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Manganese(II) as a Probe of the Active Center of Alkaline Phosphatase

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Alkaline phosphatase (AP) contains three metal ion binding sites at each active center, sites A, B, and C, forming a triangle of sides 3.9, 4.9, and 7.0 Å. When two Mn(II) ions occupy the two A sites on the AP dimer, ESR spectra at 9.1 and 35 GHz show the A sites to have both axial and rhombic distortions from cubic symmetry (D = 0.083 cm^{-1}, E = 0.013 cm^{-1}). Phosphate binding to the A site causes small but detectable changes in symmetry around the Mn(II) (D = 0.070 cm^{-1}, E = 0.017 cm^{-1}). Mn(II) in the B sites shows an ESR spectrum suggesting a regular geometry. Water ^{1}H T_1^{-1} values of solutions of apoalkaline phosphatase titrated with 1-4 Mn(II) ions/dimer show that the Mn(II) ions in the A sites carry exchanging water molecules (probably 2), while water molecules bound to the B site, if present, are in slow exchange, since the B-site Mn(II) makes little contribution to the relaxivity of the enzyme. NMRD profiles from 0.01 to 50 MHz of Mn_2E_2AP (E for "empty"), Mn_2Mg_2AP, and Mn_2Mn_2AP show the coordinated water in the A sites to be in relatively slow exchange; i.e., $\tau_M \cong 10^{-6}$ s. Phosphate binding reduces the relaxivity of all forms of the Mn(II) enzyme by approximately 50%. The phosphate ligand appears to displace one of two H_2O molecules originally present at the A site. Thus, the A-site Mn(II) appears to be 5-coordinate with three His and two H_2O ligands. While turnover of the Mn(II) enzymes is 0.3% of that of the Zn(II) enzyme, all the phosphoenzyme intermediates form and the water structure of the Mn(II) enzyme is probably similar to that of the Zn(II) enzyme.

Introduction

Alkaline phosphatase (AP)[2] from *Escherichia coli* is a dimeric zinc metalloenzyme (MW = 94000) composed of identical subunits that catalyzes the hydrolysis of phosphate monoesters via the formation of a phosphoseryl intermediate formed with Ser 102, denoted as E-P.[2-4] The enzyme containing six Cd(II) ions in the phosphorylated dimer shows three ^{113}Cd signals at 153, 72, and 3 ppm.[3-5] The crystal structures of the Zn(II) and Cd(II) enzymes show these sites, termed A, B, and C, to form a triangle 3.9 × 4.9 × 7.0 Å at each active center[6-8] (Figure 1). Site A appears to be a distorted-tetrahedral or 5-coordinate site similar to those found in a number of zinc metalloenzymes and consists of three His nitrogens from the protein and probably two water molecules to complete a 5-coordinate complex.[9] ^{35}Cl NMR spectroscopy used to detect the binding of Cl^- and HPO_4^{2-} to Zn(II) in the A site suggests that two open coordination sites are initially occupied by solvent water,[10] a finding compatible with the crystal structure, which shows a large open segment of the coordination sphere opposite the His ligands contributed by His 331, His 372, and His 412 (Figure 1). The arrangement of ligands shown in Figure 1 is taken from 3-Å data refined to 2.8 Å.[4,8] A more recent electron density map at 2.0-Å resolution suggests that, in the native Zn(II) enzyme, the carboxyl group of Asp 327 may be the third protein ligand at the A site (both oxygens coordinated), while the NH of His 372 forms a hydrogen bond to one of the carboxyl oxygens.[31] This alternative ligand arrangement does not alter the arguments presented in this paper.

Site B has ligands consisting of a nitrogen from His 370, the carboxyl of Asp 369, and the carboxyl of Asp 51. The latter carboxyl group appears to form a bridge between metal ions B and C.[7] Additional ligands at the C site are the carboxyl groups of Asp 153 and Glu 322 and the hydroxyl group of Thr 155. Bound water molecules may complete the coordination spheres of sites B and C, but their presence is not obvious at the current resolution of the crystal structure.

The first transition period metal ions have high affinity for sites A and B only, while site C appears to be normally filled by Mg(II).[3-8] Magnesium will also bind to site B in the absence of other metal ions but appears to have little affinity for site A.[11] Of the various metalloalkaline phosphatases prepared, only the Zn(II) and Co(II) enzymes catalyze the rapid hydrolysis of phosphate monoesters, with turnover numbers from 10 to 100 s^{-1} depending on conditions.[11,12] Although not detectable by the usual assay techniques, the Cd(II) enzyme has been shown by ^{31}P NMR methods to have a turnover number of approximately 0.1 s^{-1}.[3,11] The other two apparently inactive phosphatases, those formed with Cu(II) and Mn(II), are paramagnetic and hence not subject to the ^{31}P NMR methods of determining slow turnover by detecting phosphoenzyme intermediates.[3] As shown in a previous paper,[13] the Cu(II) enzyme binds phosphate in the usual metal-dependent manner but fails to phosphorylate the Ser 102.[12] On the other hand, the Mn(II) enzyme has previously been shown to both bind phosphate in a metal-dependent manner and phosphorylate Ser 102.[12,14] The present paper reports results using ESR and nuclear magnetic relaxation dispersion (NMRD) to further explore the interactions between the A- and B-site metal ions, bound water molecules, and the phosphate dianion at the active centers of Mn(II) alkaline phosphatase.

Materials and Methods

Enzymes and Chemicals. Alkaline phosphatase was prepared from *E. coli* (strain CW 3747) transduced with plasmid pH1 carrying the pho A gene, the latter kindly supplied by Jonathan Beckwith. The isolation

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(1) (a) Yale University School of Medicine. (b) University of Florence. (c) IBM T. J. Watson Laboratories.
(2) Abbreviations: AP = alkaline phosphatase, with the metal-site occupancy indicated by Mn_2Mg_2AP or Mn_2Mn_2AP, where the first metal ion symbol and subscript refer to the type and number of metal ions in the A sites, while the second metal ion symbol and subscript refer to the type and number of metal ions in the B sites. The E in the formula Mn_2E_2AP stands for "empty" and indicates that no metal ion is in the B site. E-P = the covalent phosphoenzyme formed by the phosphorylation of Ser 102; E·P = the phosphoenzyme formed by the coordination of inorganic phosphate to the A-site metal ion, often referred to as the noncovalent or Michaelis complex with phosphate.
(3) Coleman, J. E.; Gettins, P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1983**, *55*, 381.
(4) Coleman, J. E. In *Phosphate Metabolism and Cellular Regulation in Microorganisms*; Toriani-Gorini, A., Rothman, F. G., Silver, S., Wright, A., Yagil, E., Eds.; Am. Soc. Microbiology: Washington, DC, 1987; p 127.
(5) Gettins, P.; Coleman, J. E. *J. Biol. Chem.* **1983**, *258*, 396.
(6) Wyckoff, H. W.; Handschumacher, M. D.; Murthy, K. H. M.; Sowadski, J. M. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1983**, *55*, 453.
(7) Sowadski, J. M.; Handschumacher, M. D.; Murthy, K. H. M.; Foster, B. A.; Wyckoff, H. W. *J. Mol. Biol.* **1985**, *186*, 417.
(8) Wyckoff, H. W. In *Phosphate Metabolism and Cellular Regulation in Microorganisms*; Toriani-Gorini, A., Rothman, F. G., Silver, S., Wright, A., Yagil, E., Eds.; Am. Soc. Microbiology: Washington, DC, 1987; p 120.
(9) Bertini, I., Luchinat, C., Maret, W., Zeppezauer, M. Eds. *Zinc Enzymes*; Birkhauser: Boston, MA, 1986.
(10) Gettins, P.; Coleman, J. E. *J. Biol. Chem.* **1984**, *259*, 11036.
(11) Gettins, P.; Metzler, M.; Coleman, J. E. *J. Biol. Chem.* **1985**, *260*, 2875.
(12) Applebury, M. L.; Johnson, B. P.; Coleman, J. E. *J. Biol. Chem.* **1970**, *245*, 4968.
(13) Bertini, I.; Luchinat, C.; Viezzoli, M. S.; Banci, L.; Koenig, S. H.; Leung, H.; Coleman, J. E. *Inorg. Chem.* **1989**, *28*, 352.
(14) Weiner, R. E.; Chlebowski, J. F.; Haffner, P. H.; Coleman, J. E. *J. Biol. Chem.* **1979**, *254*, 9739.

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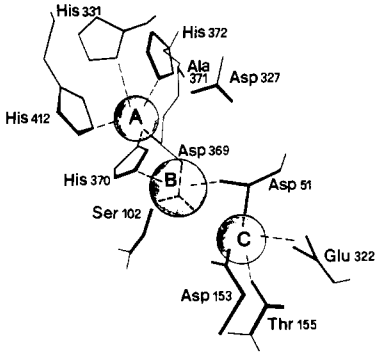

Figure 1. Diagram of the active center of alkaline phosphatase as traced from the computer graphics representation of the crystal structure of the Cd_6AP.[8]

of the enzyme followed the osmotic shock procedures previously reported.[12] Enzyme concentrations were measured spectrophotometrically at 278 nm by assuming an $\epsilon = 6.78 \times 10^4$ M^{-1} cm^{-1}, based on a MW = 94 000 calculated from the amino acid sequence.[15] The apoalkaline phosphatase was prepared by dialysis against 2 M ammonium sulfate at pH 9 as described previously.[16] Enzyme concentrations ranged from 0.5 to 2 mM. All buffer solutions, HCl, and NaOH were prepared metal-free as previously described.[12,17] Metal concentrations were determined by flame atomic absorption on an Instrumentation Laboratories Model IL157 spectrometer.

ESR spectra at 150 K and 9.15 GHz (X-band) were taken on a Varian E-4 spectrometer using a 100-kHz modulation frequency and a 10-G modulation amplitude. Temperature was controlled to within ±0.5 K with a Varian E-257 temperature controller. ESR spectra at 34.95 GHz (Q-band) were taken at the National ESR Center in Milwaukee, WI. The temperature was 123 K with a modulation amplitude of 5 G and a modulation frequency of 100 kHz. Microwave power was 12 db. Particular care was taken to avoid any extra Mn(II) exceeding the desired stoichiometry, since the Q-band spectra are particularly sensitive to even slight heterogeneity in the Mn(II) of the sample.

NMRD measurements were performed at 298 K by using a field-cycling relaxometer described elsewhere,[18,19] in the proton Larmor frequency range 0.01–50 MHz. Sample volumes were 0.5–0.8 ml, and Mn(II) was added stepwise as 1–10 μL of a stock unbuffered solution of $MnSO_4$. The enhanced relaxation of the water protons observed for solutions of Mn(II) alkaline phosphatase is expressed as relaxivity in units of mM^{-1} s^{-1} defined as follows. The experimentally observed value for the relaxation rate of the water protons induced by a given sample was corrected for the small contribution made by the protein macromolecule by subtracting the relaxation enhancement of the apoprotein at the particular field and concentration of protein used. A correction of 0.33 s^{-1} was subtracted for the water relaxation and the resulting paramagnetic contribution normalized to 1 mM concentration of enzyme dimer. The NMRD of the apoalkaline phosphatase needed for correction has been reported in detail.[13]

Results

The Mn(II) ion has five 3d electrons resulting in a ground electronic state ^6A. This orbital singlet of spin $S = {}^5/_2$ will be split by spin–orbit coupling with excited states (zero-field splitting, ZFS). The energy levels of the ground multiplet can then be described by a spin Hamiltonian as follows:[20]

$$H_S = \beta H\cdot\tilde{\mathbf{g}}\cdot S + D(S_z^2 - {}^1/_3 S(S+1)) + E(S_x^2 - S_y^2) + a/6(S_\xi^4 + S_\eta^4 + S_\zeta^4 - {}^{707}/_{16}) + S\cdot\tilde{\mathbf{A}}\cdot I \quad (1)$$

where D and E are the usual ZFS terms of axial and rhombic symmetry and a is a term of cubic symmetry. The ξ, η, and ζ are the principal axes of the cubic distortion from spherical symmetry. To minimize the number of parameters, we assume

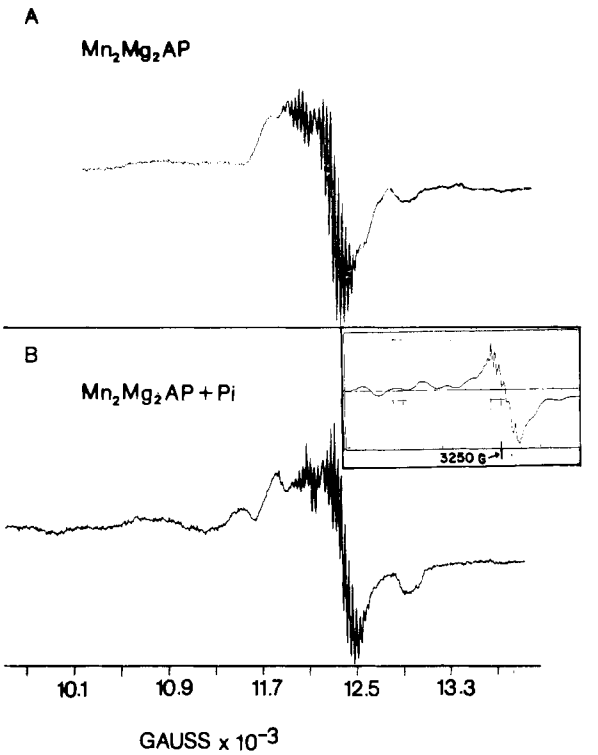

Figure 2. ESR (35 GHz) spectra of Mn_2Mg_2AP (1.5 mM) (A) and Mn_2Mg_2AP plus 2 P_i (B). Conditions: 0.01 M Tris-HCl, pH 8, −150 °C. ESR conditions are given in Methods and Materials. Inset: 9.15-GHz ESR spectrum of the sample in part B.

Table I. Hamiltonian Parameters Used for the Computer Simulation of the 35-GHz ESR Spectra of Mn(II) Alkaline Phosphatase[a]

enzyme	D, cm^{-1}	E, cm^{-1}	E/D = λ	a,[b] cm^{-1}	A, G
Mn_2Mg_2AP	0.083 ± 0.03	0.013 ± 0.005	0.16	−0.0187	44
Mn_2Mg_2AP + 2 P_i[c]	0.070 ± 0.02	0.017 ± 0.005	0.24	−0.0050	44

[a] For details of the computer program see the Appendix. [b] Of the solid-state d^5 systems studied, nearly all have positive a values. In the present case, if a were positive, D would have to be negative, which is also possible. At present, we have no way of determining which choice is correct. [c] The values for the P_i complex may represent two slightly different but closely overlapping spectra from the two A-site Mn(II) ions (see text).

that they coincide with the x, y, and z axes of the second-order terms. The cubic term is commonly neglected as being small with respect to the axial and rhombic ZFS. We have included it because we have found a small cubic term greatly improves the ability of our computer program to simulate the Q-band spectra. The final term represents the hyperfine coupling of the nuclear spin I to the electron spin S. For Mn, $I = {}^5/_2$.

Q-Band ESR Spectra of Mn(II) Alkaline Phosphatase. The unique X-band ESR spectra of Mn(II) occupying the A sites of alkaline phosphatase have been previously reported.[14] While the low-field transitions at X-band frequency are a sensitive indicator of small changes in symmetry around the metal ion occuring on phosphate binding, the Q-band ESR spectra of Mn_2Mg_2AP show that the Mn(II) ions of the A site occupy very similar environments in the presence or absence of phosphate (Figure 2). The computer simulations of the two spectra in Figure 2 show that there are only small changes in the D and E values when phosphate binds (Table I). The X-band spectrum of the sample used for the Q-band spectrum of Mn_2Mg_2AP plus 2 P_i is shown in the insert to Figure 2. This X-band spectrum with many low-field transitions is the typical spectrum that appears on titration of apoalkaline phosphatase with 0.2-2 Mn(II) ions and 1 phosphate ion per dimer.[14] As documented previously, the features of this spectrum remain constant as the amplitude of this spectrum increases in direct proportion to the added Mn(II) from 0.3 to 2 equiv of Mn(II)/mol of dimer.[14] Two Zn(II) ions occupying the A sites of the enzyme dimer prevent the appearance of this ESR spectrum. Hence, the

(15) Bradshaw, R. A.; Cancedda, F.; Ericsson, L. H.; Newman, P. A.; Piccoli, S. P.; Schlesinger, M. J.; Sriefer, K.; Walsh, K. A., *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3473.
(16) Gettins, P.; Coleman, J. E. *J. Biol. Chem.* **1983**, *258*, 408.
(17) Thiers, R. E. *Methods Biochem. Anal.* **1957**, *5*, 273.
(18) Koenig, S. H. In *Water in Polymers*; Rowland, S. P., Ed.; ACS Symposium Series 127; American Chemical Society: Washington, DC, 1980; p 157.
(19) Hallenga, K.; Koenig, S. H. *Biochemistry* **1976**, *15*, 4255.
(20) Abragam, A.; Bleaney, B. *Electron Paramagnetic Resonance of Transition Ions*; Clarendon Press: Oxford, England, 1970; pp 436–442.

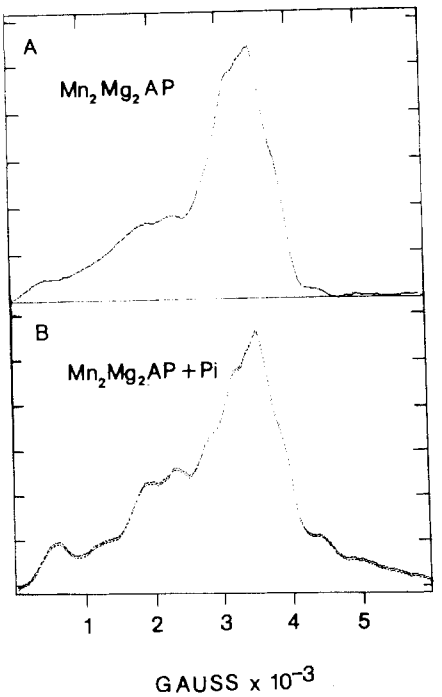

Figure 3. Absorption ESR spectra calculated from hand-digitized X-band spectra of Mn_2Mg_2AP (A) and Mn_2Mg_2AP plus P_i (B). Conditions are as in Figure 2.

spectra in Figure 2 must represent Mn(II) in the two A sites.[14] There is no evidence of spin–spin interaction in the X-band spectrum, and a large number of hyperfine lines (20+) in the 35-GHz spectra are expected from the six overlapping sets of spectra used in the computer fit (see Appendix). The $g = 2$ region cannot be fit precisely by the computer program because forbidden transitions also contribute to the fine structure in the $g = 2$ region.

Phosphate binding to Mn_2E_2AP has been shown to be negatively cooperative; i.e., in the presence of 2 equiv of inorganic phosphate, only 1 mol binds to the enzyme, as can be clearly demonstrated with ^{31}P NMR spectroscopy.[14] The phosphate ligand cannot induce a major change in the coordination complex around the A site in one monomer, since, despite the fact that the spectrum in Figure 2B represents the overlap of the spectra from both Mn(II) ions, large displacements of the two spectra have not occurred. The major changes are confined to the low-field transitions. We have hand-digitized the 9.15-GHz ESR spectra of Mn_2Mg_2AP and its phosphate complex formed by the addition of 2 equiv of inorganic phosphate ions. The resulting integrals of these spectra are plotted in Figure 3. These integrals show that other than minor changes in the intensities and energies of several of the ESR transitions, no major change occurs in the total spectral density on formation of the phosphate complex.

X-Band ESR Spectra of the Phosphate Complexes of Mn_2Mg_2AP and Mn_2Mn_2AP. We have determined the ESR spectra of numerous preparations of alkaline phosphatase containing two Mn(II) ions and four Mn(II) ions either in the presence or absence of phosphate. In no case did we observe a failure to account for the spin concentrations expected from the total Mn(II) in the protein. Representative examples of the first-derivative ESR spectra and their computer-derived first integrals for the phosphate complexes of Mn_2Mg_2AP and Mn_2Mn_2AP are shown in parts A and B of Figure 4, respectively. The ESR absorption intensity of the enzyme with Mn(II) occupying the A sites alone, 4500 arbitrary units, is slightly less than half of that for the enzyme with Mn(II) occupying both the A and B sites, 9500 arbitrary units (Figure 4). The ESR spectrum of Mn(II) in the B sites is narrower, with more spectral density near $g = 2$, which accounts for the greater than 2× intensity of Mn_2Mn_2AP compared to Mn_2Mg_2AP at $g = 2$. While the precise characteristics of the ESR signal from the Mn(II) in the B sites cannot be completely determined from the overlapping signals of the four Mn(II) enzyme, the signal is different from that shown by Mn(II) non-

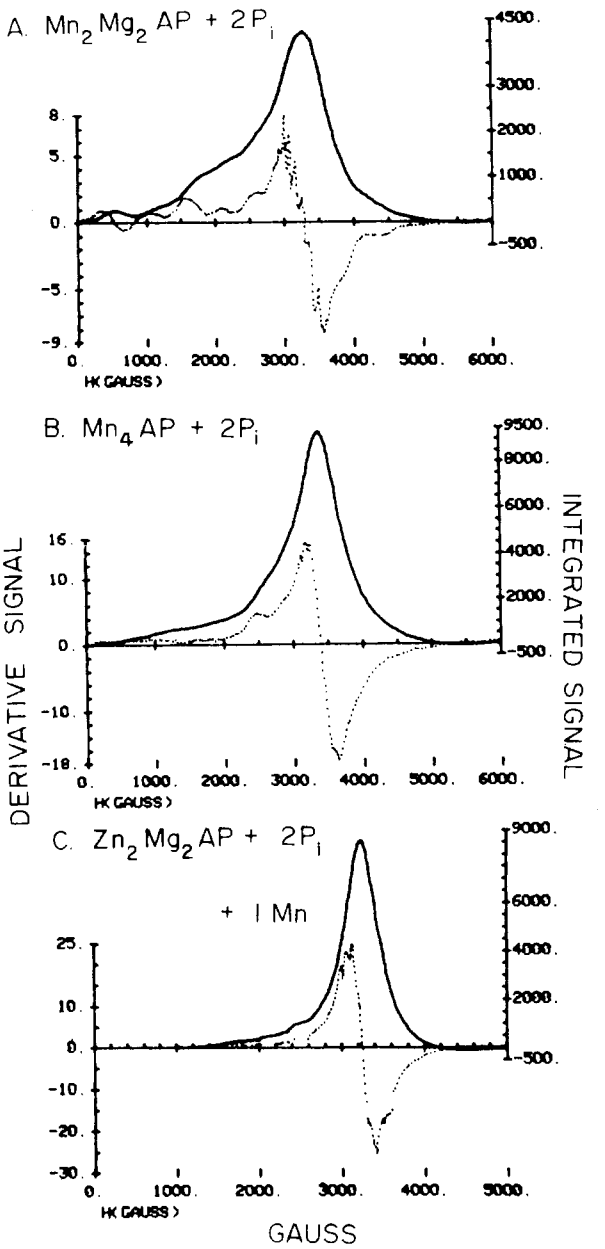

Figure 4. X-Band (9.15 GHz) ESR spectra displayed on the Prophet computer (first derivative spectra (···) and first integrals (—)): (A) Mn_2Mg_2AP plus 2 P_i; (B) Mn_2Mn_2AP plus 2 P_i; (C) Zn_2E_2AP plus 10 mM Mg(II) plus 2 P_i plus 1 Mn(II). Conditions are as in Figure 2.

specifically bound to the enzyme as shown in Figure 4C, obtained by adding Mn(II) to the Zn(II) enzyme containing 10 mM Mg(II), in which the A sites are occupied by Zn(II), and the B and C sites by Mg(II). We have often used the M_2Mg_2AP derivatives of various metal-substituted alkaline phosphatases to ensure the A-site occupancy by a IIB or transition-metal ion as well as to more completely reconstitute the active center by occupying the B site with a metal ion. Mg(II) provides a common denominator by which to compare the M_2E_2AP derivatives to a derivative with the same metal ion occupying the B site.

^1H Water NMRD in the Presence of Manganese Alkaline Phosphatase. The relaxivity of alkaline phosphatase at proton Larmor frequencies of 0.02, 0.5 and 20 MHz during the titration of the enzyme, pH 7.1, with 1–4 Mn(II) ions/dimer is plotted in Figure 5. The relaxivity rises dramatically on the addition of the first 2 equiv of Mn(II) to reach a value of 36 $(mM\ s)^{-1}$. Both Mn(II) ions contribute equally to the relaxivity. On the other hand, the second 2 equiv of Mn(II) added make little contribution to the relaxivity, which remains relatively constant, although Mn_2Mn_2AP appears to have a slightly higher relaxivity, 39–43 $(mMs)^{-1}$ (see below).

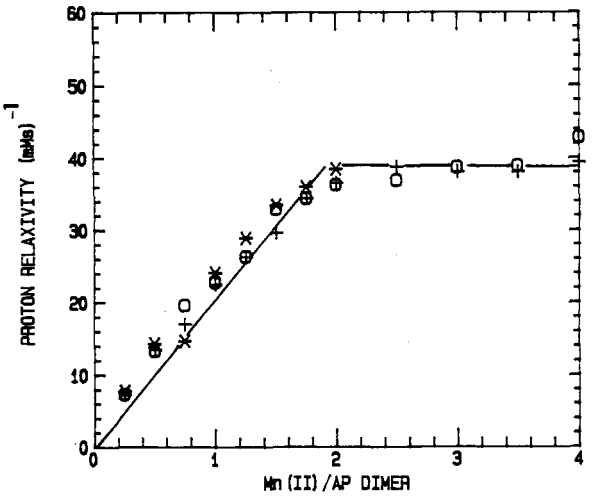

Figure 5. Relaxivity of apoalkaline phosphatase during titration with Mn(II) from 0 to 4 equiv/alkaline phosphatase dimer (pH 7.1, 25 °C). The paramagnetic contribution to relaxivity, expressed per 1 mM enzyme dimer (see Methods and Materials), is given at a field of 0.02 MHz (□), 0.5 MHz (+), and 20 MHz (✱).

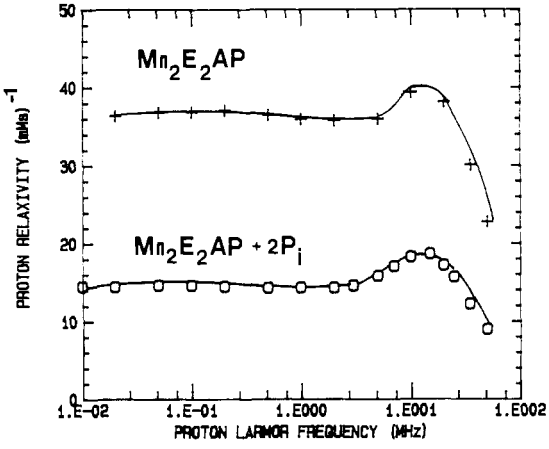

Figure 6. NMRD of bulk water protons from 0.01 to 50 MHz induced by Mn_2E_2AP (+) and Mn_2E_2AP plus 2 P_i (□) (pH 7.1, 25 °C). The relaxation data is expressed as relaxivity, the paramagnetic contribution to proton relaxation, expressed per 1 mM enzyme dimer (see Methods and Materials). The dispersion curves in this and the following figure can be fit by using the theory applied to NMRD in previous analyses[21,24] and assuming two H_2O molecules to be present at the A-site Mn(II) in the unliganded enzyme, while only one molecule of H_2O is present in the phosphate complex. The τ_M in all cases is $\sim 10^{-6}$ s.

NMRD of Mn_2E_2AP and Mn_2Mg_2AP. The NMRD profile from 0.01 to 50 MHz for the Mn(II) enzyme containing two Mn(II) bound to the A sites, Mn_2E_2AP, pH 7.1, is shown in Figure 6. The proton relaxation of Mn_2E_2AP is almost field-independent until 1 MHz when there is a small dip due to the ω_s dispersion, followed by an enhancement due to the rapid increase in the electron relaxation time, τ_s, at fields near 10 MHz.[21] The relaxivity falls rapidly above 20 MHz due to the ω_I dispersion. The NMRD profile from 0.01 to 50 MHz for Mn_2Mg_2AP is shown in Figure 7. The profile with Mg(II) in the B sites is very similar to that shown by Mn_2E_2AP, but the relaxivity at low field is somewhat higher, ~ 55 $(mMs)^{-1}$, a constant observation for the enzyme when the B sites as well as the A sites are occupied. Either Mg(II) or Mn(II) in B will cause this effect.

Effect of Phosphate Binding on the NMRD of Mn_2E_2AP, Mn_2Mg_2AP, and Mn_2Mn_2AP. Because of the sensitivity of the relaxivity of Mn(II) in proteins to the relative values of τ_s, τ_r, and τ_M, it is difficult to estimate the exact number of water molecules bound to Mn(II) in the A sites of alkaline phosphatase from the

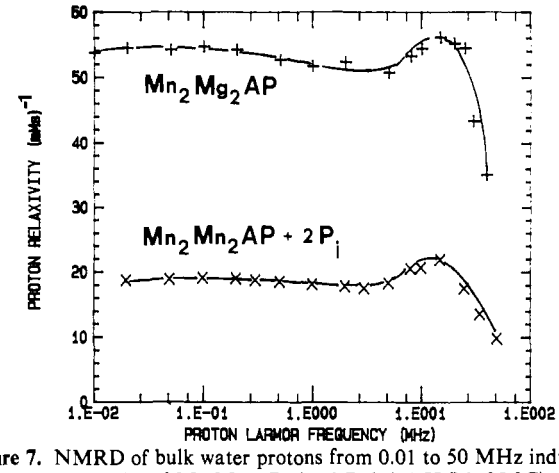

Figure 7. NMRD of bulk water protons from 0.01 to 50 MHz induced by Mn_2Mg_2AP (+) and Mn_2Mn_2AP plus 2 P_i (×) (pH 7.1, 25 °C). The relaxation data are expressed as relaxivity, the paramagnetic contribution to proton relaxation, expressed per 1 mM enzyme dimer (see Methods and Materials).

NMRD profiles of Figures 6 and 7. Previous ^{35}Cl NMR data on the Zn(II) enzyme,[10] as well as the crystal structure of the native Zn(II) protein,[6,7] suggest that the native protein contains two bound water molecules at Zn(II) in the A site, forming a 5-coordinate complex (see Figure 1). ^{31}P NMR data combined with ^{113}Cd NMR data demonstrate that the phosphate dianion in the E·P complex is coordinated to the A-site metal ion, Zn(II) or Cd(II).[3-5] From the ^{35}Cl NMR data, the phosphate dianion appears to displace one but not both monodentate ligands from solution.

Clearly, the Mn(II) enzyme contains one or more water molecules coordinated to the A-site Mn(II) in reasonably rapid exchange with bulk water (Figures 6 and 7). The change in relaxivity at three different frequencies, 0.02, 20, and 50 MHz, as a function of phosphate concentration is shown in Figure 8 for titrations of Mn_2E_2AP and Mn_2Mg_2AP with phosphate. Addition of one phosphate dianion to Mn_2E_2AP reduces the relaxivity from 36 to 14 $(mM\ s)^{-1}$. Addition of a second phosphate ion has no effect (Figure 8A). The addition of one phosphate dianion to Mn_2Mg_2AP reduces the relaxivity from 55 to ~ 39 $(mM\ s)^{-1}$, while addition of a second phosphate causes a further decrease in relaxivity to ~ 30 $(mM\ s)^{-1}$ (Figure 8B). In the case of Mn_2Mn_2AP, addition of a single phosphate dianion reduces the relaxivity from 37 to ~ 30 $(mM\ s)^{-1}$, while a second phosphate causes a further decline to ~ 20 $(mM\ s)^{-1}$. The titration of Mn_2Mn_2AP with phosphate is not included in Figure 8, but the final NMRD profile is plotted in Figure 7. The above relaxivity values refer specifically to those at 0.02 MHz, but proportional decreases in relaxivity on the addition of phosphate are observed at all fields (Figures 6 and 7). The maximum addition of phosphate to the three Mn(II) enzyme species reduces the relaxivity by $\sim 60\%$, $\sim 45\%$, and $\sim 45\%$ for the Mn_2E_2AP, the Mn_2Mg_2AP, and the Mn_2Mn_2AP species, respectively.

Effects of pH on Alkaline Phosphatase. Two alterations in the behavior of alkaline phosphatase as a function of pH need to be considered in these and other experiments. The first is the pH dependency of the E-P $\rightleftarrows$ E·P equilibrium. Generally, E-P is stable at low pH, while E·P is stable at high pH; however, there are large differences in the midpoint of this equilibrium depending on what species of metal ions are bound at the A and B sites. For example, the midpoint of the E-P $\rightleftarrows$ E·P equilibrium varies from pH 5.5 for Zn_2Zn_2AP to pH 10.0 for Cd_2Cd_2AP.[3,4,10]

The second pH-dependent function is the relative affinity of the A, B, and C sites for metal ions as they are added sequentially to the apodimer. At relatively low pH values, 6 to 7, all first transition and IIB metal ions occupy the three sites in pairs according to the following relative affinity, A > B > C. At alkaline pH the affinities of the several sites are often similar and heterogeneous occupancy is observed if less than 6 metal ions per dimer are present. For NMRD, where sequential metal site

(21) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Benjamin-Cummings: Boston, MA, 1986.

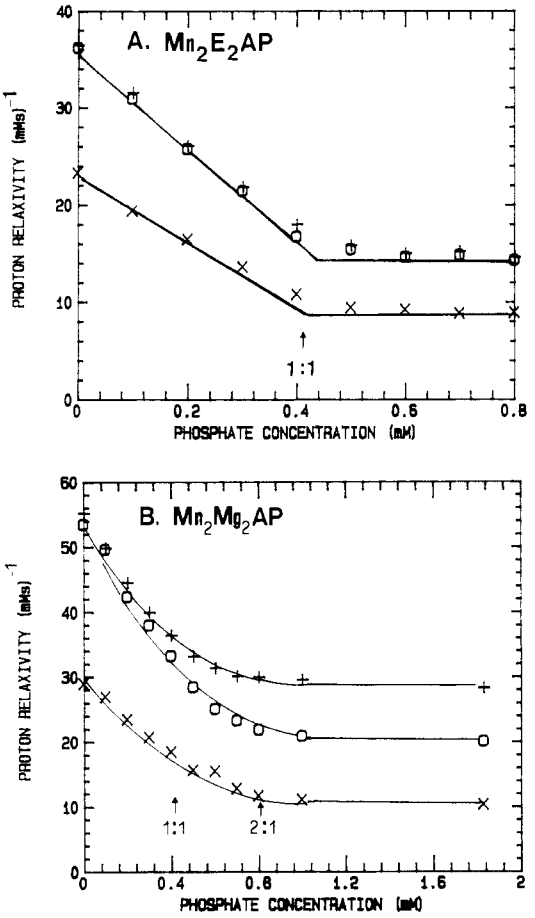

Figure 8. Effect of phosphate on the relaxivity of the Mn(II) derivative of alkaline phosphatase: (A) relaxivity at 0.02 MHz (+), 0.5 MHz (□), and 50 MHz (×) of Mn_2E_2AP as a function of phosphate concentration; (B) relaxivity at 0.02 MHz (+), 20 MHz (□), and 50 MHz (×) of Mn_2Mg_2AP as a function of phosphate concentration. Conditions are as in Figures 6 and 7.

occupancy is required, we have thus employed pH values below 7. When E·P complexes or spectra of metal sites under conditions of maximum turnover are required, alkaline pH values have been employed. Once the metal ions are bound in sites A, B, and C, there have been relatively few changes observed in the basic features of the optical, ESR, or NMR spectra as a function of pH except those reflecting the E-P ⇄ E·P equilibrium. The latter probably reflects the ionization of a coordinated water molecule at the A site.[3,4] We have not presented experiments at all pH values unless significant changes take place between pH 6 and 8.

Catalytic Activity of Mn(II) Alkaline Phosphatase. In a 1970 paper reporting the Mn(II)-dependent binding of $HO^{32}PO_3^{2-}$ to alkaline phosphatase as well as the phosphorylation of Ser 102 by the Mn(II) derivative, we reported a small but detectable activation of the apoenzyme by Mn(II) using *p*-nitrophenyl phosphate as a substrate.[12] This was in contrast to Ni(II), Cu(II), Cd(II), and Hg(II), which appeared inactive. The Cd(II) enzyme was later shown by NMR methods to have a turnover number of ~0.1 s^{-1} or about 0.1% the activity of the Zn(II) enzyme.[8,16] Since the Mn(II) complex at the active center of alkaline phosphatase can catalyze the phosphorylation of Ser 102, we have made a further careful examination of the ability of Mn(II) to activate the apoenzyme in the hydrolysis of *p*-nitrophenyl phosphate. We confirm that Mn(II) ions accelerate the rate of hydrolysis over that seen with the apoenzyme alone. By comparing this rate with that observed when Zn(II) is used to activate the apoenzyme under exactly the same conditions, we find the turnover number of the Mn(II) enzyme at pH 8 in the presence of 1 M Tris is approximately 0.2 s^{-1}, only slightly greater than that shown by the Cd(II) enzyme.

Discussion

Apoalkaline phosphatase binds 2 equiv of Mn(II) at a pair of sites of unusual symmetry (Figures 2–4). The rhombic distortion of this site in Mn_2Mg_2AP (Figures 2 and 4A; Table I) and the occupancy of two more symmetrical sites when a second pair of Mn(II) ions are added to the enzyme instead of Mg(II) (Figure 4B), indicate that the first pair of sites occupied by Mn(II) are the A sites. Mn(II) ions binding to the A sites carry exchangeable water molecules with them, as shown by the NMRD profiles (Figures 5 and 6). In the case of the Zn(II) and Cd(II) enzymes, ^{31}P NMR spectroscopy shows the phosphate to be directly coordinated to the A-site metal ion from which it is transferred to the oxygen of Ser 102.[8,16]

Conclusions Concerning Interactions of Mn(II) in the A Site with Mn(II) in the B Site and the Nature of Phosphate Binding Derived from the Electron Spin Resonance Spectroscopy. We have previously reported the changes that occur in the lowfield ESR transitions of Mn_2E_2AP or Mn_2Mg_2AP when the phosphate ligand binds.[14] The 35-GHz spectra reported here show that the phosphate-induced changes around the A-site metal ion must reflect relatively minor changes in the coordination complex (Figure 2), compatible with the replacement of a water ligand with the phosphate oxygen. The parameters used to fit the 35-GHz spectra using the spin Hamiltonian of eq 1 are given in Table I and show that E/D or λ changes from ~0.16 to ~0.25 for Mn(II) in the A sites when phosphate binds to Mn_2Mg_2AP. These λ values are slightly smaller than the maximal rhombic distortion, $\lambda = 0.33$.[22] The 35-GHz spectra and their computer simulation make possible a more satisfactory estimation of the D and E values for the unusual Mn(II) ESR spectra of alkaline phosphatase than possible previously with graphical fitting of the X-band spectra to published nomograms.[14,22] From the current fit with eq 1 including the cubic terms, D is 0.07–0.08 cm^{-1} and E is near 0.015 cm^{-1} for both the enzyme and its phosphate complex (Table I). The remaining simplifications in the computer program used to simulate the 35-GHz spectra are given in the Appendix.

When the second pair of Mn(II) ions are bound to the enzyme in the B sites, there is no evidence of spin–spin interaction between the Mn(II) ions in the A and B sites approximately 4 Å apart, since the integrated area under the absorption ESR spectrum doubles within the error of the measurement (Figure 4). Neither a change in the amplitude of the ESR signals nor additional splitting of the hyperfine structure is observed. Both the latter changes are observed for the spin-coupled Mn(II) pair in Con A (concanavalin A), where the two Mn(II) ions are 4.25 Å apart and share two carboxyl groups as bridging ligands.[23] One can conclude from this that there must not be a shared ligand between Mn_A and Mn_B. This conclusion is in agreement with the crystal structures of the Zn(II) and Cd(II) enzymes, but is in contrast to the case for the Cu(II) enzyme, in which the room-temperature ESR spectrum disappears for Cu_2Cu_2AP, and the J coupling between Cu_A and Cu_B can be estimated to be 120 cm^{-1} from magnetic susceptibility measurements, suggesting a bridging ligand between Cu_A and Cu_B.[13] In contrast to the case for the Mn(II) enzyme, phosphorylation of Ser 102 is not catalyzed by Cu_2Cu_2AP.

Analysis of the NMRD of Manganese Alkaline Phosphatase. The water protons in a solution of the manganese(II) aqua ion, $Mn(H_2O)_6$, show two relaxation dispersions, one from 0.01 to approximately 1 MHz and a second from 10 to 100 MHz.[21] The first of these can be fit by a τ of ~3×10^{-9} s and must be governed primarily by the electron relaxation time, τ_s, since the rotational correlation time, τ_r, is 10^{-11} s. This first dispersion arises from the contact term, which has a strong frequency dependence at low field. The contact term is dominant when $\tau_s \gg \tau_r$, since the dipolar contribution is reduced by the factor τ_s/τ_r, which in this case is ~10^2. The second relaxation dispersion shown by a solution of the Mn(II) aquaion can be fit by a τ of (1.5–3) × 10^{-11} s, which

(22) Dowsing, R. D.; Gibson, J. F. *J. Chem. Phys.* **1969**, *50*, 294.
(23) Antanaitis, B. C.; Brown, R. D.; Chasteen, N. D.; Freedman, J. H.; Koenig, S. H.; Lilienthal, H. R.; Peisach, J.; Brewer, C. F. *Biochemistry* **1987**, *26*, 7932.

can be identified with the rotational correlation time of the ion. At the higher fields the relaxation arises primarily from the dipolar contribution, since τ_c is dominated by τ_r and the relaxation is subject to the $\omega_s\tau_c = 1$ dispersion.

In contrast to the aqua ion, when Mn(II) is bound to a site on a large protein molecule, $\tau_r > 10^{-8}$ s, and τ_r is therefore much longer than τ_s, the dipolar interaction is the predominant contributor to the electron–nucleus relaxation. Under these conditions, the relaxivity is expected to be independent of field until it decreases above 1 MHz as the ω_s dispersion begins. The NMRD curves observed for a Mn(II) protein with rapidly exchanging water molecules in the first coordination sphere of the metal ion have been most completely analyzed for the Mn(II) complexes of Con A.[22,24] A field-independent relaxivity of $\sim$50 (mM s)$^{-1}$ is observed from 0.01 to $\sim$1 MHz with a small dip at the higher end due to the start of the ω_s dispersion. From 5 to 10 MHz, however, there is a very rapid rise in relaxivity from $\sim$50 to a maximum of $\sim$100 (mM s)$^{-1}$, followed by a rapid fall to very low relaxivity at 50 MHz.[24] The large enhancement of relaxivity can be explained by the fact that τ_s has a strong field dependence shown by the equation

$$\tau_s^{-1} = C(\tau_v/(1 + \omega_s^2\tau_v^2) + 4\tau_v/(1 + 4\omega_s^2\tau_v^2)) \quad (2)$$

When the dipolar contribution to relaxation is dominant, the rapid increase in τ_s above 1 MHz predicted by eq 2 is expected to lead to a rapid enhancement of relaxivity as observed in the case of Mn(II) Con A. This enhancement reaches a maximum, however, because as τ_s equals or exceeds τ_M, the residence time of the bound water becomes the predominant contributor to τ_c ($\tau_c^{-1} = \tau_r^{-1} + \tau_M^{-1} + \tau_s^{-1}$) and relaxivity will plateau. At fields higher than $\omega_I\tau_c = 1$, however, T_{1P}^{-1} becomes inversely proportional to ω^4, which results in the rapid fall in relaxivity observed at high fields.[21]

In order to observe the relaxation dispersion curves expected for a Mn(II) metalloprotein, as described above, the coordinated water molecules must be in fast exchange with those of the bulk solvent; i.e., τ_M must be 10^{-6}–10^{-7} s. Only Mn(II) in the A sites of alkaline phosphatase makes a contribution to the relaxation dispersion of the protein (Figure 5). As expected, the low-field relaxivity is essentially field independent except for the dip expected from the onset of the ω_s dispersion (Figures 6 and 7). On the other hand, the large enhancement due to the increase of τ_s with field, while present, is much smaller than expected. A quenching of this enhancement is expected if τ_M is relatively large, i.e., the coordinated water molecules are exchanging slower than the fast exchange limit, since $T_{1P}^{-1} = f_M/(T_{1M} + \tau_M)$, where f_M is the molar fraction of protons interacting with the paramagnetic center.

A NMRD profile similar to those observed for the various forms of Mn(II) alkaline phosphatase has been reported for Mn(II) Con A when Ca(II) is bound to a second pair of sites adjacent to the bound Mn(II) ions.[24] The binding of Ca(II) reduces the relaxivity of the Mn(II) protein and attenuates the τ_s-dependent rise in relaxivity observed at high field. The resulting NMRD profile is almost identical with those observed in Figures 6 and 7. The Mn(II)–Ca(II) Con A NMRD spectra have been interpreted best by the postulate that Ca(II) binding causes a substantial decrease in the exchange rate of the bound water at the Mn(II) sites, i.e. an increase in τ_M.[24] Mn_2E_2AP, Mn_2Mg_2AP, and Mn_2Mn_2AP all show essentially field-independent relaxivities from 0.01 to $\sim$1 MHz; hence, none of these species give rise to significant amounts of free Mn(II), in contrast to other Mn(II)-substituted Zn(II) metalloproteins, e.g. carboxypeptidase A.[21]

The Mn(II) bound to the B sites makes no contribution to the relaxation of bulk solvent (Figure 5). Thus, if there are water molecules in the coordination sphere of Mn_B, they must be in slow exchange. While the details of the coordination sphere at the B sites are not clear from the current resolution of the crystal structure,[6-8] both optical absorption spectra and magnetic susceptibility measurements of Co(II) in the B sites suggest that the B-site metal ions occupy an octahedral rather than a tetrahedral environment.[25] Since the crystal structure indicates a maximum of four protein ligands if both oxygens of Asp 369 are bound, there would appear to be bound water molecules completing the coordination sphere. The crystal structure shows the B site to be relatively occluded from solvent when both the A and B sites are occupied by metal ions.[7,8] In agreement with this finding is the observation in solution that despite the fact that Cd(II) binds to the A sites first, when ^{113}Cd(II) is bound to both the A and B sites, it is the ^{113}Cd(II) in the A site that is readily exchangeable with free ^{112}Cd(II) in solution, not the B-site ^{113}Cd(II), which is nonexchangeable.[3,26,27] Thus, it may be possible that coordinated solvent at the B site is in relatively slow exchange, compatible with the NMRD findings in Figures 5 and 6.

Phosphate Binding to Mn(II) Alkaline Phosphatase. NMRD. Binding of phosphate has dramatic effects on the relaxivity of all forms of Mn(II) alkaline phosphatase, reducing the relaxivity by approximately 50% in all cases. The simplest interpretation of these data is that the binding of each phosphate ligand displaces a coordinated water molecule from the A site. It must be the A sites, since only the A sites contribute to relaxivity (Figure 5). Extensive ^{31}P NMR data show that all species of alkaline phosphatase with both the A and B sites occupied by metal ions bind 2 phosphate dianions per dimer.[3,14,16] Thus, phosphate binding to Mn_2Mg_2AP or Mn_2Mn_2AP should displace two H_2O molecules from the A sites. Approximately half of the relaxivity remains in the phosphate complexes (Figures 6 and 7); hence, the above interpretation requires that the Mn(II) coordination complex at the A sites contain two solvent-occupied coordination positions; i.e., the original complex is 5-coordinate. An oxygen donor of the phosphate must occupy one of the original H_2O ligand sites.

In the case of the Mn(II) ion, however, a fall in relaxivity could also be due to a decrease in τ_M; i.e., phosphate binding could result in a change in the rate of exchange of the coordinated water. That radical changes do not take place in τ_M on phosphate binding to Mn(II) alkaline phosphatases is suggested by the fact that the overall NMRD profiles do not change, despite the uniform and proportional fall in relaxivity (Figures 6 and 7). In particular, the high-field τ_s-dependent rise is maintained in the phosphate complexes of both Mn_2E_2AP and Mn_2Mn_2AP. There is a larger than expected fall in the relaxivity when one phosphate binds to Mn_2E_2AP, from 36 to 14 (mM s)$^{-1}$ at 0.02 MHz (Figure 6). According to the model presented above for the M_2M_2AP species, binding of a single phosphate should displace only 25% of the bound water from the Mn_2E_2AP species. Therefore, the much larger than expected drop in relaxivity on phosphorylation of Mn_2E_2AP may represent a change in τ_M, possibly for the bound water at the nonphosphorylated site, which must undergo significant conformational change, perhaps even loss of water, since the phosphate can no longer bind. An alternative hypothesis that cannot be eliminated at present is the possibility that the final water structure at the A site is not established until a metal ion is present at the B site. This could involve either the number or the exchange rate of the A site water. This might explain the observation that all the four metal derivatives have somewhat higher relaxivities than that of Mn_2E_2AP.

Since the Mn(II) enzymes bind water as well as the phosphate dianion (Figures 2, 6, and 7) and catalyze the phosphorylation of Ser 102,[12] one might well ask why turnover at pH 8 is only 3% of that of the Zn(II) enzyme.[12] The answer may relate to the pK_a of the solvent molecule(s) coordinated to the A-site metal ion following transfer of the phosphate bound to the A-site metal ion to Ser 102. According to the mechanism of hydrolysis we have postulated for alkaline phosphatase,[4,11] the second phase of the mechanism, i.e. the dephosphorylation of Ser–OPO_3^{2-}, is catalyzed by a hydroxide (or activated water molecule) coordinated to the A-site metal ion. According to this model, the apparent pK_a

(24) Brown, R. D.; Brewer, C. F.; Koenig, S. H. *Biochemistry* **1977**, *16*, 3883.

(25) Banci, L.; Bertini, I.; Luchinat, C.; Viezzoli, M. S.; Wang, Y. *Inorg. Chem.* **1988**, *27*, 1442.

(26) Otvos, J. D.; Armitage, I. M. *Biochemistry* **1980**, *19*, 4021, 4031.

(27) Gettins, P.; Coleman, J. E. *J. Biol. Chem.* **1984**, *259*, 4991.

describing the sigmoid activity pH profile is that of the coordinated H_2O. This pK_a is also reflected in the pH dependency of the E-P $\rightleftarrows$ E·P equilibrium which moves from a midpoint of pH 5 for Zn_2Zn_2AP to pH 10 for Cd_2Cd_2AP.[3,4] Such a pH shift in the activity profile of carbonic anhydrase also occurs upon the Zn(II) to Cd(II) substitution, an enzyme in which the attack of a coordinated solvent on a carbon of the substrate has been well documented.[28] Formation of high equilibrium concentrations of the ^{32}P phosphoseryl 102 by Mn_2Mg_2AP in an early study of the enzyme,[12] showed a pH function that peaked at pH 8 rather than pH 5, as observed for Zn_2Mg_2AP.[12] This suggests that like Cd(II) in the A site, Mn(II) in the A site may induce a higher pK_a for coordinated solvent molecules.

The structure of organized water at the active center of alkaline phosphatase surrounding both the A- and B-site metal ions will be important components in understanding the details of the mechanism of action of this enzyme. Unfortunately, most structural probes, including X-ray diffraction except at the very highest resolution, offer little information on bound water. The Mn(II) probe, by virtue of its electron–nucleus relaxation, provides at least a partial insight into the nature of the water molecules bound at the active center of alkaline phosphatase. Despite the slow rate of turnover, the Mn(II) enzyme does appear to catalyze all the steps required for phosphate ester hydrolysis.

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Appendix

Computer Program for the Simulation of the 35-GHz ESR Spectra. To fit the 35-GHz ESR spectra by using the spin Hamiltonian of eq 1, the following simplifying assumptions were made: (1) The **g** and **A** tensors are taken to be isotropic. Since the ground state is an S state, deviations from isotropy will arise only from admixtures of excited states, which are expected to be small. (2) The resonance fields are calculated only to second order in perturbation theory ($D/g\beta H$ is small). Since the parameters are determined primarily by features within about 3 kG of the $g = 2$ region, the second-order calculation should be reasonably accurate. (3) The transition probabilities were calculated only to first order, and forbidden transitions were neglected. Many papers have shown that forbidden transitions must be included for accurate representation in the $g = 2$ region.[29,30] Indeed, our representations were least precise just around $g = 2$, but the unique features of the spectra further from $g = 2$ are broader and much less sensitive to modulation by forbidden transitions and were accurately fit.

The powder spectrum was calculated by doing a point-by-point integration over the angle of the magnetic field with respect to the principal axes of the ZFS tensors. The magnetic field, H_0, was kept along the z axis, and the ZFS tensors were rotated with respect to this axis. The five resonance fields (neglecting hyperfine structure) were calculated for each angle and stored in a stick intensity spectrum weighted for each transition probability and the solid angle factor, $\sin\theta$. The Gaussian derivative line shape is then folded onto the stick spectrum by using the following simple "g-strain" line width model. To first order in D, the resonance field is given by $H_r = (H_0 - Df(\theta,\psi,E/D)/g\beta$, where f is some function of the angles of H with respect to the D principal axes and H_0 is given by $h\nu/g\beta$. If we have some distribution δD of the ZFS, there will be a corresponding distribution in the resonance field given by

$$\delta H_r = (\delta D)f/g\beta = \delta D(H_r - H_0)/D$$

This model gives a line width that is proportional to the deviation in H from the $g = 2$ position. To allow for some independent variation that produces a finite line width at H_0, we used the following two-parameter line width model: $LW(H) = C(1 + (H - H_0/B)^2)^{1/2}$, where B and C both have units of gauss. The final step is to produce the six-line hyperfine structure by adding the calculated spectrum to itself six times, displacing the spectrum by $A/g\beta$ each time. This algorithm will produce a fairly smooth spectrum in terms of integration noise with about 60 integration points in θ and a corresponding number in ψ, depending on the distortion from axial symmetry.

(28) Bauer, R.; Limkilde, P.; Johansen, J. T. *Biochemistry* **1976**, *15*, 1976.
(29) Reed, G. H.; Cohn, M. J. *Biol. Chem.* **1973**, *248*, 6436.
(30) Bleaney, B.; Rubins, R. S. *Proc. Phys. Soc. London* **1961**, *77*, 103.
(31) Wyckoff, H. W. Personal communication.