the $^{31}P(^{18}O)$ and $^{31}P(^{17}O)$ NMR methods. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was determined by the $^{31}P(^{18}O)$ NMR method to be 1.3 ± 0.1 [Table I (ii)], in good agreement with the value of 1.2 ± 0.1 obtained from the previous experiment using ^{18}O-labeled ATP. In the application of the $^{31}P(^{17}O)$ NMR method, the intensity of the P_γ resonances of the ATP sample was monitored at 40.5 MHz during the course of the incubation. Although the individual unlabeled, ^{18}O-nonbridge, and ^{18}O-bridge resonances were not resolved in the P_γ doublet, the intensity of this doublet definitely increased with time as compared to the intensity of the P_α doublet (Figure 4). The intensity of the P_β triplet remained the same relative to that of the P_α doublet, as expected, since the ^{17}O atom remains bound to the β-phosphorus after bridge to nonbridge positional isotope exchange has occurred. If the increase in the area under each peak of the P_γ doublet is assumed to be due entirely to ^{17}O positional isotope exchange from the β,γ-bridge to the β-nonbridge position, the ratio of micromoles of ATP exchanged to micromoles of ADP produced was determined to be 0.89 [Table I (iii)]. This value is less accurate than that obtained by the $^{31}P(^{18}O)$ NMR method, since accurate comparisons of peak areas are particularly dependent on the quality of the base-line resolution of each peak when two separate spectra are being considered. Nevertheless, both qualitatively and semiquantitatively, the $^{31}P(^{17}O)$ NMR method shows that significant positional isotope exchange has occurred.

Conclusions

A method has been devised for synthesizing ATP highly specifically enriched with either ^{17}O or ^{18}O in the β,γ-bridge position in good yields. On the basis of the yields reported in this paper, over 6 mmols of ATP (about 3.3 g, as the disodium salt) can be synthesized from a single gram of ^{17}O- or ^{18}O-enriched water, enabling numerous positional isotope exchange studies to be carried out.

The usefulness of the $^{31}P(^{17}O)$ NMR method in obtaining reliable quantitative data for PIX experiments will undoubtedly improve as higher isotopic enrichments of $H_2{}^{17}O$ become available. Currently, $H_2{}^{17}O$ is available with 60 atom % ^{17}O enrichment, although it is in short supply. The most attractive feature of the $^{31}P(^{17}O)$ NMR method at present, as applied to PIX experiments, is that it can be performed on virtually any NMR spectrometer that can be tuned to observe ^{31}P resonances. In practice, one can screen for the PIX phenomenon using the $^{31}P(^{17}O)$ NMR method and then obtain more quantitative data on exchange rates using the $^{31}P(^{18}O)$ NMR method with a higher field NMR instrument.

The ability to synthesize pyrophosphate isotopically enriched in the bridge position may play a significant role in the application of PIX experiments to enzymes that utilize pyrophosphate. We have demonstrated that bridge- and nonbridge-labeled pyrophosphate can easily be distinguished using either ^{31}P NMR or ^{17}O NMR.

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