31P NMR of Phosphate and Phosphonate Complexes of Metalloalkaline Phosphatases*

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31P NMR spectra of phosphate and phosphonate complexes of Escherichia coli alkaline phosphatase have been obtained by Fourier transform NMR methods. One equivalent of P$_3$O$_5$ bound to Zn(II) alkaline phosphatase, pH 8, gives rise to a single 31P resonance 2 ppm downfield from that for P$_3$, and assignable to the noncovalent complex, E-P. Inorganic phosphate in excess of 1 eq per enzyme dimer gives rise to a resonance at the position expected for free P$_3$. At pH 5.1, a second resonance appears 8.5 ppm downfield from that for free P$_3$, and is assignable to the covalent complex, E-P. The large downfield shift suggests that the enzyme phosphoryl group is highly strained with an O-P-O bond angle of under 100°.

At pH 6.5, Cd(II) alkaline phosphatase forms 1 mol of a stable phosphoryl group per mol of enzyme, with a single 31P resonance 8 ppm downfield from the resonance for P$_3$. Formation of the apophosphoryl enzyme by removal of the metal ion from the Cd(II) phosphoryl enzyme shifts the 31P resonance upfield by ~2 ppm. While the metal ion induces additional strain in E-P, the protein environment of the active site serine induces much of the unusual chemical shift for E-P. The apophosphoryl enzyme is stable between pH 2 and 9. Complete pH titrations of the 31P resonance show that the enzyme phosphoserine cannot be protonated until the enzyme dissociates and unfolds below pH 3. The phosphorus nucleus in the apophosphoryl enzyme is coupled to the β protons of the serine with a coupling constant of 13 Hz, has a nuclear Overhauser enhancement ($\eta + 1$) of 1.22, and a $T_1$ of 1.5 s from which an effective rotational correlation time, $\tau_r$, of $5 \times 10^{-8}$ s can be calculated. Thus, the phosphoryl group of the apoenzyme has considerable rotational mobility relative to the protein. In contrast, the 31P nucleus of the Cd(II) phosphoryl enzyme shows a much broader 31P-(1H) resonance and has less rotational freedom.

Addition of 1 eq of P$_3$ to Co(II) or Mn(II) alkaline phosphatase results in complete disappearance of the 31P resonance, suggesting that the phosphate is bound within the first or second coordination spheres of the metal ion. The 31P resonance of a 2nd eq of P$_3$ added to the Co(II) or Mn(II) enzymes appears in the position expected for free P$_3$, and there is no paramagnetic broadening of this resonance. Thus the Co(II) and Mn(II) enzymes show absolute negative cooperativity at pH 8.0, and rapid exchange between the phosphate at the active site and free P$_3$ does not occur. In contrast, there is rapid exchange between phosphonoesterbound at the active site of the Co(II) or Mn(II) enzymes and free inhibitor.

The linewidth for P$_3$ bound to the Co(II) enzyme is significantly larger than that for bound phosphate, suggesting that phosphate may be directly coordinated to the metal ion. Coordination of the phosphate group by the metal ion, and induction by the protein of unusual strain in the bond angles of the phosphoserine intermediate, appear to play roles in the catalytic mechanism of alkaline phosphatase.

Enzyme-phosphate complexes are the major intermediates in the hydrolysis of phosphate monoesters by the Zn(II) metalloenzyme alkaline phosphatase, as demonstrated by the ejection of the alcohol in a rapid pre-steady state phosphorylation of the enzyme (1-3). Both a noncovalent complex, E-P, and a covalent complex, E-P, appear to lie on the reaction pathway (1-3). E-P has been shown to form by the phosphorylation of Ser 99 of the enzyme (4). The relative stability of E-P and E-P$^-$ suggests that the E-P$^-$ form of the enzyme is highly strained with an O-P-O bond angle of under 100°.

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*The phosphorylated serine is residue 99 from the NH$_2$-terminal threonine residue in the preliminary numbering of the sequence as it is presently available from the work of R. A. Bradshaw, P. A. Neumann, F. Cancetta, K. Schrifla, J. D. Hecht, and M. J. Schlesinger (personal communication from R. A. Bradshaw).
versus E-P varies greatly with pH; E-P is the more stable species at acid pH, E-P the more stable species at alkaline pH. As discussed in detail in the preceding paper, both the binding of phosphate and the phosphorylation and dephosphorylation of Ser 99 require a metal ion at the active site (5, 6). A powerful spectroscopic method for determining the unique chemical features of these reactive enzyme-phosphate intermediates is \(^3^P\) NMR. The \(^3^P\) NMR spectra of both phosphate and phosphonate complexes of *Escherichia coli* alkaline phosphatase are reported in this paper.

**MATERIALS AND METHODS**

**Enzymes and Chemicals—**Isolation of native alkaline phosphatase, preparation of apo- and metalloalkaline phosphatases, and determination of protein concentrations were carried out as described in the previous paper (5) with the following exception. For the NMR studies, the Co(II) and Mn(II) enzymes were prepared by the addition of slightly less than 2 eq of Me(II) per apoenzyme dimer. Titration of apoalkaline phosphatase with Me(II) ions as followed by several spectroscopic techniques shows that the first two metal ions added were tightly bound at the sites occupied by the catalytically active Zn(II) ions of the native enzyme (6-11). When using paramagnetic metal ions to reconstitute apoalkaline phosphatase, addition of more than 2 eq of Me(II) per enzyme dimer gives rise to an ESR signal significantly different from that generated by the first 2 eq of metal ion (8, 9). Concentrations of Me(II) above 2 eq per enzyme dimer also lead to broadening of the NMR lines of nuclei carried on active site ligands which is similar to that produced by free metal ions in solution (10, 11). The same phenomenon was observed in the present work with both phosphate and phosphonate as ligands. Addition of more than 2 eq of Co(II) to the enzyme resulted in the appearance of the \(^3^P\) resonances for both compounds similar to that observed with hydrated Co(II) (see under “Results”). All NMR samples and equipment were prepared metal-free following procedures described in the preceding paper (5, 11). Such precautions are particularly important because of artifacts generated in the NMR spectra due to paramagnetic contaminant. O-Phosphorosine was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade. p-Aminobenzylphosphonate was prepared by acid hydrolysis of diethyl p-aminobenzylphosphonate (Aldrich Chemical Co., Milwaukee, Wis.), and the final product was recrystallized from water.

**NMR Spectrometer—**\(^3^P\) NMR spectra were recorded on a FT-Bruker HFX-90 spectrometer operating at 36.4 MHz. Deuterium oxide (D₂O), present in the sample or in a 3-mm coaxial capillary insert, was used as a field-frequency lock. All spectra were obtained under conditions of proton noise decoupling unless otherwise indicated using the Fourier transform method. A spectral width of 5000 Hz was used with an acquisition time of 0.2 s. This sweep width was chosen to maximize the S/N improvement from a 5000-Hz bandwidth crystal filter. For all spectra shown, an interpolation expansion routine was employed providing a resolution of 1.22 Hz/point (13). Measurements were made at 25 ± 0.2°C on 0.8- to 1.0-mI samples contained in 10-mm sample tubes fitted with Vortex plugs to confine the solution within the transmitter coils. Solutions of E-P and E-P complexes ranged from 0.5 × 10⁻⁴ M to 1.2 × 10⁻⁴ M in concentration. To obtain satisfactory spectra, up to 250,000 transients were collected on individual samples. \(^3^P\) chemical shifts were determined relative to external 85% H₃PO₄ and were found to be identical for both the internal and external H lock, eliminating corrections for possible changes in magnetic susceptibility. \(^3^P\)-H coupling constants (J₃P-H) were determined by computer simulation of the observed spectra for the most simple case of an A₂X spin system using values for the spin-spin relaxation times (T₂) derived from the decoupled linewidths. Spin-lattice relaxation times (T₁) were obtained by progressive saturation (14, 15) and inversion-recovery (16) methods. Dynamic nuclear Overhauser enhancements (NOE) were measured by a previously described gating technique (17).

**RESULTS**

**\(^3^P\) NMR of Phosphate Complexes of Zn(II) Alkaline Phosphatase—**Because of the micromolar binding constant for Pₐl, alkaline phosphatase can easily become contaminated with phosphate. This phosphate can be removed by extensive dialysis. Phosphate is also removed when the metal ion is removed, since phosphate binding is metal ion-dependent, as documented in detail by Applebury *et al.* (6). Thus, all metallophosphatases made from the apoenzyme are initially phosphate-free. The \(^3^P\) NMR of a millimolar solution of the phosphate-free Zn(II) enzyme is shown in Fig. 1A under the same conditions as used to record the spectra of the phosphate complexes. Addition of 1 eq of P/mol of enzyme dimer, pH 8, results in the appearance of a single resonance 2.1 ppm downfield from the resonance position for free inorganic phosphate (Fig. 1B). Addition of a 2nd eq of phosphate per enzyme dimer, pH 8, gives rise to a second resonance at the position expected for free inorganic phosphate (Fig. 1C). The resonance from the initial enzyme-bound phosphate is una-
affected by the 2nd equivalent of P. If 1 eq of phosphate is added to the Zn(II) enzyme at pH 5.1, two resonances appear (Fig. 1D). One of these is ~2 ppm upfield from the position of the resonance for enzyme-bound phosphate observed at pH 8 and ~2 ppm downfield from the resonance position of orthophosphate at pH 5.1, which suggests that the noncovalently bound phosphate has become protonated. The second resonance is over 8 ppm downfield from the resonance position for free inorganic phosphate at pH 5.1 (Fig. 1D). The latter resonance must represent that of the covalently bound phosphate complex, E-P, since previous evidence shows that E-P becomes the predominant species at low pH (5, 6).

Effect of Co(II) and Mn(II) on 31P Resonance of Enzyme-bound Phosphate—Addition of 1 eq of phosphate to Co(II) alkaline phosphatase (pH 8.0) containing 2 g at Co(II)/mol of enzyme dimer results in complete disappearance of the 31P resonance (Fig. 1E). Under the present system of data collection, and at millimolar concentrations, the linewidth would have to be at least 300 Hz to be undetectable. The addition of a 2nd eq of P, to the Co(II) enzyme results in the appearance of a highly resolved resonance at the position expected for free inorganic phosphate at this pH (Fig. 1F). This resonance is not detectably broadened, showing that the 2nd eq of P, is not in rapid exchange with the 1st eq, and that there is negligible free Co(II) present in solution. Addition of any more than 2 eq of Co(II) to the enzyme broadens this line in a manner similar to that observed on the addition of Co(II) to a solution of inorganic phosphate. Exactly the same phenomena are observed when Mn(II) is used instead of Co(II).

31P NMR of Phosphate Complexes of Cd(II) and Apoalkaline Phosphatase—Since Cd(II) alkaline phosphatase forms a stable and well characterized phosphoryl enzyme near neutral pH (5), the 31P NMR characteristics of the phosphoryl enzyme were further explored by preparation of the phosphorylated Cd(II) enzyme. Addition of 2 eq of P, to Cd(II) alkaline phosphatase at pH 6.5 results in the appearance of two 31P resonances; one at the position of the resonance for inorganic phosphate, and the other of similar amplitude 8 ppm downfield from the resonance position for inorganic phosphate (Fig. 2A). Dialysis of the Cd(II) enzyme at pH 6.5 against metal-free buffer results in removal of the resonance at the position of inorganic phosphate and retention of the downfield resonance (Fig. 2B). As demonstrated by 31P-labeling (5), this resonance must represent the phosphorus nucleus of the Cd(II) phosphoryl enzyme.

The Cd(II) ion can be completely removed from the dialyzed Cd(II) phosphoryl enzyme by dialysis against 5 x 10^-3 M 1,10-phenanthroline to form the apophosphoryl enzyme (5). The 31P resonance of the apophosphoryl enzyme (Fig. 2C) shifts ~2.3 ppm (88 Hz) upfield from the resonance position of the Cd(II) phosphoryl enzyme (Fig. 2B), but it is still ~5 ppm downfield from the resonance for free inorganic phosphate at this pH.

Proton Coupling to Phosphorus of Cd(II) and Apoprophosphoryl Enzymes—Coupling of the 31P nucleus of phosphoserine with the 2 protons of the @ carbon is clearly illustrated by the triplet observed (V_pH = 6.1 Hz) in the 31P-1H spectrum (Fig. 3A). For the apophosphoryl enzyme, reduction in T_1 and T_2 results in a broadened resonance such that a value of V_pH cannot be obtained on inspection (Fig. 3A). Computer simulation of the undecoupled spectrum using a T_2 of 0.024 s, and assuming an A_1X spin system, permits assignment of a V_pH for the apophosphoryl enzyme of 13 Hz (Table I). The broadening of the proton-decoupled resonance in the presence of Cd(II) (T_2 = 0.016 s) compared to that of the apophosphoryl enzyme (T_2 = 0.024 s) (Fig. 2, B and C) is not due to cadmium-phosphorus spin-spin coupling. There are two isotopes of Cd with nuclear spin of 1/2 (110Cd and 111Cd), each the exchange rate of the exchangeable protons. For comparison of linewidths under different conditions see Table I.

The assumed equivalence of the @ protons which permit analysis in terms of an A_1X spin system may not be valid for the enzyme phosphoserine. Should there be a significant chemical shift difference between the @ protons, the 31P spectrum corresponds to the X of an ABX system, in which case only the sum of the proton-phosphorus couplings can be determined from the linewidth of the undecoupled spectrum. The observed linewidth, ~33 Hz, is not in conflict with conclusions drawn with respect to the structural features of the phosphoryl enzyme (see "Discussion").
Because the apophosphoryl enzyme is stable between pH 2 and 9 (5), a complete pH titration of the apophosphoryl enzyme at pH 2 is shown in Fig. 20. The $^{31}$P resonance of apophosphoryl alkaline phosphatase at pH 8.5 is -2.5 ppm further downfield than that of isolated phosphoserine at the same pH (Fig. 4). The $^{31}$P resonance of the phosphoserine group in apoalkaline phosphatase at pH 8 is -2.5 ppm further downfield than that of isolated phosphoserine at the same pH (Fig. 4). This sharp transition parallels the dissociation of the enzyme into monomers and the transition of the secondary structure to that of a random coil, as documented in detail by previous work (21).

The $^{31}$P resonance of apophosphoryl alkaline phosphatase at pH 2 is shown in Fig. 2D and compared to the phosphoseryl group of the apoenzyme as it is titrated from pH 8 to pH 2 (Fig. 3). The $^{31}$P resonance of the apoenzyme shifts only slightly between pH 8 and pH 4. The resonance shifts upfield to the resonance position of the model phosphoserine when the pH changes from 4 to 3.5 (Fig. 4). This sharp transition is supported by complete kinetic analysis of the inhibition process is completely reversible, since readjustment of the pH to 5.2 re-establishes the downfield resonance (Fig. 5F). Addition of Mg(II) does not detectably alter these resonances. Precise chemical shifts relative to that of $\text{H}_2\text{PO}_4^-$ for the various phosphate derivatives of the several forms of alkaline phosphatase are collected in Table II.

$^{31}$P NMR of Phosphate Complexes of Zn(II) Alkaline Phosphatase—The $^{31}$P resonances of apophosphoryl alkaline phosphatase at pH 7 are collected in Table II. The $^{31}$P resonances of apophosphoryl alkaline phosphatase at pH 2 is shown in Fig. 2D and compared to the phosphoseryl group of the apoenzyme as it is titrated from pH 8 to pH 2 (Fig. 3). The $^{31}$P resonance of the apoenzyme shifts only slightly between pH 8 and pH 4. The resonance shifts upfield to the resonance position of the model phosphoserine when the pH changes from 4 to 3.5 (Fig. 4). This sharp transition parallels the dissociation of the enzyme into monomers and the transition of the secondary structure to that of a random coil, as documented in detail by previous work (21).

The $^{31}$P-$^{1}$H spectra of phosphoserine and the apophosphoryl enzyme yield $T_2$ values of 0.53 and 0.024 s, respectively, from the measured linewidth (see Table I for comparison of buffer conditions). Comparison of the intensities of the gated $^{31}$P-$^{1}$H spectra (Fig. 3B) with the corresponding $^{31}$P-$^{1}$H spectra (Fig. 3C) allows direct determination of the NOE (n+1) for the phosphoserine and the apophosphoryl enzyme of 1.83 and 1.22, respectively. Determination of $T_2$ by both inversion recovery and progressive saturation gives values of 19.0 s for phosphoserine and 1.5 s for the apophosphoryl enzyme. From the NOE (assuming isotropic motion), an effective rotational correlation time, $\tau_\text{e} = \tau_\text{r}, \text{of} \ 5 \times 10^{-9} s$ can be calculated for the phosphoseryl group on the apoenzyme using published methods (19) (see footnotes to Table I).

**Ionization State of Enzyme Phosphoserine as a Function of pH**—Because the apophosphoryl enzyme is stable between pH 2 and 9 (5), a complete pH titration of the apophosphoryl enzyme can be carried out, and the chemical shifts of the $^{31}$P nucleus of the enzyme phosphoserine can be compared with the corresponding changes induced in the model compound, phosphoserine. The results are shown in Fig. 4. The $^{31}$P resonance of the phosphoseryl group in apoalkaline phosphatase at pH 8 is -2.5 ppm further downfield than that of isolated phosphoserine at the same pH (Fig. 4). This sharp transition parallels the dissociation of the enzyme into monomers and the transition of the secondary structure to that of a random coil, as documented in detail by previous work (21).
TABLE I

NMR Parameters for \( ^{31}P \) Resonance of Phosphate and Phosphonate Complexes of Apo- and Metalloalkaline Phosphates

Studies were carried out with 0.01 M Tris/0.01 M sodium acetate/0.1 M NaCl (pH 6.75)/1.2 \times 10^{-4} M phosphoserine/1.2 \times 10^{-4} M apophosphoryl enzyme/1.2 \times 10^{-4} M Cd(II) phosphoryl enzyme.

| Parameter          | Value |
|--------------------|-------|
| \( H_2 \)         | 6.1 ± 0.4 |
| \( T_1 \)         | 11.2 |
| \( T_1' \)        | 0.08 s  |
| \( T_2' \)        | 1.44 s  |
| \( T_\text{NOE} \) | 32 s   |
| \( T_\text{obs} \) | 6.5 \times 10^{-11} |
| \( T_{1\text{M}} \) | 1.24 T_1 |
| \( T_{2\text{M}} \) | 1.52 T_2 |
| \( T_\text{relax} \) | 7.3 \times 10^{-11} |

**Note:**
- \( T_{1\text{M}} \) is the dipole-dipole contribution to the observed relaxation time, \( T_{2\text{M}} \) is the anisotropic contribution to \( T_2 \).
- Variations of the theoretical NOE from 1.24 over the range of correlation times considered here are not significant.

**Footnotes:**
- c.f. Footnote 2.
- The NOE becomes dependent on \( r_s \) when \( r_s \geq 10^{-15} \text{ s} \). Consequently, in the absence of an independent determination of \( r_s \) from studies at other frequencies, we have assumed that the dipolar mechanism is the sole contribution to \( T_{\text{obs}} \). Thus, \( T_{\text{obs}} = T_{\text{relax}} \) and the \( r_s \) is taken from a theoretical curve for \( r_s \) versus \( T_\text{relax} \) constructed in a similar manner to that described previously for \( r_s \) versus \( T_\text{relax} \).

**Calculation of \( r_\text{relax} \):**

\[
\text{relax} = \frac{2.93(33\%)}{0.159} \quad (1)
\]

**Calculation of \( r_\text{relax} \):**

\[
\text{relax} = \frac{4.58(33\%)}{2.25} \quad (2)
\]

**Fig. 4.** pH dependence of \( ^{31}P \) chemical shift (\( \delta \)) for phosphoserine and apophosphoryl alkaline phosphatase (Apophosphoryl AP-P). Conditions: 0.01 M Tris/0.01 M sodium acetate/0.1 M NaCl. \( 3.0 \times 10^{-4} \text{ M phosphoserine}; \text{6.20} \times 10^{-5} \text{ M apophosphoryl enzyme}. \)

The \( ^{31}P \) linewidth as a function of the fraction of phosphonate bound to the Co(II) and Mn(II) enzymes, \( f = [\text{Et}] / [\text{Et} + \text{H}_2 \text{O}] \), is plotted in Fig. 7, and was determined by two methods. The first used a constant concentration of Co(II) enzyme and varied the phosphonate concentration. The second method utilized constant concentrations of phosphonate and apoenzyme to which fractional equivalents of Co(II) or Mn(II) were added. Data from the second method are also plotted as a function of moles of Me(II) added/mol of enzyme dimer. The first 2 eq of Me(II) bound to the apoenzyme show much less relaxation enhancement of the \( ^{31}P \) nucleus of the phosphonate than do the Co(II) or Mn(II) ions added above the 2-eq point. The relaxation observed from the first 2 eq of Me(II) is that characteristic of the metal ion at the active site.

**Calculation of Me(II)-\( ^{31}P \) Distances in Phosphonate Complexes of Mn(II) and Co(II) Alkaline Phosphatase**—The generalized Bloembergen-Solomon equations relating the metal-induced relaxation times, \( T_{1\text{M}} \) and \( T_{2\text{M}} \), to the nuclear-electron distance, \( r_s \), are given by Equations 1 and 2,

\[
\frac{1}{T_{1\text{M}}} = \frac{2\sin^2(\pi\delta/2)}{r_s^3} \left[ \frac{1 + u_0 q_1^2}{1 + u_0 q_2^2} \right] + \frac{1 + u_0 q_3^2}{1 + u_0 q_4^2} \quad (1)
\]

\[
\frac{1}{T_{2\text{M}}} = \frac{1}{1 + u_0 q_5^2} \left[ \frac{1 + u_0 q_6^2}{1 + u_0 q_7^2} \right] + \frac{1 + u_0 q_8^2}{1 + u_0 q_9^2} \quad (2)
\]

where \( q_i \) is the nuclear gyromagnetic ratio; \( \omega_e \) is the electron precession frequency; \( \omega_n \) is the nuclear precession frequency; \( A \) is the isotropic indirect hyperfine interaction; \( \tau_r \) is the correlation time for the anisotropic dipolar interaction for which \( 1/\tau_r = (1/\tau_r) + (1/\tau_m) \), where \( \tau_r \) is the rotational correlation time and \( \tau_m \) is the correlation time for the isotropic hyperfine interaction.

Applications of the above equations to the relaxation of \( ^1H \) of H\(_2\)O or other ligands by Mn(II) ions attached to macromolecules have generally assumed that the isotropic indirect hyperfine or scalar interaction is negligible (22). For Mn(II) relaxing \( ^1H \) this assumption has generally appeared to be justified, and \( T_{1\text{M}} \) has been observed to be controlled primarily by \( r_s \) (as predicted if the first (dipolar) term of Equation 2 is dominant), with the result that Mn(II) bound to the macromolecule (large \( r_s \)) is more effective in relaxing \( ^1H \) carried on...
**TABLE II**

**$^{31}$P Chemical Shifts of Phosphate Derivatives of Alkaline Phosphatase**

| $^{31}$P Chemical Shifts of Phosphate Derivatives of Alkaline Phosphatase | Model | E-P | E-P |
|---|---|---|---|
| ppm from 85% H$_3$PO$_4$ | | | |
| Zn(II) AP | 8.0 | -2.82 | -5.07 |
| | 5.5 | -0.57 | -4.20 | -8.32 |
| | 5.1 | -0.35 | -3.00 | -8.47 |
| | 4.6 | -0.22 | -2.12 | -8.55 |
| Cd(II) AP | 8.0 | -8.13 | 6.5 | -8.07 |
| | 6.5 | -8.13 | 5.3 | -8.17 |
| Apo-AP | 8.0 | -3.63 | -6.33 |
| | 6.5 | -3.13 | -6.30 |
| | 5.2 | -0.57 | -6.07 |
| | 4.0 | 0.00 | -6.00 |
| | 2.0 | +0.35 | -0.19 |

$^{31}$P, alkaline phosphatase.

*For Mn(II) attached to rapidly rotating molecules, $\tau_s \approx \tau_r$, since the $\tau_s$ for Mn(II) is generally near $10^{-8}$ s. However, for a ligand attached to a macromolecule containing Mn(II), $\tau_s$ may be sufficiently increased ($>10^{-8}$ s) for $\tau_s$ to contribute to $\tau_r$. The change in relaxation of ligand protons observed on comparing relaxation by free and bound Mn(II) is generally due to the change in $\tau_s$, even though $\tau_r$ may decrease somewhat on binding of Mn(II) to the macromolecule.*

**Fig. 5.** $^{31}$P NMR spectra obtained on interaction of phosphate with Zn(II) alkaline phosphatase: pH variation. Conditions: 0.01 M Tris/0.01 M sodium acetate/0.1 M NaCl. A, 6.20 $\times$ 10$^{-4}$ M apophosphoryl alkaline phosphatase (ApoAP-P), pH 4.6; B, sample A plus 2.48 $\times$ 10$^{-8}$ M Zn(II), pH 4.6 (see "Materials and Methods"). The pH of sample B was then adjusted to the values indicated in spectra C through F in succession.

**Fig. 6.** $^{31}$P NMR spectra obtained on binding of p-aminobenzylphosphonate to Zn(II) and Co(II) alkaline phosphatases (AP). Conditions: 0.01 M Tris/1.0 M NaCl. A, 1.0 $\times$ 10$^{-4}$ M p-aminobenzylphosphonate, pH 8.0; B, 8.51 $\times$ 10$^{-4}$ M Zn(II) enzyme/9.50 $\times$ 10$^{-4}$ M p-aminobenzylphosphonate, pH 8.0; C, 1.82 $\times$ 10$^{-4}$ M Co(II) enzyme/2.33 $\times$ 10$^{-4}$ M p-aminobenzylphosphonate, pH 6.75.
effectively than the free metal ion (Fig. 7). The simplified Bloembergen-Solomon expressions applying to Mn(II) are given in Equations 3 and 4, and to Co(II) in Equation 5. For Mn(II) \( \omega_s^2 \tau_s^2 \gg 1 \) and \( \omega_s^2 \tau_s^2 \gg 1 \), while for Co(II) \( \omega_s^2 \tau_s^2 \sim 1 \) (\( \tau_s \sim \tau_e \)) leading to the following simplified equations (23).

\[
\frac{1}{T_{1M}} = \frac{1}{T_{2M}} = \frac{4 \sin^2 \theta \sin^2 \frac{\delta}{2}}{5 \sin \theta \sin \frac{\delta}{2} + \frac{4 \sin^2 \theta \sin^2 \frac{\delta}{2}}{3 \sin \theta \sin \frac{\delta}{2} + \frac{5 \sin \theta \sin \frac{\delta}{2}}{3}} \tau_s^2
\]

(3)

The large decrease in relaxation enhancement of the \( ^3P \) of phosphonate by enzyme-bound Mn(II) compared to the hydrated ion would appear to result from a large decrease in the \( A \) value applying to the Mn(II) enzyme-phosphonate complex for the following reasons. The \( A \) value for crystalline LiMnPO₄ is 3.3 MHz (23), and \( A \) values of similar magnitude have been shown to apply to an Mn(II)-AMP and RNA complexes where Mn(II) is directly coordinated to phosphate, probably without major deviations from octahedral coordination (23). Determinations of \( T_{1M} \) from linewidths and \( T_{2M} \) from progressive saturation and inversion recovery methods for both Co(II) and Mn(II) alkaline phosphatase-phosphonate complexes are given in Table I. The temperature dependence of \( T_{1M} \) and the value of \( T_{1M}/T_{2M} \) observed for the Mn(II) enzyme indicate that the fast exchange condition obtains. The values of \( T_{1M} \) are too large for \( A \) values of the magnitude of 3.3 MHz to apply. While the second term of Equation 4 could be significantly decreased by a large enhancement of the electron spin relaxation in the enzyme-bound Mn(II), the ESR signal of the Mn(II) alkaline phosphatase has been determined in detail, and arises from Mn(II) in a rhombically distorted environment (9). Most of the observed loss of signal intensity is due to the solid state character of the spectrum and the large zero field splitting, with consequent spreading of \( g_t, g_e \) and \( g_s \) for most of the transitions, rather than a large decrease in electron relaxation time. The average \( g \) value remains \( \sim 2 \) (9). Since \( A \) values are highly dependent on the nature of the bonding (probably quite different in the enzyme from coordination in model compounds) and fall off rapidly with Me(II)-3P distance, it is not possible to make an assumption about the magnitude of \( A \) applying to the Mn(II) or Co(II) enzymes. Therefore, the only equation suitable for calculating an approximate \( r \) value is Equation 3, relating \( r^* \) to \( T_{1M} \) for the Mn(II) enzyme. The Mn(II)-3P distance calculated from Equation 3 for the Mn(II) enzyme-phosphonate complex is 7.7 Å using \( g = 2 \) and a \( \tau_s \) of \( 5 \times 10^{-16} \text{s} \). Substituting the \( r \) value of 7.7 Å into Equation 4 allows the calculation of an \( A \) value for enzyme-bound Mn(II) of 0.075 MHz. Thus, the \( A \) value is small compared to that for Mn(II) AMP, but the \( A \) term still dominates \( T_{1M} \).

For the Co(II) enzyme-phosphonate complex, \( T_{1M} \approx T_{2M} \) (Table I) showing that the condition of Equation 5 is substantially correct. This does not allow a unique solution for the Co(II)-3P distance in the Co(II) enzyme-phosphonate complex. However, assumption of an \( r \) value similar to that for the Mn(II) enzyme allows calculation of an \( A \) value of 0.63 MHz assuming \( \tau_e \) for Co(II) of \( 1.2 \times 10^{-11} \text{s} \) (24) and an average \( g \) value for Co(II) alkaline phosphatase of 3, based on the ESR.
signal at helium temperature of the enzyme containing two, Co(II) ions per dimer. As for Mn(II) enzyme, the hyperfine term dominates $T_{1\text{H}}$.

**DISCUSSION**

$^{31}$P NMR is a particularly suitable method for detecting the number and stoichiometry of the species of phosphate complexes present in alkaline phosphatase because all the chemically distinct species of bound phosphate are detected simultaneously in an enzyme sample unperturbed by the method of detection. At pH 8, phosphate-free alkaline phosphatase can bind only one noncovalently linked inorganic phosphate dianion in an unusual chemical environment (Fig. 1, A and B).

Thus, phosphate above 1 eq per enzyme dimer is either not bound to the enzyme or is bound at a site of different chemical environment so that it does not alter the chemical shift from that expected for inorganic phosphate at the same pH. This finding suggests that negative allosteric interactions exist between initially identical phosphate binding sites, one on each of the two identical subunits of the enzyme. This has previously been suggested by extensive spectral data (6, 8, 10).

The electron density map of the dimer at 3 A resolution shows that at this resolution apparent 2-fold symmetry exists between the monomers of the dimer of alkaline phosphatase.

When one phosphate ion is bound to the dimer, a second phosphate binding site of similar chemical environment is not available even at millimolar concentrations of phosphate (Fig. 1). This conclusion is strikingly confirmed by the use of $^{31}$P NMR to follow phosphate binding to the Mn(II) or Co(II) enzymes (Fig. 1). Only the first of two phosphate binds to the dimer in a position for its resonance to be broadened by the paramagnetic effect of Cu(II) or Mn(II) (Fig. 1). The second phosphate is unaffected by the paramagnetic ion, and thus clearly cannot be near the second Me(II) ion, implying absolute negative cooperativity between the active sites at neutral pH even in the presence of millimolar phosphate. We have determined the stoichiometry of phosphate binding by these NMR methods on several samples of each of the metallophosphatases discussed in this paper, and in each case only one chemically distinct phosphate binding site per dimer is present. The observed negative cooperativity may be a reflection of an underlying molecular mechanism aiding the dissociation of the product complex, $E\cdot P$, since binding of ROP to the second site may reduce the affinity of $P$, for the alternate site.

The phosphoryl enzyme formed by both the Zn(II) and Cd(II) enzymes is a most unusual phosphate monooester. The $^{31}$P resonance of this species is shifted 5 to 6 ppm downfield from the $^{31}$P resonances observed for model phosphate monoesters (Figs. 1, 2, and 5 and Table II). Assignment of this resonance to the enzyme phosphoserine is confirmed by the interchange between this species and the noncovalent enzyme-phosphate complex as a function of pH, which follows the known pH stability of $E\cdot P$ (Fig. 5) (4, 6).

Formation of the phosphorylated enzyme giving rise to the downfield resonance in stoichiometric quantities by Cd(II) further confirms this species as $E\cdot P$ (Fig. 2). While the metal ion contributes to the downfield shift of the phosphorus resonance of $E\cdot P$, the $^{31}$P resonance of the phosphate group on the apoenzyme remains 3 to 6 ppm (depending on pH) downfield from that observed for free phosphoserine (Figs. 2 and 4). Thus, the protein structure itself contributes significantly to the unusual chemical characteristics of this phosphoserine residue. The structural basis for the failure to protonate the phosphoryl residue on the apoenzyme as long as the enzyme retains its native conformation (Fig. 1), is not obvious in terms of known protein structure. Speculative suggestions might include hydrogen bonding to an adjacent hydroxyl group, in a structure similar to that occurring between the carboxyl and phenolic hydroxyl of salicylic acid, which lowers the $pK_a$ of the carboxyl (25). A distribution of positive charges around the active site seryl residue might also prevent protonation.

It has recently been proposed that an empirical correlation can be made between the chemical shift of the $^{31}$P resonance and the magnitude of the smallest O—P—O bond angle in phosphate monoesters, acyclic diesters, and five- and six-membered cyclic diesters (20). The $^{31}$P resonance shifts progressively downfield as this angle decreases from 106°. Chemical shifts observed on association of metal ions and hydrogen bonding donors with the phosphate group are accompanied by a shift in the phosphoryl $pK_a$, and fall within the range predicted by this correlation for changes in the ionization state of the phosphoryl group (20). While a divalent metal ion is in close proximity to phosphate bound to alkaline phosphatase (see below), comparison of the chemical shifts observed for the Cd(II), Zn(II), and apophosphoryl enzymes (Figs. 1 and 2) suggests that other factors must influence the chemical nature of the phosphoryl group. The magnitude of the observed chemical shifts suggests that the surrounding protein structure induces a significant compression of the O—P—O bond angle. Thus, the Zn(II) phosphoryl enzyme, with a $^{31}$P chemical shift changing that observed for five-membered cyclic diesters having an O—P—O bond angle less than 100°, appears to be a highly strained phosphate ester. The Cd(II) phosphoryl enzyme is slightly less strained, and the strain is substantially relieved in the apophosphoryl enzyme (Fig. 2). The apparent distortion of the phosphoryl group in the Zn(II) enzyme may provide the driving force for its rapid turnover (see below). Reduction of strain in the Cd(II) phosphoryl enzyme relative to the Zn(II) phosphoryl enzyme might be related to the slow turnover of the Cd(II) enzyme.

The $^{31}$P chemical shift of $E\cdot P$ is $\sim$2 ppm downfield from that observed for free $P$ (Fig. 1). This cannot be attributed simply to a shift in the $pK_a$ of $E\cdot P$, since the chemical shift difference is relatively constant from pH 4.6 to pH 8.0 (Table II). Rather, it would appear that the forces operative in inducing strain in $E\cdot P$ are also functional, albeit to a limited degree, in perturbing the structure of $E\cdot P$ in a similar manner.

**Enzyme-Phosphate Interactions as Determined by $^{31}$P NMR**—The magnitude of the $r_e$ for the apophosphoryl enzyme (Table I) suggests that the mobility of the enzyme-bound phosphoryl group is not governed by the rotational correlation time of the macromolecule $(r_e$ for a molecule of average radius 33 A, calculated from the Stokes-Einstein equation, is $3 \times 10^{-8}$ s). Thus, the data in Table I reflect the dynamic properties of a relatively mobile phosphoryl group at the active center, and can be interpreted with reference to the model compound phosphoserine.

The molecular fragment $^3\text{H}-\text{C}-\text{O}-^4\text{P}$ has been demonstrated to show an angular dependence for $^3\text{P}$.H spin-spin coupling similar to that given by the Karplus equation for vicinal $^3\text{H}-^3\text{H}$ coupling (26–28). Thus, direct assessment of the fractional population of the minimum energy rotomers (I, II, and III) can be made on the basis of the reported values of $J_{\text{HH}}$.

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for the gauche ($\phi(31P\ 'H) = -60^\circ$) and transe ($\phi(31P\ 'H) = 180^\circ$) couplings, 3 ± 2 Hz and 25 ± 3 Hz, respectively (27). For phosphoserine, $\nu_{P} = 6.1 Hz$, indicating that the model compound exists predominantly (~70%) as the gauche-gauche rotomer II, in accord with the conformational preferences predicted for linear hydrocarbons and observed for other phosphate esters (28–30). In contrast, the value of $\nu_{P}$ for the apophosphoryl enzyme, 13 Hz, indicates that II is only a minor contributor (~8%) to the rotomer population, and that either one or both of the trans-gauche rotomers, I and III, becomes dominant. This restriction to a less favorable conformer implies that interactions between the phosphoryl group and the surrounding enzyme structure limit the rotational freedom of the phosphoseril residue. This is also reflected in the magnitude of $T_{1P}$, (0.024 s) compared to that of the model compound (0.53 s), suggesting that a single rotomer (I or III) is preferentially populated.

The further reduction in $T_{1P}$ for the Cd(II) phosphoryl enzyme (0.016 s) implies that additional constraints are placed on the mobility of the phosphoryl group when the metal ion is present in the enzyme. This result, along with the downfield chemical shift induced by the presence of Cd(II), can be explained if direct coordination between the metal ion and the phosphoserine residue exists, limiting the mobility and inducing increased angular strain.

The presence of the metal ion near the $31P$ nucleus also provides the basis for a rationalization of the magnitude of $\nu_{P}$ for the Cd(II) phosphoryl enzyme. The apparent decrease in $\nu_{P}$ to 7 Hz from the value of 13 Hz for the less immobilized apophosphoryl enzyme is as predicted for electron withdrawal from phosphorus and implies that the normal angular dependence of $\nu_{P}$ has been altered by metal coordination. All these factors are consistent with the presence of the phosphoryl group within the first coordination sphere of the metal ion.

Unfortunately, we have not found conditions where the usual methods required for the measurement of metal-nuclear distances by NMR can be applied to the phosphate complexes. The resonances of the phosphate bound at the active sites of the paramagnetic metalloalkaline phosphatases are all broadened beyond detection, and the bound phosphate is in slow exchange with free phosphate (Fig. 1). A phosphate resonance is not detectable until free phosphate is present, and the resonance of free phosphate is not broadened until Me(II) above 2 $\mu$g at/mol of enzyme dimer is present (Fig. 7). This prevents the measurement of $T_{1M}$ for the phosphoryl group. The Mn(II)-$31P$ distance of 7.7 Å calculated for the phosphate complex of the Mn(II) enzyme as compared to 3.3 Å for this distance in LnMnPO$_4$ (23) suggests the possibility that phosphorus is bound in the outer coordination sphere of the metal ion, perhaps by a slowly exchanging water molecule in between. However, the Mn(II)-$31P$ distance for phosphate is for a ligand bound over 10$^4$ times less tightly than phosphate, and we are reluctant to assume that this is directly applicable to the phosphate complex, especially since phosphate binding appears likely to induce conformational changes in the enzyme. While a phosphate oxygen may be bound no nearer than the second coordination sphere, the linewidth for phosphate-bound to the Co(II) enzyme (at least 300 Hz) is considerably greater (and may be much greater) than the linewidth for phosphate-bound to the Co(II) enzyme (170 to 190 Hz), suggesting that $r$ may be much smaller for bound phosphate.

Although present data do not rule out an inner sphere coordination of phosphate to the metal ion, Zukin and Hollis (32) have made a number of estimates of metal-water and metal-phosphate distances based on the relaxation of the protons of solvent water by alkaline phosphatases containing four Mn(II) and four Co(II) ions, and suggest an outer sphere coordination for the phosphate anion. These data, however, are not sufficient to exclude inner sphere coordination by slowly exchanging phosphate. Details of these distance calculations are covered in Ref. 32.

The above findings make possible the following suggestions concerning the mechanism of action of alkaline phosphatase. ROP binds to the active site with at least one of the negatively charged oxygen atoms within the first coordination sphere of the Zn(II) ion (IV), or alternatively with a water molecule between the phosphate and the Zn(II). The Zn(II)-O= P or Zn(II)-O-H-O-P bonding decreases the electron density on the phosphorus, which potentiates a nucleophilic attack of the seryl hydroxyl in an apical position (IV). A positively charged cage around the phosphate binding site could also aid binding and reduce the negative charge on phosphorus. Previous evidence strongly suggests that the phosphorylation and subsequent dephosphorylation of Ser 90 proceed via nucleophilic displacement mechanisms (3).

$E\cdot P$ is slightly strained (Fig. 1), which suggests that $E\cdot$ROP (IV) may also be strained on the way to the formation of the highly strained phosphoserine intermediate (V) (Figs. 1 and 2). The strain would greatly facilitate the departure of RO. Such strain is believed to be responsible for the observation that hydrolysis of the methyl ester external to the ring occurs 10$^4$ times faster in methyl ethylene phosphate than it does in acyclic methyl esters of phosphate (33). Likewise, the resultant phosphoserine intermediate is highly strained, a strain induced by the surrounding protein structure and increased by inner or outer sphere coordination to the metal ion (Fig. 2). Thus, contrary to many assumptions, the enzyme phosphoserine intermediate must be considerably less stable than free phosphoserine, and should readily undergo hydrolysis, as water attacks in the apical position opposite to the seryl residue (V), to produce $E\cdot$P (VI). There must be additional pH-dependent changes in protein structure, perhaps the ionization of an adjacent group in the active center, which make the phosphoserine of the Zn(II) enzyme even more unstable at alkaline pH, accounting for its very rapid dephosphorylation.

While the $31P$ NMR spectra give detailed information on the phosphorus environment at the active center of alkaline phosphatase, the energetics of the overall system require
comment. The mechanism represented by IV, V, and VI could be driven through the high energy intermediate (V) by a slightly greater stability of VI relative to IV. However, at low pH, V is present in higher equilibrium concentrations than VI (Fig. 5) even though the $^{31}$P NMR suggests more strain around phosphorus in V. While the strain around phosphorus may not reflect the overall stability of the intermediate, the precise reasons for the relative stability of $E\cdot P$ and $E\cdot P$ as a function of pH remain a puzzling feature of the mechanism.

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