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Characterization of the Properties of the Multiple Metal Binding Sites in Alkaline Phosphatase by Carbon-13 Nuclear Magnetic Resonance†

James D. Otvos‡ and Ian M. Armitage*

ABSTRACT: Carbon-13 nuclear magnetic resonance (^{13}C NMR) of *Escherichia coli* alkaline phosphatase labeled biosynthetically with β,β-[γ-^{13}C]dideuteriohistidine has been used to determine the number and identity of the histidine residues that participate in metal ion coordination at the three classes of binding sites in this dimeric Zn^{2+} metalloenzyme. Detailed ^{13}C NMR titrations of the apoenzyme with $^{113}Cd^{2+}$ and Mg^{2+}, in conjunction with parallel ^{113}Cd NMR measurements [Otvos, J. D., & Armitage, I. M. (1980) *Biochemistry* (third of three papers in this issue)], permitted the assignment of four histidine residues as ligands to the "catalytic", or A site, metal ions, two coordinated via their N^{π} imidazole nitrogens and two via N^{τ}. In addition, a fifth histidyl ligand, coordinated through N^{τ}, was shown to be located at the "structural", or B, sites on the dimer. The "regulatory", or C, sites do not contain histidyl metal ligands. Unambiguous identification of the three histidines coordinated to metal ion via N^{τ} was provided by the observation of resolved ^{113}Cd–^{13}C spin–spin coupling (^3J = 12–19 Hz) in their γ-carbon resonances. Once assigned, the ^{13}C resonances of the five histidyl metal ligands were used to monitor the relative affinities of the A, B, and C sites for Cd^{2+} and Zn^{2+}. At pH 6.3, Cd^{2+} was found to bind to the A sites at least 10 times tighter than to the B or C sites, which have roughly equal affinities. In marked contrast, Zn^{2+} was found to have similar affinities for the A and B sites at both pH 6.3 and 8.0. The affinity of the C sites for Zn^{2+} and Mg^{2+} was shown to be at least an order of magnitude lower. The binding constants of all three sites for Cd^{2+} and Zn^{2+} are greater than 10^5 M^{-1}. Evidence is also presented that suggests that the A, B, and C sites may be located in close proximity to one another in the monomers.

Escherichia coli alkaline phosphatase is a dimeric zinc metalloenzyme which requires the occupation of three distinct pairs of metal binding sites to produce maximal catalytic activity and structural stability (Bosron et al., 1977; Brown et al., 1974; Hull & Sykes, 1976; Chlebowski & Mabrey, 1977). One class of sites appears to have a higher affinity for metal ion than the others and has been termed "catalytic", since the first pair of metal ions added to apoenzyme has been shown to be the minimal requirement for the induction of catalytic function (Applebury et al., 1970). Metal binding to these tight sites, located ~32 Å apart across the twofold dimer axis (Knox & Wyckoff, 1973), is also thought to play the major role in determining the formation and stability of the tertiary and quaternary structures of the enzyme (Chlebowski & Mabrey, 1977; Trotman & Greenwood, 1971).

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510. *Received February 26, 1980.* This work was supported by Grant AM 18778-04 from the National Institutes of Health and by Grants PCM 76-82231 and PCM 77-18941 from the National Science Foundation.

‡ Present address: Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, WI 53201.

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Absorption spectroscopy of the Co^{2+}-substituted enzyme and ESR[1] of the Mn^{2+} enzyme suggest that the ligand geometry about the catalytic metal ions is distorted tetrahedral or five-coordinate (Applebury & Coleman, 1969b; Anderson et al., 1976; Haffner et al., 1974). Other than the probable participation of several histidine residues in metal ligation (Tait & Vallee, 1966; Taylor & Coleman, 1972; Csopak & Falk, 1974), the coordination sphere of the catalytic metal ions has not been further defined.

In addition to the catalytic Zn^{2+}, the functional importance of an additional pair of Zn^{2+} and Mg^{2+} ions has been recognized. Binding of these additional metal ions to the Zn_2^{2+} enzyme[2] causes catalytic activity to increase by about sevenfold (Bosron et al., 1977), ligand binding stoichiometry to increase from 1 to 2 equiv per dimer (Otvos et al., 1979), and structural stability to be markedly enhanced (Chlebowski & Mabrey, 1977; Anderson et al., 1975). The second pair of Zn^{2+} ions binds relatively tightly ($K_D \ll 10^{-6}$ M) (Bosron et al., 1977) to a class of sites that has been termed "structural". The coordination geometry about these structural sites appears to be octahedral-like (Anderson et al., 1976), although no information is available concerning the number or identity of its protein ligands. A third class of sites, also possessing octahedral-like coordination geometry (Anderson et al., 1976), binds Mg^{2+} with greater affinity than Zn^{2+} (Bosron et al., 1977). These sites, termed "regulatory", are presumably occupied by the 1–2 equiv of Mg^{2+} found in the native Zn^{2+} enzyme (Bosron et al., 1975). While Mg^{2+} exerts an influence on the structural stability and specific activity of the Zn^{2+} enzyme, neither the physical characteristics of its binding site nor the specific role played by the bound metal ion have been characterized.[3]

The absence of a suitable physical technique to assess the distribution of Zn^{2+} among the three classes of metal binding sites as a function of overall enzyme metal content has presented a major obstacle in attempts to define the relationship between structure and function in alkaline phosphatase. Because of its filled d shell, Zn^{2+} is "spectroscopically silent", and information regarding its binding characteristics has necessarily had to be inferred from spectroscopic studies based on its replacement by chromophoric and/or paramagnetic metal ions (Coleman & Chlebowski, 1979). Unfortunately, these metals may be expected to differ from Zn^{2+} in their relative affinities for the various binding sites because of differences in their preferences for particular ligands or coordination geometries. In an attempt to develop a probe capable of directly monitoring Zn^{2+} binding to the multiple sites in alkaline phosphatase, we have extended the ^{13}C NMR studies reported in the preceding paper (Otvos & Browne, 1980) on enzyme labeled biosynthetically with [γ-^{13}C]histidine. The previous data suggested that at least three histidine residues participate in metal ion ligation. Detailed metal ion titration studies now indicate that a total of five histidine residues are coordinated to the A- and B-site metals[3] located at the active site. Unambiguous assignment of several of these histidyl metal ligands has been made possible by the ability to resolve ^{13}C–^{113}Cd spin couplings in the $^{113}Cd^{2+}$-substituted enzyme. By utilizing the ^{13}C resonances of the A- and B-site histidine residues as probes of the occupancy of these sites, we are able to show that Zn^{2+} exhibits markedly different binding characteristics than previously supposed.

Materials and Methods

Enzyme Preparations. β,β-[γ-^{13}C]Dideuterio-DL-histidine (90% enriched from Merck and Co., Inc.) was biosynthetically incorporated into alkaline phosphatase using a histidine auxotroph of *E. coli* strain C-90 (kindly provided by Dr. Brooks Low). Bacteria were grown to late stationary phase in a medium identical with that described previously (Malamy & Horecker, 1964), except for the replacement of the Difco peptone with 5×10^{-4} M inorganic phosphate and the addition of 30 μg/mL of the labeled DL-histidine. Alkaline phosphatase was released by osmotic shock and isolated as previously described (Applebury et al., 1970). A typical yield of enzyme from a 10-L growth was about 300 mg.

Enzyme concentrations were determined spectrophotometrically at 280 nm using $E_{1cm}^{0.1\%} = 0.77$ (Browne & Otvos, 1976). For molar calculations, a molecular weight of 95 000 was used.[4] Apophosphatase was prepared by dialysis of the Zn^{2+} enzyme at 4 °C against four changes of 5×10^{-3} M *o*-phenanthroline (100-fold volume excess) in 0.01 M Tris-HCl, 0.01 M NaOAc, and 0.1 M NaCl, pH ~5, for 2 days, followed by at least eight changes of the same buffer, pH 6.5, to assure quantitative removal of the chelator. Upon reconstitution with four Zn^{2+} and two Mg^{2+} ions, apoenzyme prepared in this manner gave specific activities of 2800 ± 200 units (micromoles of *p*-nitrophenyl phosphate hydrolyzed per hour per milligram of protein in 1 M Tris-HCl, pH 8, 22 °C). Concentration of apoenzyme to NMR volumes (1–2 mL) was carried out at 4 °C in a metal-free Amicon ultrafilter using a PM-10 membrane. Me^{2+} phosphatases were prepared by direct addition of stoichiometric amounts of $MgSO_4$, $ZnSO_4$, and $MnSO_4$ (spectrograde from Johnson Matthey Chemicals, Ltd., London) and $^{113}CdCl_2$ and $^{112}CdCl_2$ (prepared from the 96% isotopically enriched oxides from Oak Ridge Laboratory) to the apoenzyme. All NMR measurements were performed on enzyme solutions in 0.01 M Tris-HCl, 0.01 M NaOAc, and 0.1 M NaCl at the pH values indicated.

NMR Methods. ^{13}C NMR spectra were recorded at 22.6 MHz on an extensively modified Bruker HFX-90 spectrometer (Armitage et al., 1978) and at 50.3 MHz on a Bruker CXP-200 spectrometer. All measurements were made at ambient temperature (25 ± 2 °C) under conditions of broad-band proton decoupling. The spectrometers were internally locked on the deuterium resonance of D_2O contained in a 3-mm coaxial capillary inserted into the 10-mm NMR sample tubes. Chemical shifts are reported in parts per million downfield from the ^{13}C resonance of Me_4Si. The following parameters were typically used: spectral width = 5000 Hz, data points = 4096 or 8192, pulse repetition rate = 1.5 s, pulse angle =

[1] Abbreviations used: ESR, electron spin resonance; NMR, nuclear magnetic resonance; CD, circular dichroism; MCD, magnetic circular dichroism; ^{13}C(D) AP, β,β-[γ-^{13}C]dideuteriohistidine alkaline phosphatase; Me^{2+}, divalent metal ion.

[2] The number of metal ions added to apoalkaline phosphatase is indicated by the subscript number following the metal symbol; e.g., Zn_2^{2+} identifies apoenzyme to which 2 g-atoms of Zn^{2+} per mol of enzyme has been added.

[3] The previous categorization of the three pairs of metal binding sites in alkaline phosphatase as "catalytic", "structural", and "regulatory" was a purely operational designation rather than a functional one. All three classes of metal ion confer on the enzyme different degrees of structural stability and catalytic efficiency, which vary in a complex manner according to the overall metal content of the protein. Without a better understanding of the exact roles played by the metal ions in the catalytic mechanism, we believe it is preferable to adopt a nomenclature that does not imply a specific function for the different classes of metal ion. For this reason, we will henceforth refer to the "catalytic", "structural", and "regulatory" sites as the A, B, and C sites, respectively.

[4] This molecular weight value was calculated from recent sequence data that indicates the presence of about 450 amino acid residues per monomer (R. A. Bradshaw, personal communication).

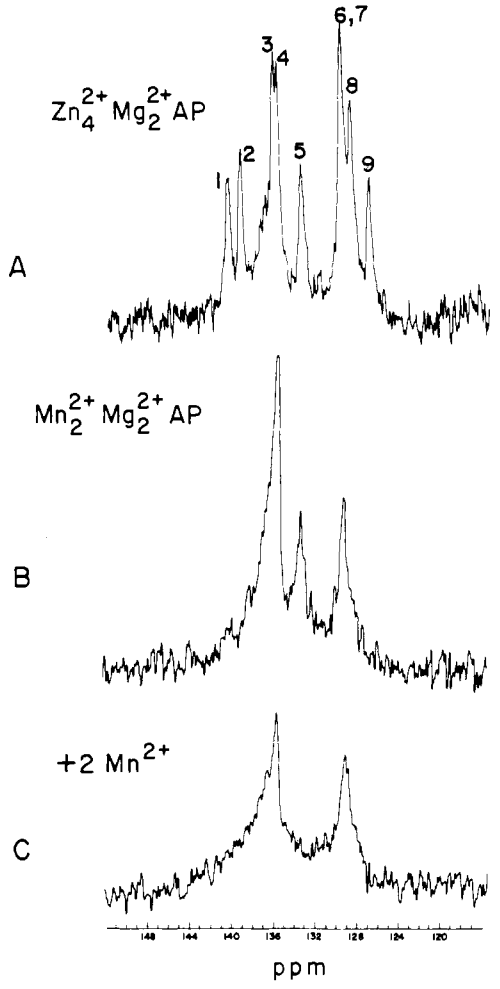

FIGURE 1: The influence of Mn^{2+} ion on the ^{13}C NMR spectrum of $^{13}C(D)$ AP at 22.6 MHz. The enzyme concentration was 1.8 mM in 0.01 M Tris, 0.01 M NaOAc, and 0.1 M NaCl, pH 8.0: (A) apophosphatase reconstituted with 4.2 equiv of Zn^{2+} and 2.1 equiv of Mg^{2+}; (B) apophosphatase reconstituted with 2.2 equiv of Mg^{2+} and 2.1 equiv of Mn^{2+}; (C) same as B plus 2.1 equiv of Mn^{2+}. 45°, acquisitions = 10 000–30 000, digital broadening = 2–5 Hz.

Results

In the preceding paper (Otvos & Browne, 1980) it was shown that the ^{13}C NMR spectrum of β,β-[γ-^{13}C]dideuteriohistidine alkaline phosphatase ($^{13}C(D)$ AP) contains 8 or 9 resolved resonances (depending on experimental conditions) arising from the 10 histidine residues located in each of the symmetrically disposed subunits of the dimer. The two most downfield resonances (His-1 and -2) and the most upfield resonance (His-9) were tentatively assigned to histidines coordinated to active-site metal ion(s) via their N^{τ} and N^{π} nitrogen atoms,[5] respectively (see Figure 1A for numbering scheme). His-7 and -8 were also suggested to be located in or near the active site, although the evidence for their involvement in metal ligation was less certain. All of the ^{13}C NMR experiments that led to these conclusions were conducted on either apoenzyme or holoenzyme generated by dialysis of apoenzyme against excess Zn^{2+} and Mg^{2+}. For this reason, it was not possible to determine whether the suspected histidyl ligands were coordinated to Zn^{2+} or Mg^{2+}, or whether the metals involved were located at the A, B, or C metal binding sites.

[5] We use the convention that N^{τ} in histidine is the nitrogen atom closest to the point of attachment of the imidazole ring to the β carbon.

In an initial attempt to resolve these questions, ^{13}C NMR titrations of apoenzyme with Mg^{2+}, Zn^{2+}, and Cd^{2+} were performed at 22.6 MHz (Coleman et al., 1979). From this data, it was possible to make firm conclusions only about His-1 and -2, both of which were shown to function as ligands to the A site metal. This assignment was relatively straightforward because of the fact that the unusual downfield chemical shifts of these residues can be unambiguously attributed to N^{τ} metal ligation (Otvos & Browne, 1980). In contrast, because of the complexity of the spectral changes in the upfield region of the spectra induced by metal ion binding, it was impossible to draw similar conclusions regarding the participation of His-7, -8, and -9 in metal coordination (His-3, -4, -5, and -6 have previously been shown not to be metal ligands (Otvos & Browne, 1980)).

Titration of $^{13}C(D)$ AP with Mn^{2+} at 22.6 MHz. To help clarify which of the upfield [^{13}C]histidine resonances correspond to metal ion ligands, a titration was performed with paramagnetic Mn^{2+} ion in place of Zn^{2+}. The influence of the unpaired electron spin of Mn^{2+} on the relaxation rates of the γ carbons of nearby histidine residues can be expected to broaden beyond detection those resonances that arise from histidines located within approximately 10 Å of the enzyme-bound metal. Therefore, assuming that a close chemical shift correspondence exists between the Zn^{2+} and Mn^{2+} enzymes, any resonances from histidine residues coordinated to Mn^{2+} will be missing from the spectrum. The ^{13}C NMR spectrum of apoenzyme reconstituted with 2 equiv each of Mg^{2+} and Mn^{2+} is shown in Figure 1B. Under these conditions it has been demonstrated by ESR that the Mn^{2+} ions selectively occupy the pair of A sites on the dimer (Weiner et al., 1979). For comparison purposes, the fully reconstituted Zn^{2+} enzyme spectrum is shown in Figure 1A. It is clear from Figure 1B that the only resonances that are not excessively broadened by A-site Mn^{2+} are His-3, -4, -5, and -6. This observation confirms the results in the preceding paper (Otvos and Browne, 1980), which suggested that these histidine residues were not located at the active site. As expected, His-1 and -2 are not observed in the spectrum in Figure 1B, consistent with their previous assignment as ligands to A-site metal. The inability to detect resonance intensity corresponding to His-7, -8, and -9 is also consistent with, but not proof of, participation of these residues in A-site metal coordination. Since any histidine located within about 10 Å of the A-site Mn^{2+} will experience severe line broadening, the possibility cannot be excluded that His-7, -8, and -9 are unliganded residues located in close proximity to the A site.

Addition of a second pair of Mn^{2+} ions to the Mg_2^{2+},Mn_2^{2+} enzyme results in the selective abolition of His-5 (Figure 1C). Mn^{2+} coordination to this histidine residue can be ruled out by previous data showing it to undergo normal pH titration behavior (Otvos & Browne, 1980). Therefore, resonance 5 would appear to arise from a pair of unliganded histidines in the dimer which happen to be located in close proximity to the second pair of bound Mn^{2+} ions, though it is not known whether it is the B or C binding sites that are occupied by Mn^{2+} under these conditions.

Titration of $^{13}C(D)$ AP with $^{113}Cd^{2+}$ at 50.3 MHz. Several properties of $^{113}Cd^{2+}$ suggest that its binding to the [^{13}C]-histidine enzyme may allow unambiguous assignments to be made of the histidine residues in the enzyme which participate in metal ion coordination at the A, B, and C sites. Most importantly, as a diamagnetic spin $^1/_2$ nucleus, $^{113}Cd^{2+}$ may be expected to provide ^{113}Cd–^{13}C spin–spin coupling in the γ-carbon resonances of those histidines to which it is directly

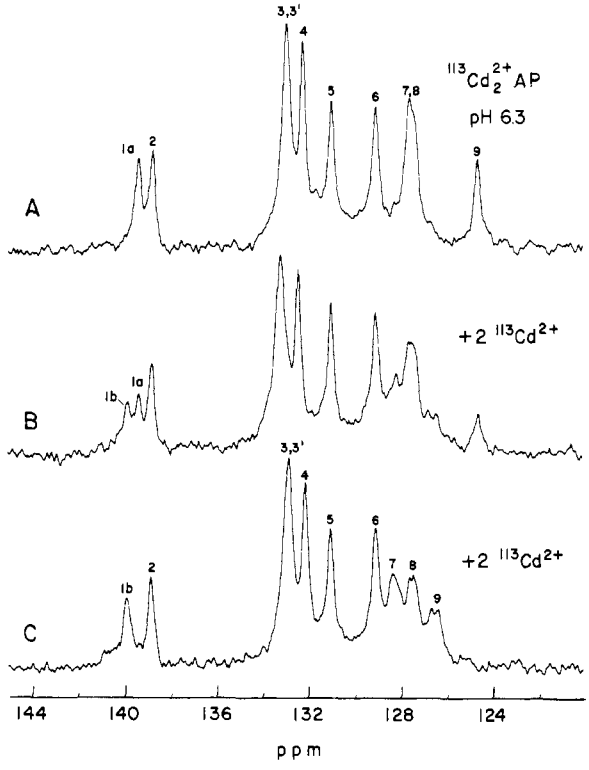

FIGURE 2: ^{13}C NMR spectra at 50.3 MHz of $^{13}C(D)$ apophosphatase titrated with $^{113}Cd^{2+}$ at pH 6.3: (A) 1.4 mM apoenzyme reconstituted with 2 equiv of $^{113}Cd^{2+}$; (B) A plus 2 equiv of $^{113}Cd^{2+}$; (C) B plus 2 equiv of $^{113}Cd^{2+}$. The spectra (10000 transients each) were acquired 12–16 h after metal ion addition.

coordinated. In addition, the $^{113}Cd^{2+}$ ion itself can be detected by NMR, thereby providing an independent method by which to monitor its equilibrium distribution among the three classes of metal binding sites under various conditions (Otvos & Armitage, 1980). By using this information to complement the ^{13}C NMR results, it should be possible to assign each histidyl metal ligand to a particular metal binding site in the protein. Finally, titration with Cd^{2+} may be expected to generate enzyme species that closely resemble those formed by the native Zn^{2+} because of their preferences for similar coordination geometries and ligands.

Titration of apoenzyme with 2-equiv increments of $^{113}Cd^{2+}$ gives rise to the ^{13}C spectra shown in Figure 2. These and all subsequent spectra were acquired at 50.3 MHz, about double the frequency used previously, in order to improve spectral resolution in the upfield region of the spectrum. The enzyme was maintained at pH 6.3 so that the ^{13}C NMR results would be directly comparable to those obtained in parallel ^{113}Cd NMR titrations performed at this pH (Otvos & Armitage, 1980).

Addition of the first pair of $^{113}Cd^{2+}$ ions to the apodimer generates the clean, well-resolved spectrum shown in Figure 2A. The simplicity of this spectrum in itself indicates that the members of each pair of symmetrically disposed histidine residues in the dimer experience identical environments in the two subunits. Therefore, the $^{113}Cd_2^{2+}$ enzyme must be homogeneous as the result of selective metal binding to only one of the three pairs of metal binding sites in the dimer. Confirmation of this fact is provided by the observation that the integrated areas of the resonances from His-1 and -2 do not increase upon binding of additional $^{113}Cd^{2+}$ to the enzyme (Figures 2B and 2C). Since these two histidines exhibit unusual downfield chemical shifts only as the result of metal ion coordination to their N^{π} nitrogen atoms, it follows that the high-affinity A sites of which they are a part are fully occupied in the $^{113}Cd_2^{2+}$ enzyme. The same conclusion was reached in a parallel ^{113}Cd NMR experiment which showed that the first two $^{113}Cd^{2+}$ ions added to apoenzyme bind to sites that have indistinguishable chemical environments (Otvos & Armitage, 1980).

In view of our assignment of His-1 and -2 to A-site $^{113}Cd^{2+}$ ligands, the question arises as to why one is unable to resolve ^{113}Cd–^{13}C spin–spin coupling in their γ-carbon resonances. The answer presumably lies in the very small magnitude of the two-bond coupling constant that results from $^{113}Cd^{2+}$ coordination to the N^{π} imidazole nitrogen. It is well known that two-bond ^{13}C–^{1}H spin couplings are generally much weaker than those which arise from similar one-bond or three-bond interactions (Stothers, 1972). This trend also appears to hold for spin coupling to ^{113}Cd, based on ^{13}C NMR spectra of several $^{113}Cd^{2+}$ cryptates that exhibit resolved three-bond ^{113}Cd–^{13}C spin splittings of 5–12 Hz, but no resolvable splittings arising from the two-bond interactions (unpublished results). Given this relationship, one might predict that the three-bond coupling arising from coordination of $^{113}Cd^{2+}$ to histidine N^{τ} would be detectable. This expectation is borne out by the ability to resolve ^{113}Cd–^{13}C spin–spin couplings in the upfield resonances of the fully reconstituted $^{113}Cd^{2+}$ enzyme spectrum (Figure 2C). Although the spin splittings in the spectrum of the $^{113}Cd_2^{2+}$ enzyme in Figure 2A are obscured because of resonance overlap, subsequent experiments to be presented below will show that His-7 and -8, like His-1 and -2, participate as protein ligands to the A-site metal ion. In view of the above, the absence of spin coupling to His-9 in Figure 2A is a clear indication that this residue, which exhibits an unusually high-field chemical shift of 124.6 ppm, is not coordinated to A-site $^{113}Cd^{2+}$. Since an upfield shift of this magnitude is not observed in enzyme with any other metal stoichiometry (Zn^{2+} or Cd^{2+}), a unique local environment must be created for His-9 by the simultaneous occupation of the A sites and vacancy of the B and C sites. It might be speculated that ring current contributions from one or more nearby aromatic residues may be responsible for this abnormal upfield chemical shift.

The ^{13}C spectrum of the enzyme to which a second pair of $^{113}Cd^{2+}$ ions was added (Figure 2B) is more complex than the spectrum of the $^{113}Cd_2^{2+}$ enzyme. The observation that the number of resonances in the spectrum exceeds the number of histidine residues contained in a single subunit (10) provides convincing evidence that the $^{113}Cd_4^{2+}$ enzyme is heterogeneous. Subsequent binding of a third pair of $^{113}Cd_2^{2+}$ ions is apparently sufficient to restore the enzyme dimer to a symmetric state, judging from the generation of the simplified, homogeneous $^{113}Cd_6^{2+}$ enzyme spectrum in Figure 2C. $^{113}Cd^{2+}$ addition in excess of 6 equiv induces no further spectral changes. The above results suggest that the relative affinities of the B and C sites for Cd^{2+} are not sufficiently different to ensure the selective occupancy of one pair or the other in the $^{113}Cd_4^{2+}$ enzyme. Instead, there is competition between the two pairs of sites for the available $^{113}Cd^{2+}$ until both pairs become fully populated in the $^{113}Cd_6^{2+}$ enzyme. The resonances of His-1 and -9 provide convenient monitors of the equilibrium distribution of Cd^{2+} between the B and C sites in enzyme containing submaximal amounts of metal ion. Both resonances shift downfield upon Cd^{2+} binding to the B and/or C sites: His-1 shifts from 139.3 to 139.8 ppm and His-9 shifts from 124.6 to 126.5 ppm, where it is split into a doublet as the result of spin coupling to $^{113}Cd^{2+}$ located at the B or C sites. The coexistence in Figure 2B of approximately equal amounts of

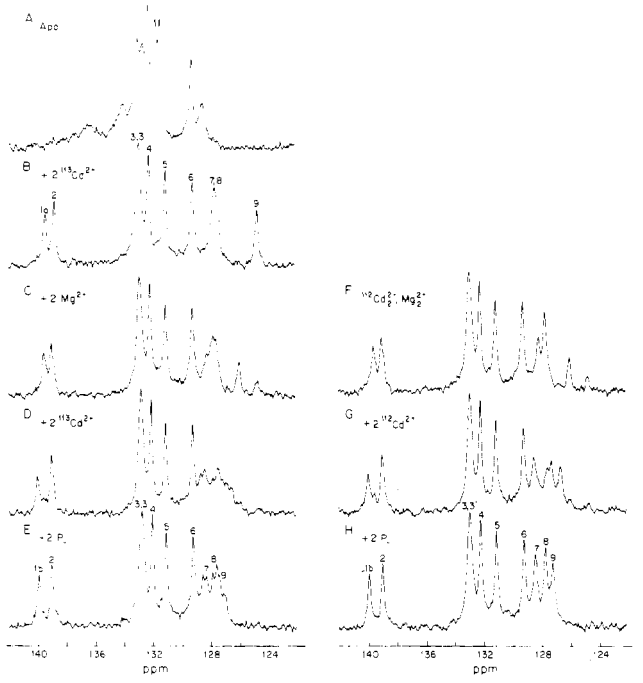

FIGURE 3: Comparison of the effects of $^{113}Cd^{2+}$ and $^{112}Cd^{2+}$ on the ^{13}C NMR spectra at 50.3 MHz of ^{13}C(D) apophosphatase titrated with metal ion and phosphate at pH 6.4. Spectra A–E are of 1.3 mM apophosphatase to which the indicated quantities of $^{113}Cd^{2+}$, Mg^{2+}, and inorganic phosphate were added to the sample used to obtain the preceding spectrum. Spectra F, G, and H are of enzyme containing the same metal and phosphate stoichiometries as those which gave rise to C, D, and E, respectively, except that $^{112}Cd^{2+}$ was substituted for $^{113}Cd^{2+}$ in the former three samples. Each spectrum required 14000 transients. The enzyme samples used to obtain spectra E and H were shown by ^{31}P NMR to be covalently phosphorylated at both subunits of the dimer.

both forms of His-1 and -9 indicates that (1) $^{113}Cd^{2+}$ is distributed equally among the competing B and C sites of the dimer in the $^{113}Cd_4^{2+}$ enzyme and (2) dissociation of B- and C-site $^{113}Cd^{2+}$ is slow on the NMR time scale, implying relatively tight binding to both sites.

In the spectrum of the fully reconstituted $^{113}Cd_6^{2+}$ enzyme in Figure 2C it is seen that His-7, -8, and -9 exhibit resonances that are either broad or split into resolved doublets. We have suggested that the origin of these effects is the three-bond ^{113}Cd–^{13}C spin coupling which is expected to result from the coordination of $^{113}Cd^{2+}$ to the N^{τ} nitrogen atoms of these histidine residues. The experiment shown in Figure 3 was designed to confirm the existence of ^{113}Cd–^{13}C spin–spin splittings in these resonances as well as to identify the metal binding sites at which the liganded histidines are located. Two metal titrations of apoenzyme were conducted in parallel, one employing $^{113}Cd^{2+}$ (Figures 3A–E) and the other using $^{112}Cd^{2+}$ (Figures 3F–H). Unlike $^{113}Cd^{2+}$, $^{112}Cd^{2+}$ has no nuclear spin and hence will not exhibit spin–spin interactions with the [γ-^{13}C]histidine residues in the protein.

As previously discussed, the initial pair of $^{113}Cd^{2+}$ ions added to apoenzyme at pH 6.3 binds selectively to the two identical A sites on the dimer, thereby generating a characteristic homogeneous ^{13}C spectrum with fully developed intensity in the low-field histidine resonances 1 and 2 (Figure 2A). In the starting point of the experiment in Figure 3, an identical $^{113}Cd_2^{2+}$ enzyme spectrum is observed (Figure 3B). Addition of 2 equiv of Mg^{2+} to the $^{113}Cd_2^{2+}$ and $^{112}Cd_2^{2+}$ enzyme gives rise to the spectra shown in Figures 3C and 3F, respectively. Since the chemical shifts and integrated areas of His-1 and -2 are unaffected by Mg^{2+} binding, it can be inferred that A-site Cd^{2+} is not displaced by Mg^{2+}. Instead, the two Mg^{2+} ions, like the second pair of $^{113}Cd^{2+}$ ions in Figure 2B, apparently distribute themselves among the vacant B and C sites on the dimer. The resulting heterogeneity in the Cd_2^{2+},Mg_2^{2+} enzyme would provide the simplest explanation for the appearance in Figures 3C and 3F of two resonances at 125.9 and 124.6 ppm attributable to His-9. The only spectral differences that can be detected between the $^{113}Cd^{2+}$ and $^{112}Cd^{2+}$ enzymes occur in the region of His-7 and -8. Mg^{2+} binding to the $^{112}Cd_2^{2+}$ enzyme causes His-7 to shift downfield by about 0.5 ppm, allowing it to be clearly resolved from His-8 (Figure 3F). In the corresponding spectrum of the $^{113}Cd^{2+}$ enzyme in Figure 3C, the downfield movement of His-7 allows partially resolved splittings to be detected in both resonances 7 and 8. Since the two Cd^{2+} ions in the enzyme are known to be located exclusively at the A sites, the splittings in Figure 3C must therefore arise from direct ^{113}Cd–^{13}C spin coupling of His-7 and -8 to A-site $^{113}Cd^{2+}$.

Addition of a second pair of $^{113}Cd^{2+}$ or $^{112}Cd^{2+}$ ions to the two enzyme samples generates the relatively complicated spectra in Figures 3D and 3G, respectively. Once again, from the presence of several resonances exhibiting submaximal amplitudes in the spectral regions occupied by His-1, -7, -8 and -9, it may be concluded that the Cd_4^{2+},Mg_2^{2+} enzyme sample does not consist of a homogeneous population of symmetric dimers. Mg^{2+} and Cd^{2+} must therefore have insufficiently different affinities for the B and C sites to allow one metal ion or the other to selectively occupy a particular class of binding sites. Interestingly, as shown by the simplified, "homogeneous" ^{13}C spectra in Figures 3E and 3H, the addition of 2 equiv of inorganic phosphate to the enzyme appears to alter the relative affinities of the sites for Cd^{2+} and Mg^{2+} such that the equilibrium distribution of the two metals between the three pairs of binding sites becomes homogeneous. Under these experimental conditions, inorganic phosphate is known to covalently phosphorylate a specific serine residue at both active sites on the dimer (Otvos et al., 1979). The ^{113}Cd NMR spectrum of this $^{113}Cd_4^{2+}$,Mg_2^{2+} diphosphoryl enzyme clearly shows that the two pairs of $^{113}Cd_2^{2+}$ ions in the protein are bound exclusively to the A and B sites (Otvos & Armitage, 1980). Furthermore, the chemical shifts of the A- and B-site $^{113}Cd^{2+}$ resonances indicate that the C sites in the enzyme are occupied by Mg^{2+}. From this information, it may be concluded that the splitting of resonance 9 in Figure 3E results from spin coupling of His-9 to $^{113}Cd^{2+}$ located at the B sites rather than the C sites.

One of the ^{113}Cd NMR results presented in the accompanying paper (Otvos & Armitage, 1980) suggested an additional experiment which would serve not only to verify the participation of His-7, -8, and -9 in A- and B-site metal coordination, but also to confirm the assignments which were made in that paper of the A- and B-site $^{113}Cd^{2+}$ resonances. The experiment is based on the observation that exposure of the $^{113}Cd_6^{2+}$ diphosphoryl enzyme to an excess of another isotope of Cd^{2+} results in a loss of ^{113}Cd resonance intensity only in those signals assigned to A- and C-site $^{113}Cd^{2+}$ (Figure 4 in Otvos & Armitage, 1980). Presumably owing to the extremely high stability of the B-site $^{113}Cd^{2+}$ complex in the phosphorylated enzyme, isotope exchange at the B site does not appear to occur, at least on the same time scale as at the A and C sites. As shown in Figure 4, the same phenomenon can be monitored by ^{13}C NMR of the [γ-^{13}C]histidine enzyme. The spectra resulting from diphosphorylation of the $^{113}Cd_6^{2+}$ and $^{112}Cd_6^{2+}$ enzymes are shown in Figures 4A and 4B, respectively. Under these conditions (pH 6.2) it is possible for the first time to

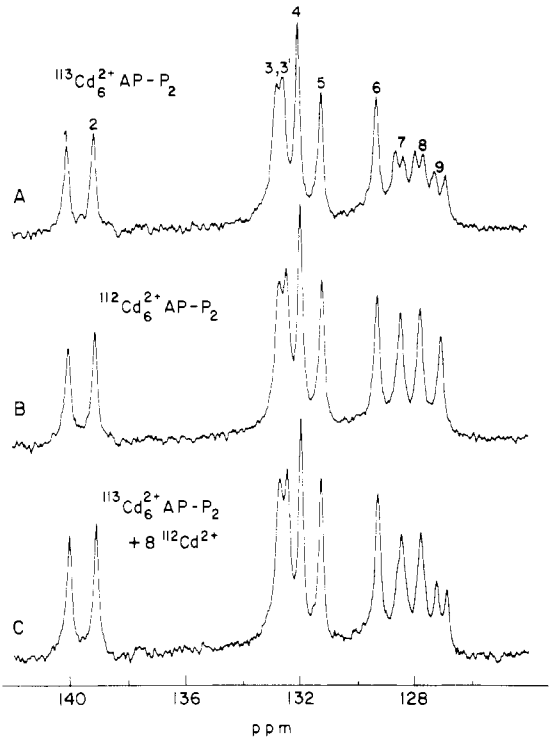

FIGURE 4: The effect of selective Cd^{2+} isotope exchange at the A and C sites on the ^{13}C NMR spectrum at 50.3 MHz of phosphoryl $^{13}C(D)$ AP at pH 6.2: (A) 1.3 mM apophosphatase reconstituted with 6 equiv of $^{113}Cd^{2+}$ and 2 equiv of P_i; (B) 1.3 mM apophosphatase reconstituted with 6 equiv of $^{112}Cd^{2+}$ and 2 equiv of P_i; (C) sample A after addition of 8 equiv of $^{112}Cd^{2+}$, equilibration for 72 h, and removal of excess Cd^{2+} by ultrafiltration. The ^{113}Cd NMR spectrum of this sample indicated that about two thirds of the $^{113}Cd^{2+}$ originally located at the A and C sites, but none of the $^{113}Cd^{2+}$ at the B sites, had been displaced by $^{112}Cd^{2+}$. Spectra A–C required 24 000 transients each.

resolve two resonances corresponding to His-3 and -3′, thereby allowing separate resonances to be detected from all 10 pairs of histidine residues in the symmetric dimer. The spin–spin splittings in His-7, -8, and -9 are also clearly resolved in Figure 4A; the three-bond ^{113}Cd–^{13}C coupling constants for these histidines are measured to be 12, 14, and 19 Hz, respectively. Incubation of the $^{113}Cd_6^{2+}$ diphosphoryl enzyme for 3 days in the presence of 8 equiv of $^{112}Cd^{2+}$, followed by removal of excess Cd^{2+} by ultrafiltration, gives rise to the ^{13}C spectrum in Figure 4C. As anticipated, spin coupling is retained in resonance 9 and abolished in resonances 7 and 8, consistent with our previous assignment of histidine 9 as a B-site metal ligand and His-7 and -8 as A-site ligands. A ^{113}Cd NMR spectrum of the same enzyme sample confirmed that $^{113}Cd^{2+}$ at the B sites had undergone no detectable isotope exchange, while the majority of the $^{113}Cd^{2+}$ at the A and C sites had been replaced by $^{112}Cd^{2+}$.

Phosphate-Induced Metal Ion Redistribution in the Cd_2^{2+} Enzyme. Previous studies have indicated that the Cd_2^{2+} enzyme can be phosphorylated at only one of its two active sites (Chlebowski et al., 1977; Otvos et al., 1979). A ^{113}Cd NMR investigation into the structural basis for this "half-sites" reactivity revealed that enzyme phosphorylation appears to be accompanied by migration of one of the two A-site Cd^{2+} ions to a B site in a slow process that requires several days for completion (Otvos & Armitage, 1980). Since the ^{13}C resonances of the histidine residues that participate as ligands at the A and B sites should serve as sensitive monitors of the metal ion occupancy of these sites, the experiment shown in Figure 5 was performed to confirm the interpretation of the ^{113}Cd NMR results. Spectra of the enzyme taken 15 and 94

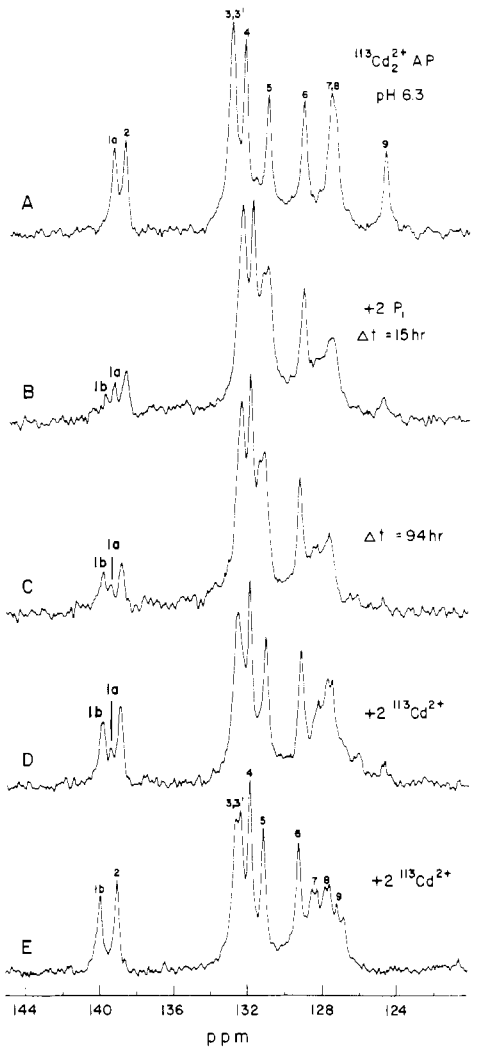

FIGURE 5: The effect of enzyme phosphorylation of the ^{13}C NMR spectrum of the $^{113}Cd_2^{2+}$ enzyme. Each spectrum was acquired at 50.3 MHz using 10 000 transients: (A) 1.4 mM $^{13}C(D)$ apophosphatase reconstituted with 2 equiv of $^{113}Cd^{2+}$; (B) spectrum acquired 15 h after addition of 2 equiv of P_i to A; (C) same as B, acquired 94 h after P_i addition; (D) C plus 2 equiv of $^{113}Cd^{2+}$; (E) D plus 2 equiv of $^{113}Cd^{2+}$.

h after addition of phosphate to the homogeneous $^{113}Cd_2^{2+}$ enzyme are presented in Figures 5B and 5C, respectively. Several features of these spectra offer unambiguous support for a phosphate-induced metal migration. First, if dissociation of Cd^{2+} from half the A sites does indeed occur, one would anticipate a corresponding decrease in the integrated areas of His-1 and -2, since it is only as a result of A-site metal coordination that these resonances exhibit their characteristic low-field chemical shifts. Such a decrease in resonance intensity is readily observed in the spectra in Figures 5B and 5C, accompanied by a corresponding increase in intensity in the spectral region occupied by unliganded histidine residues at this pH (131–133 ppm). Evidence for the gradual population of B sites by Cd^{2+} is also provided in Figures 5B and 5C by the time-dependent increase in the intensity of resonance 1b, which parallels the disappearance of resonance 1a. Combined with the complementary ^{113}Cd NMR results, which show that $^{113}Cd^{2+}$ occupies A and B sites rather than A and C sites in the $^{113}Cd_2^{2+}$ monophosphoryl enzyme, it is now possible to attribute this small downfield shift of His-1 (which was also noted in Figures 2 and 3) to the influence of Cd^{2+} binding at B sites rather than C sites. The fact that resonance 1a continues to be observed in Figure 5C is an indication that the

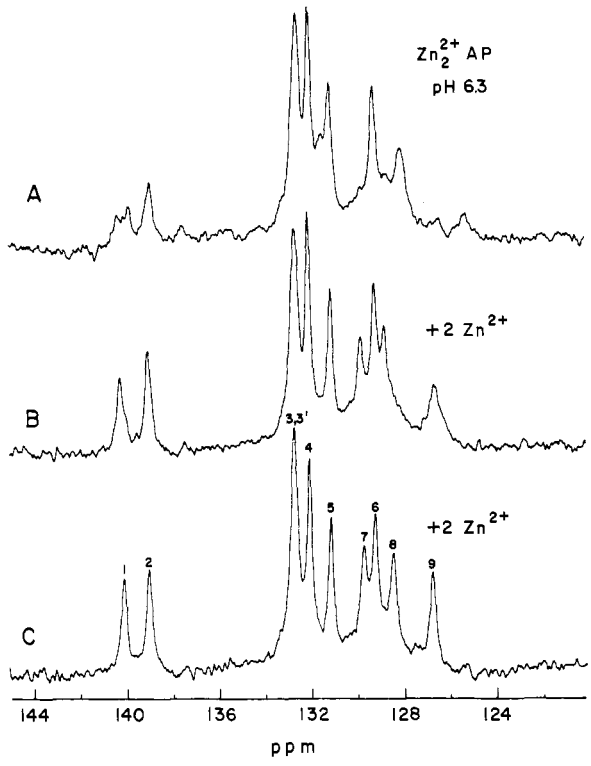

FIGURE 6: ^{13}C NMR spectra at 50.3 MHz of $^{13}C(D)$ apophosphatase titrated with Zn^{2+} at pH 6.3: (A) 1.4 mM apoenzyme reconstituted with 2 equiv of Zn^{2+}; (B) A plus 2 equiv of Zn^{2+}; (C) B plus 2 equiv of Mg^{2+}. Each spectrum (13 000 transients) was acquired 10 h after metal ion addition.

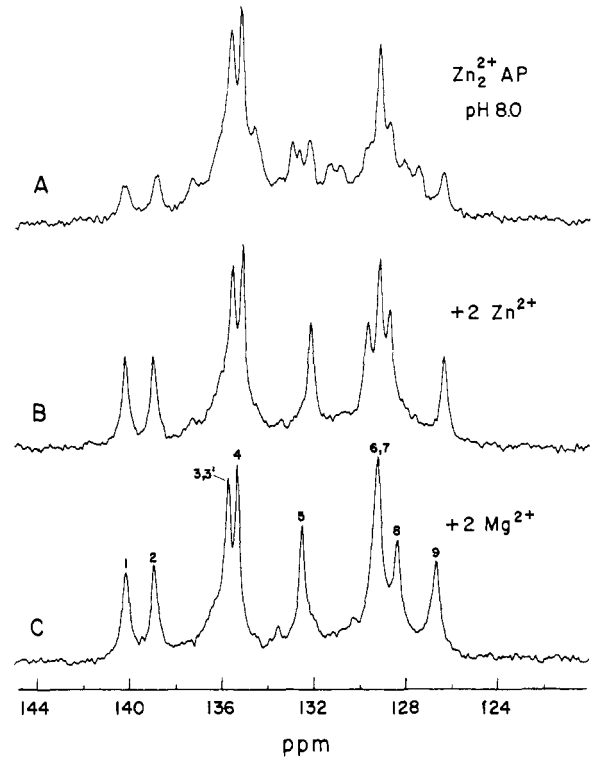

FIGURE 7: ^{13}C NMR spectra at 50.3 MHz of $^{13}C(D)$ apophosphatase titrated with Zn^{2+} and Mg^{2+} at pH 8.0: (A) 1.4 mM apoenzyme reconstituted with 1.9 equiv of Zn^{2+}; (B) A plus 1.9 equiv of Zn^{2+}; (C) B plus 1.9 equiv of Mg^{2+}. Each spectrum (28 000 transients) was acquired 12 h after metal ion addition.

equilibrium metal ion distribution has not quite been achieved even after 94-h incubation. Additional evidence for both the existence and nature of the metal migration is provided by the time-dependent disappearance of the His-9 resonance at 124.6 ppm. His-9 has previously been shown to exhibit this unusual high-field chemical shift only when located in subunits containing Cd^{2+} at the A sites and no metal ion bound to the B sites. In apoenzyme and fully reconstituted enzyme, His-9 is located at least 2 ppm to lower field. The gradual disappearance of the 124.6-ppm resonance can therefore be taken as evidence that the metal redistribution that accompanies enzyme phosphorylation generates a sample consisting of equal populations of aposubunits and subunits containing Cd^{2+} bound to its A and B sites. A detailed discussion of the driving force for the metal migration and its mechanistic significance is reserved for the accompanying paper (Otvos & Armitage, 1980). The spectral changes induced by the sequential binding of additional 2-equiv aliquots of $^{113}Cd^{2+}$ are shown in Figures 5D and 5E. Once again, the spectra clearly indicate that a homogeneous enzyme is generated only when stoichiometric amounts of Cd^{2+} are available to occupy all three pairs of metal binding sites on the dimer.

Titration of $^{13}C(D)$ AP with Zn^{2+} at 50.3 MHz. The assignment of resonances 1, 2, 7, 8, and 9 to histidyl metal ion ligands at the A and B sites of the $^{113}Cd^{2+}$ enzyme now makes possible their utilization as nonperturbing monitors of the binding properties of the native Zn^{2+} ion. Two titrations of apoenzyme with Zn^{2+} were performed, one at pH 6.3 (Figure 6) and the other at pH 8 (Figure 7). Both series of spectra indicate that Zn^{2+} differs markedly from Cd^{2+} with respect to its relative affinity for the three pairs of metal binding sites in the protein. In particular, Zn^{2+} is shown to exhibit considerably less selectivity for A-site binding than was previously shown for Cd^{2+}. This fact is apparent from two aspects of the Zn_2^{2+} enzyme spectra in Figures 6A and 7A: (1) the intensities of the A-site histidyl resonances 1, 2, 7, and 8 are not fully developed, indicating that only a fraction of the A sites are occupied by Zn^{2+}, and (2) the spectra are complex (more than 10 resonances are detected), demonstrating that the Zn_2^{2+} enzyme is heterogeneous as a result of nonselective Zn^{2+} binding to competing classes of sites on the dimer. A direct measure of the fractional occupancy of the A sites in the Zn_2^{2+} enzyme at both pH values may be obtained by comparing the integrated areas of resonances 1 and 2 in Figures 6A and 7A with their corresponding areas in the spectra of the fully reconstituted enzymes in Figures 6C and 7C, respectively. It is found that only 50% of the A sites contain Zn^{2+} at pH 8.0, while about 80% of the sites are occupied at pH 6.3. Since it has previously been shown that metal ion is bound less tightly to the enzyme at low pH values (Applebury & Coleman, 1969a), it is likely that the observed increase in selectivity for A-site binding at pH 6.3 is due to a decrease in the affinity of the competing site(s) for Zn^{2+} rather than to an increase in affinity for the A sites.

Judging by the relative simplicity of the spectra in Figures 6B and 7B, it is clear that binding of a second pair of Zn^{2+} ions restores the enzyme dimer to a symmetric state, presumably by saturating the remaining unoccupied A and B sites in the enzyme. This metal distribution is inferred by the observation that each of the resonances assigned to an A- or B-site histidyl ligand exhibits its maximum intensity in the two spectra of the Zn_4^{2+} enzyme. From the above titration results, one may conclude that at both pH values Zn^{2+} exhibits similar affinities for the A and B sites, but a significantly lower affinity for the C sites. This finding correlates well with previous equilibrium dialysis measurements at pH 8 which showed that the enzyme binds 4 equiv of Zn^{2+} with high affinity ($K_D \ll 10^{-6}$ M) and an additional 2 equiv of Zn^{2+} or Mg^{2+} somewhat

less tightly (Bosron et al., 1977).

Aside from resonances 3, 4, and 5, which undergo pH titration shifts (Otvos & Browne, 1980), the only resonance that is affected by the pH difference between the Zn_4^{2+} enzyme samples is His-9. For reasons that are currently unknown, this resonance at pH 6.3 is broader and is located 0.3 ppm to lower field than at pH 8.0. When a third pair of Zn^{2+} ions is bound at pH 6.3, presumably at the C sites, the line width of His-9 is reduced and His-7 and -8 are shifted upfield by 0.1 and 0.4 ppm, respectively (Figure 6C). Additional Zn^{2+} causes no further alterations in the spectrum. Similar spectral changes accompany C-site binding by Mg^{2+} at pH 8.0: His-7 and -8 shift upfield by 0.5 and 0.4 ppm, respectively, and His-9 shifts downfield by 0.4 ppm (Figure 7C). It is significant that the only resonances whose chemical shifts differ in Figures 6C and 7C are His-3, -4, -5, and -7. Since each of these shift differences can be attributed solely to the pH difference between the two samples (Otvos & Browne, 1980), it may be concluded that the local environments of the A and B sites as monitored by His-7, -8, and -9 are indistinguishable despite occupation of the C sites by Zn^{2+} in one case and Mg^{2+} in the other.

Although not shown here, data from other Zn^{2+} titrations of the enzyme at alkaline pH indicate that the [γ-^{13}C]histidine enzyme can also provide information concerning quaternary structure. Under most conditions, alkaline phosphatase exists as a dimer. However, at alkaline pH in the presence of relatively high concentrations of free Zn^{2+} (10^{-4} M) it has been reported that a reversible self-association of the enzyme occurs to form a tetramer (Reynolds & Schlesinger, 1969b). This process is readily monitored by ^{13}C NMR, since we observe a dramatic increase in the line widths of all ^{13}C resonances whenever Zn^{2+} is added to enzyme which already contains its full complement of metal ion (6 equiv). Lowering the pH to below 7 or removing the excess Zn^{2+} by dialysis causes the line widths to return to their original values. Because of this sensitive relationship between ^{13}C line width and the distribution of enzyme between dimeric and tetrameric states, we are able to conclude that, despite the millimolar protein concentrations that are necessary to carry out the NMR measurements, the enzyme existed as a dimer in all the experiments reported in this paper.

Discussion

In recent years it has become apparent that much of the uncertainty that has existed regarding several fundamental structural and catalytic properties of alkaline phosphatase was the result of a lack of awareness of the complexity of the enzyme. This has been particularly true with respect to attempts aimed at elucidating the function(s) of the enzyme-bound metal ions. Following early reports that full catalytic activity could be generated by the binding of Zn^{2+} to a single pair of high-affinity sites on the dimer (Simpson & Vallee, 1968; Csopak & Szajn, 1973), the properties of these "catalytic" binding sites became the subject of active investigation by a variety of techniques. In most studies, which usually involved replacement of Zn^{2+} with various transition-metal probes, the uniqueness of the "catalytic" sites was reinforced by the observation of distinct spectral characteristics associated with the binding of the first pair of metal ions to the apoenzyme (for a review, see Coleman & Chlebowski, 1979). At about the same time, based on reports indicating that four, rather than two, Zn^{2+} were required to produce maximal activity (Reynolds & Schlesinger, 1969a; Petitclerc et al., 1970; Trotman & Greenwood, 1971), the importance of a second pair of "structural" binding sites began to be recognized. More recently, the existence of yet a third class of sites, apparently occupied by Mg^{2+} in the native enzyme, was demonstrated (Anderson et al., 1975; Bosron et al., 1977). Metal binding to these "regulatory" sites was found to increase catalytic activity even further.

It is now clear that in order to reach a full understanding of the catalytic mechanism of alkaline phosphatase, it will be necessary to define the roles played by all three of the distinct classes of metal ions in the enzyme. This task is made extremely formidable by the absence of techniques capable of selectively monitoring the occupancy of each set of sites during the course of a metal-activity titration. Spectroscopic methods (absorption, CD, MCD, ESR) used to follow the titration of apoenzyme with chromophoric and/or paramagnetic transition metal ions generally fail to provide the necessary information because the resolution is insufficient to allow the separate spectral contributions from metal bound to more than one class of sites to be uniquely distinguished. In addition, it does not appear that the relative affinities of the multiple sites for Zn^{2+} can be reliably extrapolated from information obtained using nonnative metal ions (Anderson et al., 1976).

In the ^{13}C NMR studies described in this paper, we demonstrate a method for assessing the binding characteristics of Zn^{2+} to the three pairs of sites in the enzyme which benefits, rather than suffers, from the diamagnetism of this d^{10} ion. The information is obtained by exploiting our capability to detect individual ^{13}C resonances arising from each pair of symmetrically disposed histidine residues in alkaline phosphatase labeled in vivo with β,β-[γ-^{13}C]dideuteriohistidine (Otvos & Browne, 1980). Using this ^{13}C-enriched protein, we have found it possible to monitor the equilibrium distribution of Zn^{2+} under various conditions simply by observing the characteristic ^{13}C spectral changes that accompany metal coordination to the five histidine residues which we have been able to show are located at two of the three metal binding sites in each subunit. In discussing the different classes of metal binding sites in this and the following paper (Otvos & Armitage, 1980), we have chosen to abandon their historical designations as "catalytic", "structural", and "regulatory" and refer to them instead as A, B, and C sites, respectively. This was done in order to avoid the suggestion that specific functions have been identified for any of these metal ions when, in fact, there is currently no conclusive evidence for such assignments.

Identification of the five histidine residues that participate in metal ion ligation in each monomer was achieved by investigating the ^{13}C NMR properties of the enzyme substituted with $^{113}Cd^{2+}$. Use of this spin $^1/_2$ isotope of cadmium permitted unambiguous assignments to be obtained for the following two reasons: (1) well-resolved ^{113}Cd–^{13}C spin–spin coupling could be detected in the ^{13}C resonances of those histidine residues that are coordinated to $^{113}Cd^{2+}$ via their N^{τ} nitrogen atoms (the coupling arising from $^{113}Cd^{2+}$ ligation to N^{π} is not resolvable, presumably because of the smaller magnitude of the two-bond ^{113}Cd–^{13}C coupling constant), and (2) the precise distribution of $^{113}Cd^{2+}$ among the A, B, and C sites at various stages of the metal titration studies could be independently assessed by ^{113}Cd NMR (Otvos & Armitage, 1980). An additional factor that served to simplify the assignment problem was the selectivity exhibited by cadmium for binding to the A sites in the Cd_2^{2+} enzyme under the conditions employed (pH 6.3). As a result, it was a relatively straightforward matter to demonstrate that four histidine residues function as metal ligands at the high-affinity A sites, two coordinated via their N^{π} nitrogens (His-1 and -2) and the other two via N^{τ} (His-7 and -8). The presence of a fifth histidyl metal ligand at either the B or C site on the monomer

was indicated by the observation of resolved spin splitting in resonance 9 in the spectrum of the $^{113}Cd_6^{2+}$ enzyme (Figure 2C). By analogy to the native Zn_4^{2+},Mg_2^{2+} enzyme, where the C sites are defined as those occupied by Mg^{2+}, His-9 has been assigned as a B-site ligand since the ^{113}Cd–^{13}C spin coupling in its γ-carbon resonance persists in the spectrum of the $^{113}Cd_4^{2+},Mg_2^{2+}$ diphosphoryl enzyme (Figure 3E). A ^{113}Cd NMR spectrum of this enzyme species, which exhibited only A- and B-site resonances, confirmed that the four $^{113}Cd^{2+}$ ions were bound exclusively to the pair of A and B sites on the dimer and not to the C sites.

Once assigned, the ^{13}C resonances corresponding to the five pairs of histidyl metal ligands in the dimer can serve in two ways to monitor the relative affinities of the A, B, and C sites for Cd^{2+} and Zn^{2+}. First, since the characteristic signals arising from coordinated and uncoordinated histidines are always found to be in slow exchange, the integrated areas of the five resonances will directly reflect the fractional occupancy of the A and B sites in enzyme containing substoichiometric amounts of metal ion. Secondly, since each histidyl ligand is related to its counterpart in the opposite subunit by the twofold symmetry of the dimer, a partially reconstituted sample will produce a "simple" spectrum consisting of single two-carbon resonances from each pair of residues only if the enzyme is homogeneous as a result of site-selective saturation of a particular class or classes of sites on the dimer. We have used the above criteria to demonstrate that the first pair of Cd^{2+} ions added to apoenzyme at pH 6.3 binds exclusively to the A sites, while the second and third pairs distribute themselves approximately equally among the B and C sites (Figure 2). The affinity of Cd^{2+} for the A sites must therefore be at least an order of magnitude greater than for the B and C sites, which in turn bind Cd^{2+} with roughly equal affinities. Interestingly, the relative stabilities of the complexes of Zn^{2+} with the A, B, and C sites were found to be significantly different. At pH 8, the spectrum of the Zn_2^{2+} enzyme (Figure 7A) clearly indicates that there is little, if any, difference in the affinity of zinc for the A and B sites. The increase in A-site selectivity which was noted at pH 6.3 (Figure 6A) suggests that the relative stability of the B-site complex with Zn^{2+} is lessened somewhat as the pH is lowered. This is consistent with equilibrium binding studies which demonstrated an increased affinity of the enzyme for stoichiometries of Zn^{2+} in excess of 2 g-atoms/mol at pH $\geq$7 (Applebury & Coleman, 1969a). The same situation apparently holds for cadmium binding as well, judging by our observation that at alkaline pH Cd^{2+} binds less selectively to the A sites than at lower pH values (unpublished results). At both acid and alkaline pH, the ^{13}C NMR data on the Zn^{2+} enzyme clearly demonstrate that binding of either Zn^{2+} or Mg^{2+} to the C sites must be at least an order of magnitude weaker than to the A or B sites, in agreement with a previous report (Bosron et al., 1977).

In addition to supplying information regarding the *relative* affinities of the multiple sites for Cd^{2+} and Zn^{2+}, the ^{13}C NMR titrations allow one to at least place lower limits on their *absolute* affinities. This capability stems from our observation that all metal-induced spectral changes were found to be complete following addition of 6 equiv of metal ion per dimer. Using a conservative estimate of our detection limit, we conclude from this that the six metal binding sites are at least 90% saturated in the Me_6^{2+} enzyme samples. Coupled with a knowledge of the enzyme concentrations used in the titration experiments ($\sim$1 mM), we can calculate that the binding constants associated with Cd^{2+} or Zn^{2+} complexation with all three classes of sites must be greater than 10^5 M^{-1}.

An important contribution to our current understanding of the properties of alkaline phosphatase was the demonstration that the native enzyme contains 1–2 equiv of Mg^{2+} in addition to 4 equiv of Zn^{2+} (Bosron et al., 1975). Subsequent studies indicated that Mg^{2+} significantly affects the catalytic activity, structural stability, and spectral characteristics of the Zn^{2+} and Co^{2+} enzymes, particularly in the presence of substoichiometric quantities of metal ion (Anderson et al., 1975, 1976; Bosron et al., 1977). For example, Mg^{2+} binding increases the specific activity of the Zn_2^{2+} enzyme 5-fold, but that of the Zn_4^{2+} enzyme only 1.4-fold (Bosron et al., 1977). There is some evidence to suggest that the structural basis for effects such as these is increased occupancy of certain "essential" binding sites brought about by a Mg^{2+}-induced alteration in the equilibrium distribution of metal ion among the available sites in the dimer (Anderson et al., 1976). However, because of a lack of information concerning the relative affinities of the three classes of sites for Mg^{2+}, it could not be stated whether the driving force for metal redistribution is direct competition of Mg^{2+} with the other metals for several binding sites or an actual increase in affinity of metal for the "essential" sites, which results indirectly via protein conformational changes induced by selective Mg^{2+} binding to one specific class of sites (by definition, the C sites).

From the influence of Mg^{2+} on the ^{13}C NMR spectra of the cadmium enzyme (Figure 3) and the zinc enzyme (Figure 7 and unpublished data), we are now able to conclude that Mg^{2+} regulates the overall distribution of metal ion in enzyme containing submaximal metal stoichiometries by its ability to compete effectively with other metals for the B and C sites, but not the A sites. This is best illustrated by the "complex" nature of the spectra in Figures 3F and 3G of the Cd_2^{2+},Mg_2^{2+} and Cd_4^{2+},Mg_2^{2+} enzymes, respectively, which demonstrates that the two enzyme samples are heterogeneous as a result of the failure of either Mg^{2+} or Cd^{2+} to bind selectively to the available B and C sites in the dimer. Similarly, because of its ability to interact with B, but not A sites, Mg^{2+} addition to the Zn_2^{2+} enzyme at pH 8 induces a migration of most of the B-site Zn^{2+} to the vacant A sites, thereby causing A-site occupancy to increase from 50 to about 90% (unpublished result). Upon subsequent binding of a second pair of Zn^{2+} ions, however, Mg^{2+} is apparently displaced from the B sites and bound instead to the C sites, since a spectrum identical with the one shown in Figure 7C is obtained. It is interesting to note that spectra of freshly isolated native [^{13}C]histidine enzyme samples are also identical with the spectrum of the Zn_4^{2+},Mg_2^{2+} enzyme in Figure 7C,[6] suggesting that the protein in vivo most likely contains Zn^{2+} bound to the A and B sites and Mg^{2+} to the C sites.

Having demonstrated the utility of ^{13}C NMR as a probe of metal ion distribution in alkaline phosphatase, we are currently performing more detailed Zn^{2+} and Mg^{2+} titrations of the [^{13}C]histidine enzyme in conjunction with measurements of catalytic activity in order to help unravel the relationship between structure and function in this complicated system. Our expectation is that it will prove difficult on the basis of activity correlations alone to draw any firm conclusions regarding the roles of the individual classes of metal ion in the enzyme. The reason for this feeling is the tremendous amount of complexity that is introduced by the need to consider not only the state of occupany of the three types of sites in the

[6] The presence of variable amounts of "endogenous" phosphate in the native enzyme preparations is also reflected in their ^{13}C NMR spectra by the characteristic chemical shift changes in His-7 and -8 that accompany noncovalent phosphate binding (Otvos & Browne, 1980).

individual subunits, but also the possible influence on the catalytic parameters that might be exerted by the metal composition and distribution in the other subunit of the dimer. From our results thus far, activity has not been found to correlate in a simple manner with the occupancy of any one class of binding sites. Instead, it appears that metal occupancy of the A and B sites constitutes the minimal requirement for the induction of catalytic function, a result which is supported by evidence cited in the accompanying paper (Otvos & Armitage, 1980). Metal binding to the C sites appears to affect the overall efficiency of catalysis in ways that have yet to be defined in any detail.

The ^{13}C NMR studies presented herein also provide some insight into the spatial relationships that exist between the A, B, and C sites in the enzyme. Two lines of evidence suggest that the A and B sites are located in close proximity to one another in the monomer. First, it was shown in Figure 1 that selective binding of Mn^{2+} ion to the A sites induced severe paramagnetic broadening not only in those resonances arising from the four A-site histidyl ligands, but also in the signal corresponding to B-site histidine, indicating that this residue must be located within about 10 Å of the A-site metal ion. We have also observed that two of the A-site ligands, His-1 and -7, undergo characteristic downfield chemical shift changes upon Cd^{2+} binding to the B sites (Figure 3), suggesting, though not proving, that the A and B sites are quite close. In view of these findings, it is interesting to note that recent X-ray diffraction data indicates that there are two metal ions in the subunit which are located approximately 5 Å apart (H. W. Wyckoff, personal communication). Since the protein ligands to these metal ions have not yet been identified from the X-ray data, it is not possible to unambiguously confirm that these metals are located at the sites we have identified as the A and B sites, although such an assignment would appear reasonable. No X-ray information is currently available regarding the location of the C sites. However, from the distinct chemical-shift changes that are induced by C-site metal binding in the ^{13}C resonances corresponding to several A- and B-site ligands (His-7, -8, and -9), as well as in the A- and B-site $^{113}Cd^{2+}$ resonances (Otvos & Armitage, 1980), it is not unreasonable to suggest that the C sites are also located relatively near to the A and B sites.

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