^{15}N and ^{17}O NMR Studies of the Proton Binding Sites in Imidodiphosphate, Tetraethyl Imidodiphosphate, and Adenylyl Imidodiphosphate

Mark A. Reynolds,[1a] John A. Gerlt,*[1b,2] Peter C. Demou,[1b] Norman J. Oppenheimer,[1a,3] and George L. Kenyon*[1a]

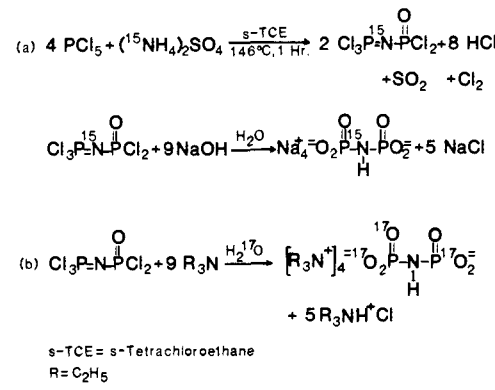
Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, and the Department of Chemistry, Yale University, New Haven, Connecticut 06511. *Received October 4, 1982*

Abstract: ^{15}N- and ^{17}O-enriched samples of imidodiphosphate (PNP), its tetraethyl ester, and 5'-adenylyl imidodiphosphate (AMP-PNP) have been prepared. The ^{15}N NMR spectra of both PNP and AMP-PNP reveal the presence of approximately 70-Hz $^{1}H-^{15}N$ coupling constants for the fully ionized samples, demonstrating an imido tautomeric structure in both cases. For AMP-PNP this coupling persists in the presence of a stoichiometric amount of Mg^{2+}. The ^{17}O NMR chemical shifts of the resonances associated with the phosphoryl oxygens have been assigned. The effect of pH on the resonances for PNP and AMP-PNP is similar to that observed for ATP (Gerlt, J. A.; Demou, P. C.; Mehdi, S. *J. Am. Chem. Soc.* **1982**, *104*, 2848) and its thiophosphate and phosphonate structural analogues (Gerlt, J. A.; Reynolds, M. A.; Demou, P. C.; Kenyon, G. L. *J. Am. Chem. Soc.*, preceding paper in this issue), indicating that protonation of the tetraanion of PNP occurs exclusively on the oxygens and that protonation of the tetraanion of AMP-PNP occurs exclusively on the γ-phosphoryl oxygens. Although ^{17}O NMR demonstrates that protonation of the monoanion of the tetraethyl ester of PNP occurs predominantly on nitrogen, the corresponding ^{15}N NMR chemical shift change was only 2.50 ppm. Thus, ^{15}N NMR chemical shift changes cannot be used reliably to ascertain the sites of protonation in imidodiphosphates.

5'-Adenylyl imidodiphosphate (AMP-PNP, Ib) was synthesized in 1971 by Yount and his co-workers and has been used widely in enzymological studies by virtue of its close structural similarity to ATP. Since the P-N-P bridge in AMP-PNP is relatively inert to hydrolysis both in basic solution and in the presence of enzymes that catalyze the hydrolysis or transfer of the γ-phosphoryl group of ATP, this compound has proven to be a useful competitive inhibitor of kinases and adenosine triphosphatases. The ability of enzymes to effectively bind AMP-PNP has been rationalized by its being isosteric with ATP, since the crystal structures for imidodiphosphate (PNP) and pyrophosphate show that the bond angles and lengths of the P-N-P and P-O-P linkages are nearly identical.[5] However, the solution structures of PNP and AMP-PNP have not been examined, and the possibility does exist that if the pK_a of the N-H proton in the bridge were sufficiently low the proton could reside on oxygen rather than nitrogen (Figure 1a);[6] the stability of these imino structures could be explained by the presence of intramolecular hydrogen bonding as illustrated in Figure 1b. In addition, since enzymatic reactions that utilize ATP as a substrate almost always require it in the form of its complex with Mg^{2+}, coordination of AMP-PNP with Mg^{2+} could stabilize the imino tautomers; direct coordination of Mg^{2+} with the nitrogen of the P-N-P bridge in AMP-PNP has been suggested on the basis of ^{31}P NMR studies.[7]

The ^{31}P NMR spectral properties of AMP-PNP as a function of both pH and Mg^{2+} concentration have been reported, and these demonstrate a behavior markedly different from that of ATP.[7,8]

Scheme I



For example, upon protonation the resonance associated with the β-phosphorus of AMP-PNP shifts upfield more than the resonance associated with the γ-phosphorus; protonation of ATP leads to a larger upfield shift for the resonance associated with the γ-phosphorus. These data may suggest a fundamentally different ionization behavior for AMP-PNP as compared to ATP and could support the existence of the hypothetical imino structures shown in Figure 1b. However, Jaffe and Cohn have recently summarized a large body of experimental data which lead to the conclusion that chemical shift changes observed in ^{31}P NMR spectroscopy cannot be used reliably to identify sites of protonation in polyphosphates.[9]

To resolve the problem of the structure of the P-N-P bridge in PNP and AMP-PNP in aqueous solution, we have utilized both ^{15}N and ^{17}O NMR to ascertain the sites of proton binding. We have synthesized ^{15}N- and ^{17}O- enriched samples of PNP and its tetraethyl ester and of AMP-PNP. Our results provide strong evidence for the existence of the imido tautomer of the P-N-P bridge in both PNP and AMP-PNP. Our experimental observations on the tetraethyl ester of PNP indicate that, in contrast to other nitrogen acids, the ^{15}N NMR chemical shift of the bridging nitrogen is surprisingly insensitive to ionization of the directly bonded proton.

(1) (a) University of California. (b) Yale University
(2) NIH Research Career Development Awardee (CA-00499), 1978-83; Alfred P. Sloan Foundation Fellow, 1981-83.
(3) NIH Research Career Development Awardee (CA-00587), 1979-1984.
(4) Yount, R. G.; Babcock, D.; Ballantyne, W.; Ohala, D. *Biochemistry 1971*, **10**, 2484.
(5) Larsen, M.; Willet, R.; Young, R. G. *Science (Washington, D.C.)* **1969**, *166*, 1510.
(6) The predominance of an imino tautomer in a P(O)-N(H)-P(O) bridge system was proposed for the tetramethyl and tetraethyl esters of PNP by using infrared spectroscopy: (a) Kabachnik, M. K.; Gilyarov, V. A.; Popov, E. M. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1961**, *6*, 1022; (b) Kireev, V. V.; Kolesnikov, G. C.; Titov, S. S. *Zh. Obshch. Khim.* **1970**, *40*, 2105. In addition, these esters have been found to be reasonably acidic; the pK_a values for the tetramethyl and tetraethyl esters of PNP have been measured as 2.6 and 3.7, respectively: (c) Riesel, L.; Pich, G.; Ruby, C. *Z. Anorg. Allg. Chem.* **1977**, *430*, 227.
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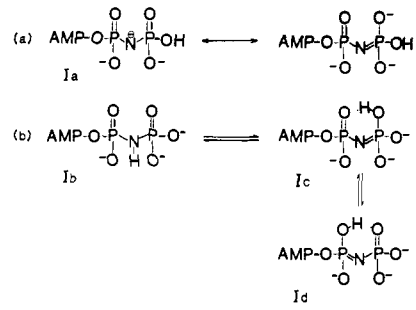

Figure 1. (a) Proposed resonance structures for AMP-PNP^{4-} where O-protonation is favored over N-protonation. (b) Proposed tautomers of AMP-PNP^{4-} showing internal H bonding (···).

Materials and Methods

$H_2{}^{17}O$ (13% ^{16}O, 52% ^{17}O, 35% ^{18}O) was obtained from Monsanto. [^{15}N]Ammonium sulfate was purchased from MSD Isotopes in 99% isotopic purity. Unlabeled AMP-PNP and tetrasodium PNP were purchased from Boehringer-Mannheim. AMP was the product of Sigma. [$^{17}O_1$]AMP was described in a previous publication.[10] QAE-Sephadex was purchased from Pharmacia Chemicals. All other chemicals were of the finest quality commercially available and were used without further purification.

^{15}N and unlabeled trichloro((dichlorophosphoryl)imino)phosphorane were prepared according to the procedure of Emsley and co-workers[11] (Scheme I) in yields of 66% and 67%, respectively. It was found that improved yields are obtained when the ammonium sulfate is finely ground in a mortar and pestle prior to use.

Tetrasodium [^{15}N]imidodiphosphate decahydrate was prepared by treating [^{15}N]trichloro((dichlorophosphoryl)imino)phosphorane (0.540 g, 2 mmol) with 1 N NaOH (19.0 mL, 19.0 mmol) in an ice bath for 2 h. This sample was purified by anion-exchange chromatography at 2–4 °C on QAE-Sephadex A-25 (HCO_3^-) by using a 3-L linear gradient of 0.1–0.7 M trimethylammonium bicarbonate, pH 8.6, as eluent. Fractions were assayed for acid-labile phosphate according to the procedure of Ames.[12] Imidodiphosphate is eluted from the column by 0.4 M triethylammonium bicarbonate under these conditions. The pooled fractions were concentrated to a syrup on a rotary evaporator using a vacuum pump and a dry ice/ethanol trap with a bath temperature below 25 °C. One milliliter of tributylamine was added to the syrup, and residual water and triethylammonium bicarbonate were removed by repeated evaporation of 25-mL aliquots of absolute methanol under reduced pressure. The resulting syrup was transferred to a 30-mL glass centrifuge tube with three 2-mL rinses of methanol. The sodium salt of [^{15}N]imidodiphosphate 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 15 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 (0.795 g, 89%).

Tetraethyl [^{15}N]imidodiphosphate was synthesized as previously described[13] except that 2 equiv of $^{15}NH_3$ and 1 equiv of triethylamine were used in the synthesis of diethyl phosphoramidate.

Tetrasodium [^{17}O]imidodiphosphate decahydrate was prepared by dropwise addition of a solution of trichloro((dichlorophosphoryl)imino)phosphorane (0.538 g, 2 mmol) in 2 mL of dry methylene chloride to a stirred mixture of 1-mL $H_2{}^{17}O$, triethylamine (2.51 mL, 18 mmol), and 2-mL dry methylene chloride at 0 °C. The mixture was stirred for 0.5 h at 0 °C and then for 2 h at room temperature. The methylene chloride was removed by rotary evaporation using a water aspirator and a bath temperature of 20 °C. The resulting viscous solution was purified by chromatography on a column of QAE-Sephadex A-25 (HCO_3^-). Purification was accomplished as described for [^{15}N]imidodiphosphate to give needles (0.605 g, 67%). This materials was approximately 25% enriched with ^{17}O in each of the oxygens as judged by integration of the ^{31}P NMR spectrum of the [β,γ-^{17}O]AMP-PNP prepared from this sample.

Tetraethyl [^{17}O]imidodiphosphate was synthesized from diethyl [^{17}O]phosphoramidate as previously described for the unlabeled ester.[13] The labeled precursor was prepared as follows. A mixture of freshly distilled triethylamine (7.0 mL, 50 mmol) and $H_2{}^{17}O$ (1 mL, 55 mmol) was cooled to 0 °C, and diethyl chlorophosphite (7.0 mL, 49 mmol) was added dropwise with vigorous stirring. When the addition was complete, the reaction was allowed to warm to room temperature and stirring was continued overnight. Triethylammonium hydrochloride was removed by filtration, and the product diethyl [^{17}O]phosphite was purified by distillation at 73 °C and 14 torr. The yield was 5.8 g (85%). The labeled phosphite was converted to diethyl [^{17}O]phosphorochloridate by the action of triethylamine and CCl_4. A mixture of diethyl [^{17}O]phosphite (5.73 g, 41.2 mmol) and CCl_4 (12.67 g, 82.4 mmol) was cooled to O °C, and triethylamine (0.66 mL, 4.74 mmol) was added dropwise with stirring. The reaction mixture was allowed to warm to room temperature, and stirring was continued for 3 h. Precipitated triethylammonium hydrochloride was removed and the product was purified by vacuum distillation at 70 °C and 4 torr (lit.[14] bp 64 °C (6–7 torr)). The yield was 5.58 g (78%). The diethyl [^{17}O]phosphorochloridate was added dropwise to an ice-cold, stirred solution of concentrated NH_3 in H_2O (29.6% NH_3 by weight, 5.54 g, 96.5 mmol). The reaction mixture was allowed to warm to room temperature, and stirring was continued for 1 h. Water was added, and the product diethyl [^{17}O]phosphoramidate was extracted with CH_2Cl_2. The dried CH_2Cl_2 solution was evaporated to dryness to afford 2.84 g (69%) of the desired product, which was used to prepare tetraethyl [^{17}O]imidodiphosphate.

[β,γ-^{15}N]-, [α-$^{17}O_1$]-, and [β,γ-^{17}O]AMP-PNP were prepared from either the corresponding labeled PNP or from [$^{17}O_1$]AMP in a procedure that closely paralleled that of Yount and co-workers[4] in yields of 38%, 38%, and 40%, respectively. The purification procedure for these samples of AMP-PNP differed from that reported by Yount in two respects: QAE-Sephadex A-25 (HCO_3^-) was used in place of DEAE-Sephadex A-25 (HCO_3^-), and a linear gradient of 0.1–0.7 M triethylammonium bicarbonate, pH 8.6, was used for elution instead of a gradient of 0.0–0.4 M triethylammonium bicarbonate, pH 7–8. (AMP-PNP is more stable at the higher pH.) All three labeled samples of AMP-PNP had identical R_f values (0.34) when compared to authentic unlabeled AMP-PNP by using polyethylenimine cellulose TLC with 1.2 N LiCl as eluent.[15]

The [α-$^{17}O_1$]AMP-PNP is predicted to have an ^{17}O enrichment of 32% in one of the α-nonbridging oxygens. Integration of the ^{31}P NMR resonances of [β,γ-^{17}O]AMP-PNP relative to an internal standard of AMP was consistent with an ^{17}O-enrichment of approximately 25% in each of the oxygens derived from [^{17}O]PNP.

Sample Preparation. The sodium salt of [β,γ-^{15}N]AMP-PNP or [^{15}N]PNP was dissolved in 1.7 mL of 20% D_2O to give a final solute concentration of about 70 mM. For pH titration experiments, EGTA was added to a final concentration of 0.1 mM. In experiments involving Mg^{2+}, the nucleotide solution was freed from adventitious divalent metal ions by shaking with Chelex-100 (Na^+) and filtering. Mg^{2+} was added from a stock solution prepared by dissolving $MgCl_2{\cdot}6H_2O$ in distilled deionized water.

The ^{17}O-labeled samples were either percolated through columns of Chelex-100 (tetraethylammonium) and lyophilized or used directly as their sodium salts to prepare 2-mL samples of 40 mM solutes dissolved in 20% D_2O containing 1 mM EGTA; the 10-mm NMR tubes were made metal free as previously described.[10]

NMR Measurements. ^{15}N NMR spectra were obtained at 10.14 MHz on a Varian XL-100 NMR spectrometer. A spectral width of 2000 Hz and 4096 data points were used to acquire the free induction decay; a time delay between pulses of 3 s was employed. Five hundred transients were usually collected. An exponential line-broadening factor of 3 Hz was applied to the total free induction decay prior to Fourier transformation. In most cases, spectra were taken with broad-band ^{1}H decoupling. When it was desired to measure ^{1}H–^{15}N coupling, a gated method was used that turned off the decoupler during data acquisition. Chemical shifts are measured relative to [^{15}N]ammonium sulfate used as an external standard.

A ^{15}N NMR spectrum of the tetraethyl ester of [^{15}N]PNP could not be obtained in aqueous solution, presumably the result of a very long T_1 for the ^{15}N nucleus. We therefore employed an indirect method to determine the magnitude of the ^{15}N NMR chemical shift change produced by protonation of the monoanion of this compound. The line width of the ^{31}P NMR resonance of the labeled tetraethyl ester was measured as a function of a narrow-band ^{15}N decoupling frequency; these spectra were obtained without ^{1}H decoupling so the ^{31}P resonance was relatively broad. The line width of the resonance was calculated by assuming a Lorentzian line shape, and plots were made of the line widths as a function of the ^{15}N decoupling frequency at pH values of 6.22 and 1.88, where the ester is monoanionic and neutral, respectively. The results obtained by this procedure are shown in Figure 2. As the ^{15}N decoupling

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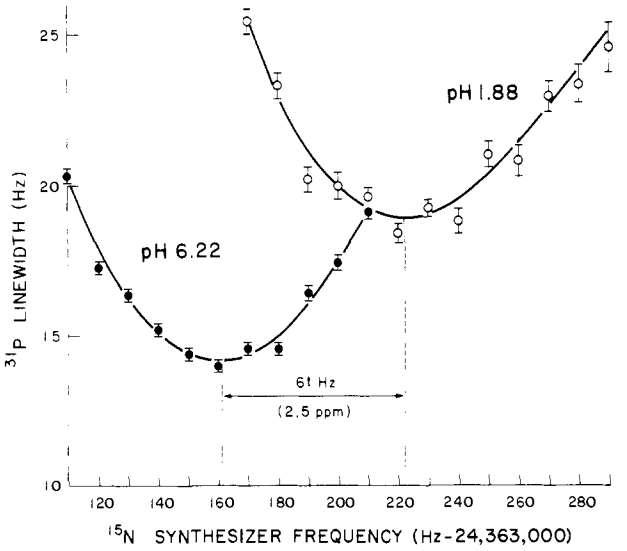

Figure 2. Determination of the ^{15}N NMR chemical shift change produced by protonation of the monoanion of the tetraethyl ester of PNP. Experimental details are given in the text.

frequency was varied, the ^{31}P line width at each pH value passed through a minimum value. After calibration of the ^{15}N decoupling frequency by performing a similar experiment with [^{15}N]PNP, which has an observable ^{15}N NMR resonance in aqueous solution, the ^{15}N decoupling frequency at the minimum was assigned to the frequency of the resonance which could not be directly observed.

^{17}O NMR spectra at 36.6 MHz and 67.8 MHz were obtained with Bruker WH-270 and WM-500 NMR spectrometers, respectively, as described in the previous article.[16] Chemical shifts are measured relative to natural abundance $H_2{}^{17}O$ (0.57 ppm downfield of natural abundance ^{17}O in the 20% D_2O used as solvent).

^{31}P NMR spectra were taken either at 32 MHz on a Varian CFT-20 NMR spectrometer or at 40.5 MHz on a Varian XL-100 NMR spectrometer. Broad-band ^1H decoupling was used on both instruments. A sweep width of 2000 Hz, 4096 data points, and a probe temperature of 29 °C was used on the CFT-20 spectrometer; a sweep width of 1000 Hz, 4096 data points, and a probe temperature of 25 °C was used on the XL-100 spectrometer. Chemical shifts are measured relative to 85% H_3PO_4, with positive shifts being downfield of the reference.

Data Analysis. Values for the pK_as and chemical shifts associated with species differing in state of protonation were obtained with the computer programs described in the previous paper.[16]

Results and Discussion

The synthesis of tetrasodium [^{15}N]PNP was achieved by a more direct route than that previously reported by one of our laboratories[13] (Scheme Ia); [^{17}O]PNP was prepared by a similar route (Scheme Ib). That the known trichloro((dichlorophosphoryl)imino)phosphorane could be directly converted to PNP by hydrolysis was not previously recognized.

^{31}P NMR pH Titrations. In the previous article we described ^{31}P NMR pH titration studies on pyrophosphate and its methylene bridging analogue which revealed that ^{31}P NMR could not be used reliably to quantitate the degree of charge neutralization of the phosphoryl oxygens in these molecules.[16] In the present study we measured the ^{31}P NMR chemical shift of PNP as a function of pH, and the results are presented in Figure 3. Protonation of both the tetra- and trianionic species results in upfield changes in the chemical shift of the ^{31}P NMR resonance, but the magnitudes of the shift changes are significantly different (0.21 and 1.86 ppm, respectively). The pK_as derived from these data are 10.26 ± 0.24 and 7.42 ± 0.03; these are in excellent agreement with those obtained by potentiometric titration (10.22 and 7.32).[17] Although the pK_as measured by ^{31}P NMR are consistent with those obtained by more conventional techniques, an explanation for the absolute magnitudes of the chemical shift changes induced

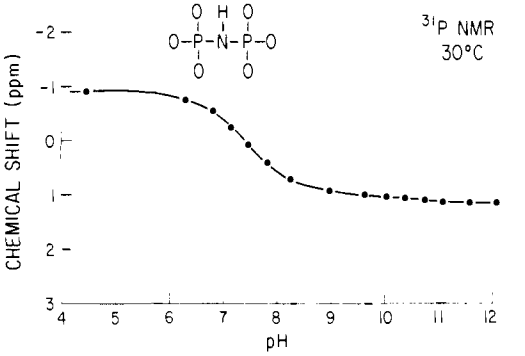

Figure 3. pH titration curve for PNP as determined by ^{31}P NMR at 29 °C.

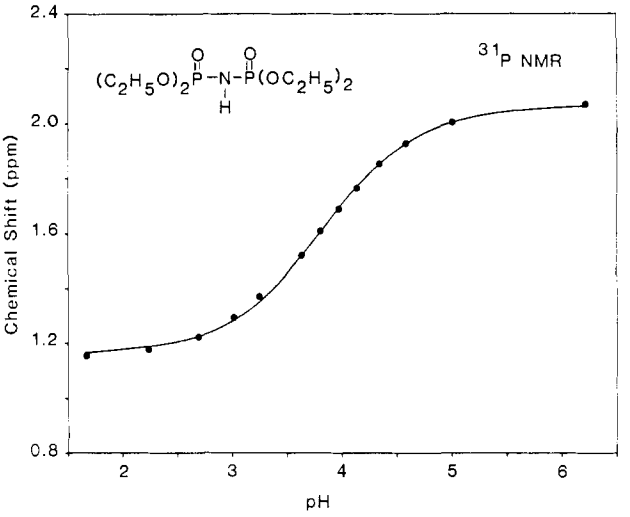

Figure 4. pH titration curve for the tetraethyl ester of PNP as determined by ^{31}P NMR at 25 °C.

by protonation remains ambiguous given the possibility for contribution by the imino tautomer of PNP (Figure 1).

The ^{31}P NMR pH titration data for AMP-PNP have been reported[7,8] and confirmed in our laboratories. We find that upon protonation at 29 °C the resonance associated with the β-phosphorus shifts upfield 3.28 ppm whereas the resonance for the γ-phosphorus shifts upfield 1.09 ppm. The pK_as obtained from the chemical shift changes are 8.23 ± 0.02 and 8.26 ± 0.01, respectively; these are in good agreement with the value of 8.41 measured by potentiometric titration.[18,19]

We have also studied the ^{31}P NMR pH titration behavior of the tetraethyl ester of PNP, and the data are shown in Figure 4. Protonation of the monoanionic species is accompanied by an upfield change in chemical shift of 1.2 ppm. The pK_a derived from these data is 3.8, and this is in excellent agreement with the value obtained by potentiometric titration (3.7).[6c]

^{15}N NMR Studies. Proton-decoupled ^{15}N NMR spectra of PNP at pH 11.00 and at pH 7.00 are shown in Figure 5. The chemical shift of the ^{15}N NMR resonance shifts upfield 3.35 ppm upon the addition of two protons to the tetraanionic species. At each pH the resonance is a triplet as the result of the one-bond ^{15}N–^{31}P coupling constant; from these spectra this coupling constant is found to be 27 Hz.

Proton-decoupled ^{15}N NMR spectra of AMP-PNP at pH 11.75 and at pH 7.63 are reproduced in Figure 6. As the pH is decreased from 11.75 to 7.17, an upfield change in the chemical shift of 1.1 ppm is observed; it was not possible to obtain chemical shift data below pH 7.17 due to the appearance of hydrolysis products that complicate the spectra at the field strength used in these studies. At high pH the ^{15}N NMR resonance appears as a doublet of doublets due to the different one-bond ^{15}N–^{31}P coupling constants to the β- and γ-phosphorus nuclei that can be assigned from the ^{31}P NMR spectrum as 22 Hz and 32 Hz, respectively. At

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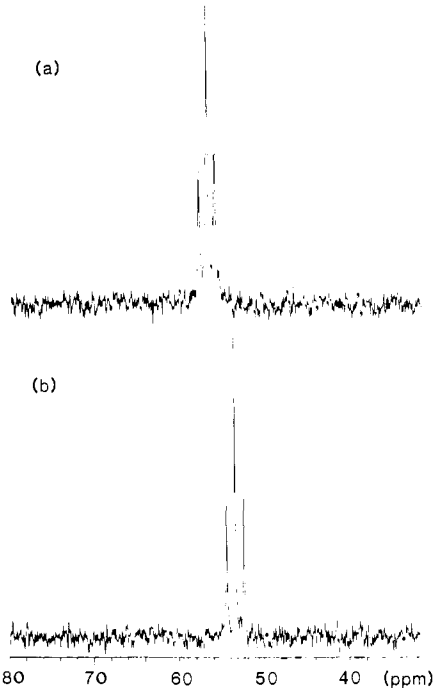

Figure 5. Proton-decoupled ^{15}N NMR spectra for [^{15}N]PNP at (a) pH 11.22 and (b) at pH 7.00.

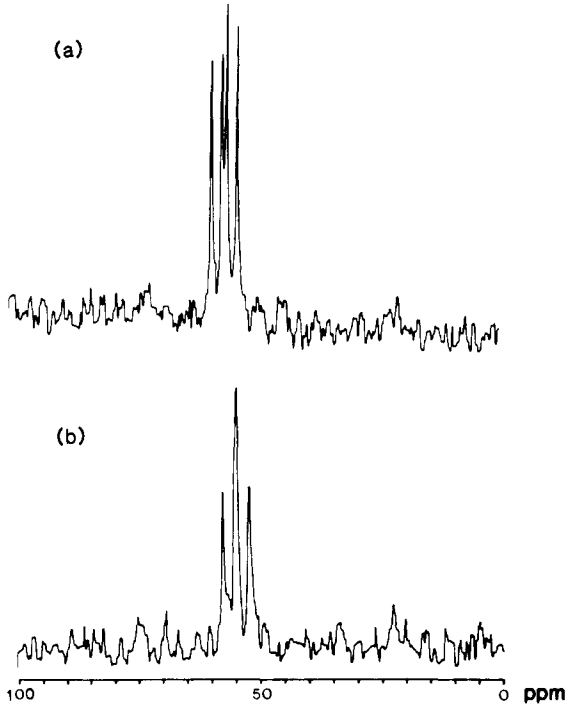

Figure 6. Proton-decoupled ^{15}N NMR spectra for [β,γ-^{15}N]AMP-PNP at (a) pH 11.75 and (b) pH 7.63.

lower pH values, the coupling constants become equivalent and equal to 27 Hz, giving rise to a triplet in the ^{15}N NMR spectrum.

The change in chemical shift resulting from the protonation of the monoanion of the tetraethyl ester of PNP was determined indirectly by observing the effect of ^{15}N decoupling frequency on the ^{31}P NMR spectrum as was described in the Experimental Section. The data shown in Figure 2 demonstrate an upfield change in chemical shift of about 2.50 ppm when the monoanion is protonated.

Proton-coupled ^{15}N NMR spectra were obtained for each of the labeled materials, and the spectra are shown in Figures 7–9. In each of these figures the top spectrum is that with ^{1}H coupling and the bottom is that with ^{1}H decoupling. At pH 11.00, the

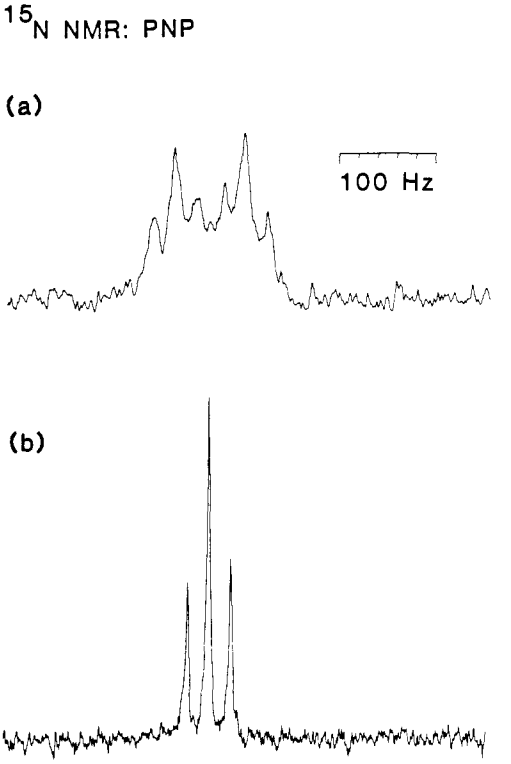

Figure 7. ^{15}N NMR spectra of PNP at pH 11.00 (a) without and (b) with proton decoupling.

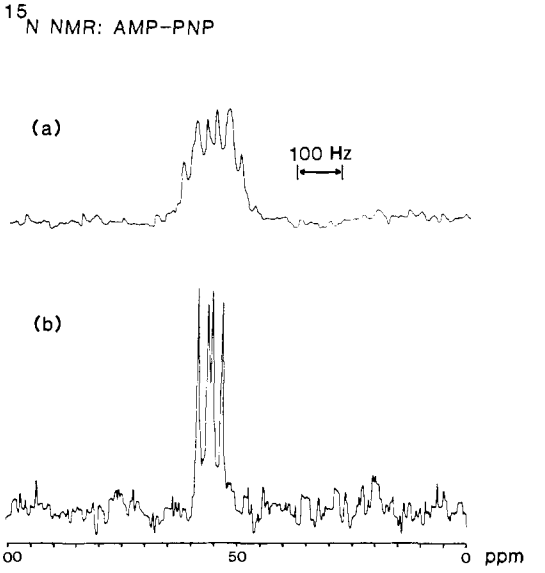

Figure 8. ^{15}N NMR spectra of AMP-PNP at pH 11.75 (a) without and (b) with proton decoupling.

one-bond ^{1}H–^{15}N coupling constant for PNP is 73 Hz (Figure 7). At pH 11.75, the one-bond ^{1}H–^{15}N coupling constant for AMP-PNP is 71 Hz (Figure 8). The ^{1}H NMR chemical shift of the imido proton in the ^{15}N-enriched sample of AMP-PNP was estimated by using narrow-band ^{1}H heteronuclear decoupling; it was found to be about 4.4 ppm downfield from the reference sodium 3-(trimethylsilyl)tetradeuteriopropionate. The detection of the one-bond ^{1}H–^{15}N coupling in PNP and AMP-PNP demonstrates that at alkaline pH the imido tautomer of the P–N–P bridge predominates in both of these compounds (e.g., structure Ib in Figure 1b).

Given the problem encountered with obtaining an ^{15}N NMR spectrum of the tetraethyl ester of PNP in aqueous solution, we turned to $CDCl_3$ as the solvent and recorded the spectra shown in Figure 9. The ^{1}H-coupled ^{15}N NMR resonance of the tetraethyl ester of PNP is a broadened triplet, with the triplet being the result

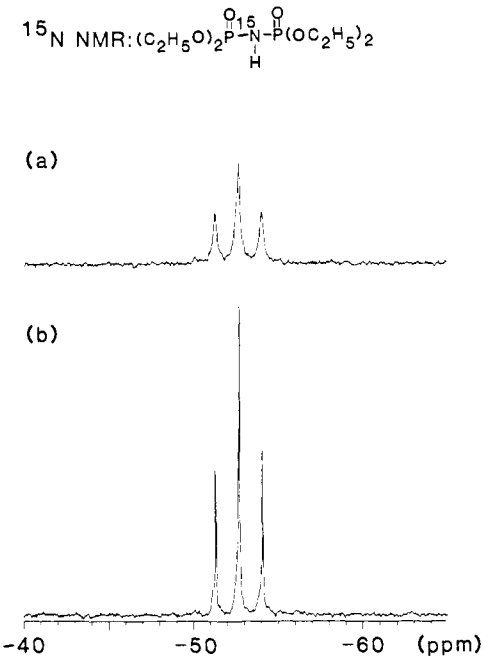

Figure 9. ^{15}N NMR spectra of the tetraethyl ester of PNP in $CDCl_3$ solution (a) without and (b) with proton decoupling.

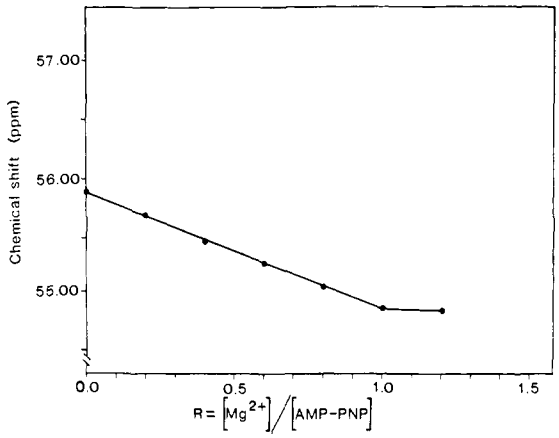

Figure 10. Dependence of the ^{15}N NMR chemical shift of [β,γ-^{15}N]-AMP-PNP on the ratio R = [Mg^{2+}]/[AMP-PNP] at pH 9.4.

of the one-bond ^{15}N–^{31}P coupling constant of 33.6 Hz (Figure 9). The absence of a sizable ^{1}H–^{15}N coupling may be interpreted as resulting either from the predominance of the imino tautomer or from rapid exchange of the relatively acidic proton (pK_a = 3.8) in the imido tautomer; ^{17}O NMR data discussed in the next section cause us to favor the latter explanation.

We have also determined the effect of Mg^{2+} on the ^{15}N NMR chemical shift of the resonance for [β,γ-^{15}N]AMP-PNP at pH 9.4, and the data in Figure 10 demonstrate that an upfield change in chemical shift occurs as the ratio of [Mg^{2+}]/[AMP-PNP] (= R) increases to 1.0; the maximum chemical shift change is 1.0 ppm. Further addition of Mg^{2+} causes no change in the ^{15}N NMR chemical shift. In the absence of ^{1}H decoupling and at equal concentrations of Mg^{2+} and AMP-PNP, a spectrum analogous to that shown in top of Figure 7 was observed (data not shown) with the one-bond ^{1}H–^{15}N coupling constant being 70 Hz. These data demonstrate that the imido tautomer of the P–N–P bridge predominates in the Mg^{2+}–AMP-PNP complex.

^{17}O NMR pH Titrations. The ^{17}O NMR pH titration curve of [^{17}O]PNP was measured at 30 °C, and the data are shown in Figure 11. Equal upfield chemical shift changes are found for protonation of both the tetra- and trianionic species (9.6 and 8.8 ppm, respectively). With the assumption that charge neutralization occurs exclusively on the phosphoryl oxygens, the magnitudes of the upfield shifts per charge neutralized are 58 and 53

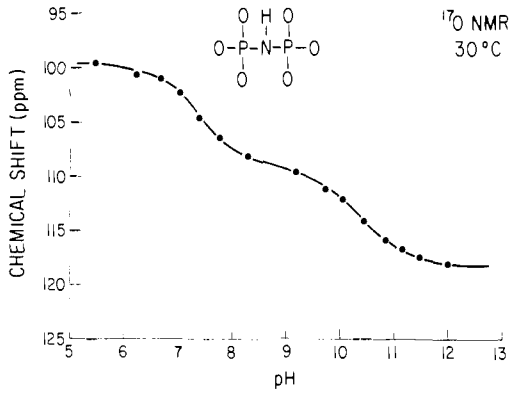

Figure 11. pH titration curve for [^{17}O]PNP as determined by ^{17}O NMR at 30 °C in the presence of broad-band ^{31}P decoupling.

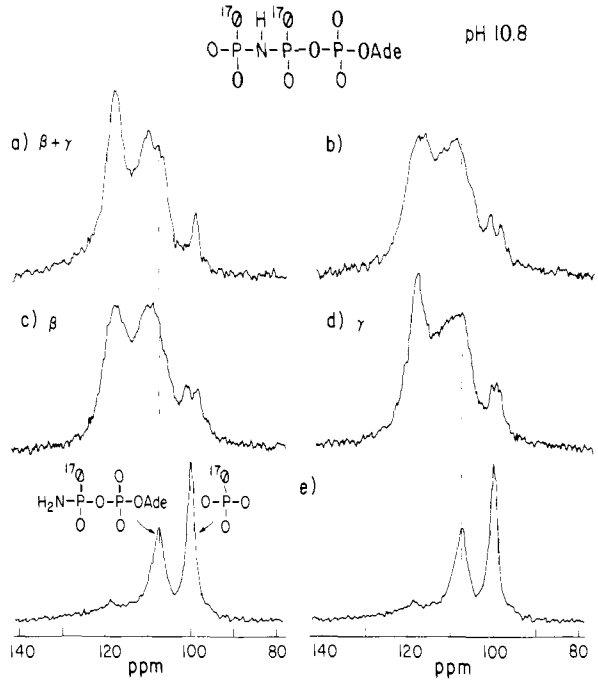

Figure 12. ^{17}O NMR spectra at 36.6 MHz, 50 °C, and pH 10.8 of [β,γ-^{17}O]AMP-PNP used to assign the resonances. The spectra were taken with the following ^{31}P decoupling conditions: (a) decoupling of both the β- and γ-nuclei; (b) no decoupling; (c) decoupling of only the β-nucleus; and (d) decoupling of only the γ-nucleus. The spectra labeled "e" are of the hydrolysis products, obtained in the presence of broad-band ^{31}P decoupling.

ppm, respectively; these values are in good agreement with those determined for a variety of phosphates and phosphate analogues,[10,16] thereby confirming the assumption that protonation occurs on the oxygens. The pK_a values obtained from this titration curve, 10.37 ± 0.12 and 7.41 ± 0.10, are in good agreement with those obtained by ^{31}P NMR. Therefore, the ^{17}O NMR data indicate that the ^{31}P NMR pH titration of PNP must be considered anomalous, thereby providing yet another example of the inability of ^{31}P NMR to quantitate charge neutralization of phosphoryl oxygens.

Although AMP-PNP is less stable than ATP and its thiophosphate and phosphonate analogues, ATPγS and AMP-PCP, we have been able both to assign the resonances in the tetraanionic species of [β,γ-^{17}O]AMP-PNP at 50 °C and also to determine complete ^{17}O NMR pH titration data at this temperature. The assignments of the resonances in the doubly labeled sample were accomplished by selective decoupling of the directly bonded ^{31}P nuclei; this technique was used in the research described in the previous article to make the chemical shift assignments of the resonances of [β,γ-^{17}O]AMP-PCP.[16] The essential spectra were obtained at pH 10.8 with a sample of the tetraethylammonium

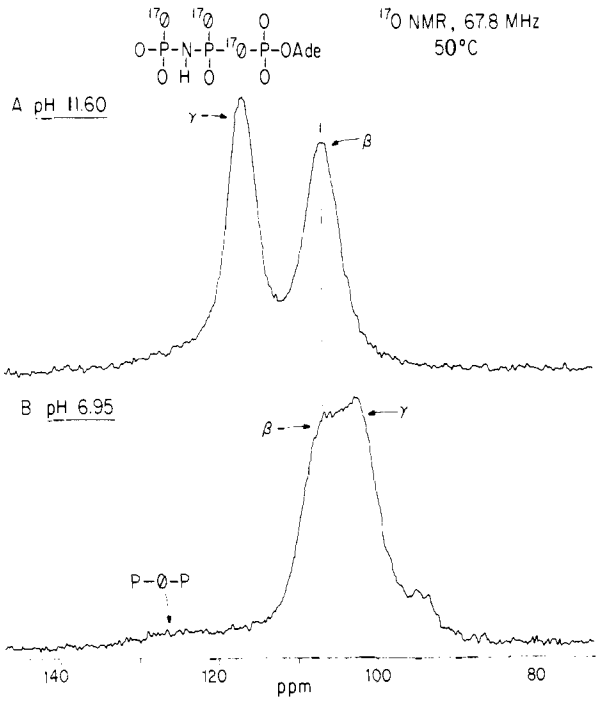

Figure 13. ^{17}O NMR spectra at 67.8 MHz and 50 °C of the sodium salt of [β,γ-^{17}O]AMP-PNP in the tetraanionic (pH 11.60, top spectrum) and trianionic (pH 6.95, bottom spectrum) states of ionization. The spectra were obtained without ^{31}P decoupling.

salt of [β,γ-^{17}O]AMP-PNP, and these are shown in Figure 12. The spectra reveal the presence of small amounts of the hydrolysis products, [β-^{17}O]ADP-NH_2 (107 ppm) and inorganic phosphate (100 ppm). The chemical shift of the resonance associated with ADP-NH_2 is very similar to that of the resonance assignable to the β-nonbridging oxygens in AMP-PNP; however, the amount of ADP-NH_2 present in the sample does not interfere with the assignments. The decoupling conditions used to acquire these spectra are as follows: spectrum a, broad-band ^{31}P decoupling of both the β- and γ-nuclei; spectrum b, no ^{31}P decoupling; spectrum c, selective ^{31}P decoupling of the β-nucleus; and spectrum d, selective ^{31}P decoupling of the γ-nucleus. A ^{31}P- decoupled spectrum of the hydrolysis products is shown in spectrum e. Decoupling of only the β-nucleus (spectrum c) produces a sharpening of the resonances at about 110 ppm whereas decoupling of only the γ-nucleus (spectrum b) produces a sharpening of the resonance at about 118 ppm. This behavior allows the downfield resonance to be assigned to the γ-oxygens and the upfield to the β-oxygens.

Using the sodium salt of the labeled AMP-PNP we have been able to determine the effect of protonation of the tetraanionic species of AMP-PNP on the chemical shifts of the various phosphoryl oxygens. The 67.8-MHz ^{17}O NMR spectra of the [β,γ-^{17}O]AMP-PNP obtained at 50 °C are shown in Figure 13. The spectrum at pH 11.60 is that of the tetraanion and the spectrum at pH 6.95 is that of the trianion; the resonance which was assigned to the γ-phosphoryl oxygens shifts upfield approximately 16 ppm upon protonation of the molecule whereas that assigned to the β-phosphoryl oxygens is essentially pH independent. The resonance associated with the β,γ-bridging oxygen atom can be observed at about 125 ppm in the spectrum taken at pH 6.95, with this assignment being made by comparison with the chemical shift of the bridging oxygen in pyrophosphate. (The weak doublet at about 94 ppm in the spectrum obtained at pH 6.95 can be attributed to inorganic phosphate that resulted from hydrolysis of a small amount of this doubly labeled sample.)

We determined the complete ^{17}O NMR pH titration behavior of the samples of AMP-PNP at 36.6 MHz, and the results are shown in Figure 14. The data for the β- and γ-phosphoryl oxygens were obtained with the sodium salt of the doubly labeled sample at 50 °C, and those for the α-phosphoryl oxygens were

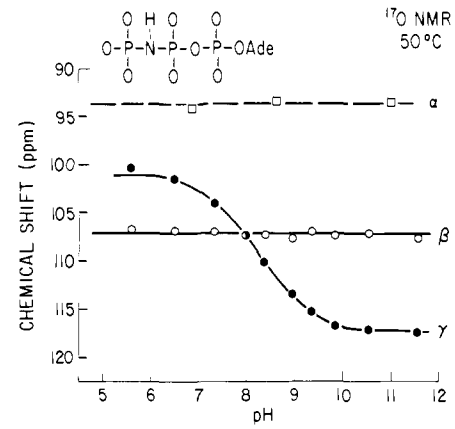

Figure 14. pH titration curves for AMP-PNP as determined by ^{17}O NMR in the presence of broad-band ^{31}P decoupling. Conditions are described in the text.

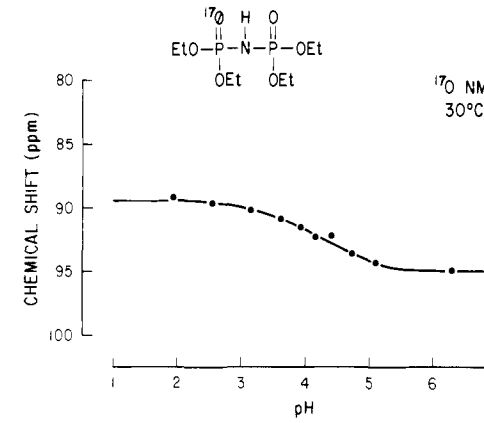

Figure 15. pH titration curve for the tetraethyl ester of [^{17}O]PNP as determined by ^{17}O NMR at 30 °C in the presence of broad-band ^{31}P decoupling.

obtained with the tetraethylammonium salt of [α-$^{17}O_1$]AMP-PNP at 30 °C. As expected on the basis of the spectra shown in Figure 13, only the chemical shift of the resonance associated with the γ-phosphoryl oxygens is sensitive to pH, with the upfield shift occurring on protonation being 15.6 ppm. The change in chemical shift for the γ-phosphoryl oxygens is 47 ppm per charge neutralized, a value slightly smaller than those found for PNP but in the range expected for full protonation occurring only at this position. The pK_a derived from the pH titration data is 8.22 ± 0.03, which is in good agreement with those determined by both ^{31}P NMR and potentiometric titrations.[18] We conclude that the ^{17}O NMR pH titration behavior of AMP-PNP is in accord with protonation occurring only on the γ-phosphoryl oxygens, thereby confirming the conclusion reached from ^{15}N NMR that the imido tautomer of AMP-PNP predominates.

We initially considered the small changes in ^{15}N NMR chemical shift that were documented earlier for PNP and AMP-PNP (Figures 5 and 6) as evidence for protonation occurring on oxygen rather than nitrogen, but in view of the small chemical shift change observed on the protonation of the monoanion of the tetraethyl ester of PNP (Figure 7), we must regard the results of the ^{15}N NMR pH titrations as equivocal. One possible explanation for the small ^{15}N NMR chemical shift change observed for the tetraethyl ester of PNP is that the imino tautomer is the predominant species in solution; this is consistent with our inability to detect a large one-bond ^{1}H–^{15}N coupling constant for this

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(19) Similar observations regarding the relative magnitudes of the ^{31}P NMR chemical shift changes induced by protonation were observed in 5'-adenylyl *N*-methylimidodiphosphate which may be considered to be a locked imido tautomer of AMP-PNP (structure Ib in Figure 1a): Reynolds, M. A.; Kenyon, G. L., unpublished observations.

species in $CDCl_3$ solution. We have further investigated this question by examining the ^{17}O NMR pH titration of the tetraethyl ester of PNP labeled in one nonesterified phosphoryl oxygen; the data are shown in Figure 15. Protonation of the monoanion is accompanied by an upfield change in chemical shift of 5.4 ppm; the pK_a obtained from these data is 4.21 ± 0.04, a value which is in fair agreement with that obtained by ^{31}P NMR pH titration and potentiometric titration.[6c] The change in chemical shift per charge neutralized for the labeled oxygen is 10.8 ppm, a value considerably smaller than that expected if protonation were occurring exclusively on the oxygen.[10,16] This chemical shift change may be interpreted as resulting from either a minor but significant contribution by the imino tautomer to the solution structure of the tetraethyl ester of PNP or resonance stabilization of the negative charge of the monoanion by charge delocalization on the phosphoryl oxygens. Even though we cannot unequivocally distinguish between these explanations, we believe that the small change in ^{15}N chemical shift observed upon protonation of the monoanion is a reasonably accurate reflection of the magnitude of the chemical shift change caused by charge neutralization of this type of nitrogen. This value, approximately 2.50 ppm, is considerably smaller than those documented for protonation of other types of nitrogen atoms.[20-22]

Summary

The ^{15}N and ^{17}O NMR studies reported in this article are consistent with proton binding to imidodiphosphates, including AMP-PNP, occurring only through interactions with the phosphoryl oxygens, with no participation by the nitrogen atom in the P-N-P bridge. Furthermore, our results indicate that AMP-PNP and PNP both exist in solution primarily as the imido tautomers. The NMR data described in this and our previous papers[10,16] provide an unambiguous characterization of the sites of proton binding in ATP and its widely used structural analogues. Finally, we have found that protonation of the nitrogen anion of the tetraethyl ester of PNP is accompanied by an unusually small change in the ^{15}N NMR chemical shift.

Note Added in Proof. The crystal structure of the tetraphenyl ester of imidodiphosphate was recently solved by Mr. Kent R. Myers and Professor Joseph R. Murdoch, University of California, Los Angeles. In the solid this imidodiphosphate ester exists as the imido tautomer, a result in keeping with the solution studies reported in this article. We are grateful to Mr. Myers and Professor Murdoch for allowing us to quote their results prior to publication.

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Registry No. [β,γ-^{15}N]-AMP-PNP, 86993-96-6; [α-$^{17}O_i$]-AMP-PNP, 86993-97-7; [β,γ-^{17}O]-AMP-PNP, 86993-98-8; [$^{17}O_i$]-AMP, 86993-99-9; Na_4[^{15}N]PNP, 81068-46-4; Et_4[^{15}N]PNP, 86993-90-0; Na_4[^{17}O]PNP, 86993-91-1; Et_4[^{17}O]PNP, 86993-92-2; [^{15}N]dichlorophosphoryltrichloroiminophosphorane, 50535-66-5; dichlorophosphoryltrichloroiminophosphorane, 13966-08-0; diethyl [^{17}O]phosphoramidate, 86993-93-3; diethyl chlorophosphite, 589-57-1; diethyl [^{17}O]phosphite, 86993-94-4; diethyl [^{17}O]phosphorochloridate, 86993-95-5.

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Electron Paramagnetic Crystallography of Cobalt(II) and Copper(II) Carboxypeptidase A

L. Charles Dickinson* and J. C. W. Chien*

Contribution from the Department of Chemistry and Materials Research Laboratories, University of Massachusetts, Amherst, Massachusetts 01003. *Received December 23, 1982*

Abstract: Electron paramagnetic resonance spectra of single crystals of cobalt(II) carboxypeptidase A (CoCPA) and copper(II) carboxypeptidase A (CuCPA) have been obtained and solved for the orientation of the principal values of the ***g*** and hyperfine (***A***) tensors. The largest *g* and *A* canonical values for both species are found to be near the perpendicular to the plane containing the His-196 and 69δ-histidine nitrogen atoms and the metal ion. Further, the orientation of the three respective components of the ***g*** tensors of the CoCPA and CuCPA deviates no more than 30° from being parallel. This is taken to indicate that the distortions of the ligand fields in CuCPA and CoCPA are similar in their influence upon the d orbitals, each of which is at least half-filled in both copper(II) and high-spin cobalt(II). The copper hyperfine tensor is precisely parallel to the ***g*** tensor in CuCPA, and the splitting pattern in CuCPA is consistent with two equivalent nitrogen atoms. Cobalt hyperfine splitting is seen only along the direction of the maximum *g* value in CoCPA. The presence of "extra" sites in the CoCPA crystals suggests that conformational substates or ligand variations are possible. The two important results are that the ligand field strongly influences the directions of the ***g*** and ***A*** tensors, which are nearly the same for the d^7 CoCPA and the d^9 CuCPA. Secondly, the lack of enzyme activity in CuCPA may be related to the absence or inaccessibility of conformational substates.

Electron paramagnetic resonance (EPR) spectra have been employed for structural determination of metal sites and spin-labels in metalloenzymes and metal ion activated enzymes over the past 25 years. However, the bulk of these studies have involved frozen solution spectra that are ambiguous, especially when there is considerable electron density on the ligands. Many assignments of principal values for ***g*** and ***A*** tensors based on frozen solution data were shown to be wrong when single-crystal EPR results

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