

Determination by Cadmium-113 Nuclear Magnetic Resonance of the Structural Basis for Metal Ion Dependent Anticooperativity in Alkaline Phosphatase[†]

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ABSTRACT: Cadmium-113 nuclear magnetic resonance (¹¹³Cd NMR) has been used to probe the binding characteristics of ¹¹³Cd²⁺ to the three classes of metal binding sites in *Escherichia coli* alkaline phosphatase to help elucidate the molecular origin of the metal ion dependent "half-sites" reactivity exhibited by this dimeric Zn²⁺ metalloenzyme [Otvos, J. D., Armitage, I. M., Chlebowski, J. F., & Coleman, J. E. (1979) *J. Biol. Chem.* 254, 4707-4713]. In the absence of phosphate, the first two ¹¹³Cd²⁺ ions added to the apodimer give rise to a single ¹¹³Cd resonance (169 ppm), indicating selective binding to the pair of symmetrically disposed A sites. Resonances arising from additional ¹¹³Cd²⁺ bound to the B and C sites cannot be observed; B- and/or C-site occupation also renders the A-site ¹¹³Cd resonance undetectable. Both these observations have been attributed to severe chemical exchange broadening in the A-, B-, and C-site ¹¹³Cd signals induced by an unknown modulation process(es). Interestingly, covalent phosphorylation of the active-site serine residues abolishes this exchange modulation, allowing three separate resonances to be detected and assigned to ¹¹³Cd²⁺ located at each of the three

classes of metal binding sites in the enzyme. By varying the metal composition of the phosphorylated enzyme, we have characterized the correlations that exist between the chemical shifts and intensities of these ¹¹³Cd resonances and the metal occupancies of the A, B, and C sites in the individual subunits. This information has allowed us to conclude that the half-sites phosphorylation of the Cd²⁺ enzyme is accompanied by a slow migration of half the Cd²⁺ originally located at the A sites to the B sites on the phosphorylated subunits. The driving force for this metal redistribution, which at equilibrium leaves half the subunits devoid of metal ion and thereby incapable of binding phosphate, is apparently the dramatic stabilization of the complex of Cd²⁺ with the B sites, which was demonstrated to occur in those subunits that become phosphorylated. From the kinetics of both phosphorylation and metal redistribution in Cd²⁺ enzyme, we suggest that population of the A and B sites in a subunit, rather than the A site alone, constitutes the minimum requirement for induction of catalytic function in alkaline phosphatase. The spin relaxation properties of the enzyme-bound ¹¹³Cd²⁺ ions are also briefly discussed.

Escherichia coli alkaline phosphatase is a dimeric zinc metalloenzyme that requires the occupation of three distinct pairs of metal binding sites¹ for full expression of its catalytic activity and structural stability (Bosron et al., 1977; Brown et al., 1974; Chlebowski & Mabrey, 1977). We have recently demonstrated that the metal ion content also influences the expression of negative cooperativity by this enzyme (Otvos et al., 1979b). By directly monitoring the amount of phosphate bound covalently or noncovalently to the enzyme using ³¹P NMR, we have shown that enzyme containing 2 equiv of metal ion can bind only a single mole of phosphate per enzyme dimer, hence exhibiting half-of-the-sites reactivity with respect to ligand binding. In contrast, enzyme containing its full complement of metal ion tightly binds 2 mol of phosphate to indistinguishable sites on the dimer. To investigate the molecular basis for this dependence of phosphate binding stoichiometry on the metal ion content of the enzyme, we have used ¹¹³Cd NMR of the ¹¹³Cd²⁺-substituted enzyme to probe the environments of the bound metal nuclei as a function of metal content and ligand binding.

The spin 1/2 isotope of cadmium, ¹¹³Cd, can, by isotopic enrichment, be made sensitive enough to detect satisfactory NMR signals from ¹¹³Cd²⁺ substituted at the Zn²⁺ sites of a number of metalloenzymes (Armitage et al., 1978). With a chemical shift range of >800 ppm, ¹¹³Cd NMR holds great

promise for providing a sensitive measure of the identity, coordination geometry, and conformational fluctuations of its protein ligands. In alkaline phosphatase, ¹¹³Cd NMR potentially offers the only method capable of simultaneously providing solution information about the occupancy and environment of all three classes of metal binding sites in the protein. The Cd²⁺-substituted enzyme is an active enzyme, but is >10² times slower than the Zn²⁺ enzyme as a molecular catalyst (Chlebowski & Coleman, 1976). This has presented an advantage in studying the nature of the reaction intermediates, since the slow turnover of the Cd²⁺ enzyme allows the covalent phosphoryl-enzyme intermediate to exist at high equilibrium concentrations near neutral pH (Chlebowski et al., 1977). Cd²⁺ is also more suitable as an analogue of Zn²⁺ than the transition metal ions, since it prefers similar coordination geometries and, like Zn²⁺, exhibits limited susceptibility to expansion of its coordination sphere.

Our preliminary ¹¹³Cd NMR results on alkaline phosphatase containing two ¹¹³Cd²⁺ ions have been reported (Chlebowski et al., 1977). In the absence of ligand a single resonance was observed, consistent with the occupation of the two A sites,¹ which are identical due to the twofold symmetry of the dimer. Upon covalent phosphorylation at a single active site, the single cadmium resonance split into two peaks, both of which had

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¹ The three pairs of metal binding sites in alkaline phosphatase, which have previously been designated as the "catalytic", "structural", and "regulatory" sites, will be referred to as A, B, and C sites, respectively (Otvos & Armitage, 1980). The A sites correspond to those containing four histidyl nitrogen ligands and possessing distorted tetrahedral or 5-coordinate geometry. The B and C sites are characterized by octahedral-like geometries and contain one and zero histidyl nitrogen ligands, respectively.

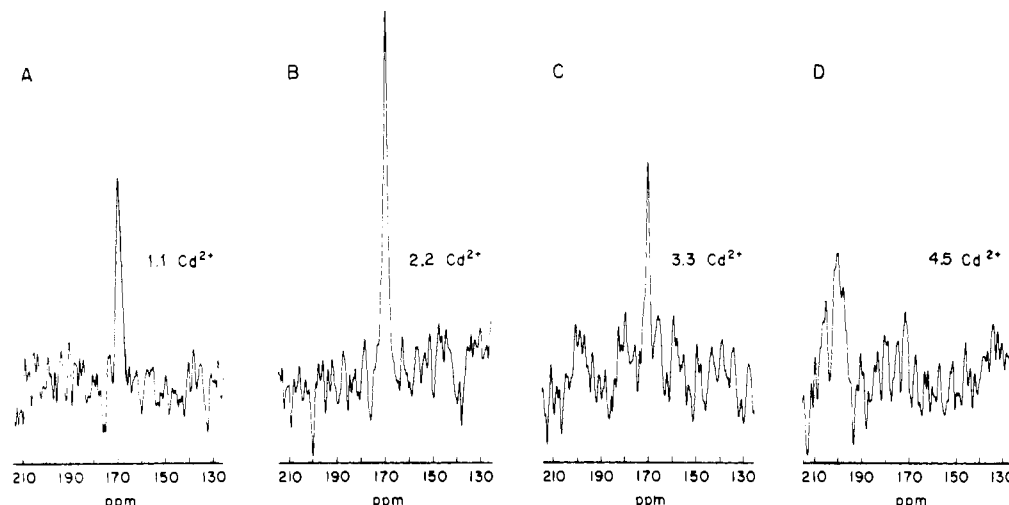


FIGURE 1: ^{113}Cd NMR spectra at 44.37 MHz of apophosphatase titrated with $^{113}\text{Cd}^{2+}$ at pH 6.2. The enzyme concentration was 1.6 mM, and aliquots of 0.37 M $^{113}\text{CdCl}_2$ were added to give the indicated stoichiometries. Each spectrum was acquired using a pulse angle of 35° , recycle time of 1.0 s, and 24 500 transients (6.8 h).

chemical shift values that were different from the original resonance. This result suggested that the environments of both A-site Cd^{2+} ions in the dimer were altered upon phosphorylation of only one subunit, perhaps as a result of the ligand-induced conformational change that had been proposed to account for the half-of-the-sites reactivity of the Cd_2^{2+} enzyme² (Chlebowski et al., 1977). An extension of these ^{113}Cd NMR studies to include enzyme containing its full complement of Cd^{2+} as well as Mg^{2+} is reported in this paper. Individual resonances have been detected and assigned to $^{113}\text{Cd}^{2+}$ bound to all three classes of metal binding sites in the protein. These resonances provide a sensitive monitor of the location and affinities of the metal ions bound to the enzyme and potentially provide the first experimental method for assessing the role played by each class of metal ion in the reaction mechanism.

The ^{113}Cd NMR data indicate that enzyme phosphorylation induces dramatic changes in the affinities of the different metal binding sites for Cd^{2+} , which is, in turn, responsible for altering the distribution of Cd^{2+} among the sites when substoichiometric amounts of metal ion are present. In light of this new information, we have altered our original interpretation of the origin of the spectral changes produced upon phosphorylation of the Cd_2^{2+} enzyme. The ^{113}Cd NMR results, combined with the ^{13}C NMR data presented in the previous paper (Otvos & Armitage, 1980), also suggest rational explanations for discrepancies that have appeared in previous reports concerning properties of the native Zn^{2+} enzyme such as metal content, phosphate content, catalytic activity, and negative cooperativity.

Materials and Methods

Enzyme Preparations. Alkaline phosphatase was isolated from *E. coli* (strain C90) by osmotic shock followed by purification by DEAE-cellulose chromatography (Applebury et al., 1970). Enzyme concentrations were determined spectrophotometrically at 280 nm with $E_{1\text{cm}}^{0.1\%} = 0.77$ (Browne & Otvos, 1976). For molar calculations, a molecular weight of 95 000 was used.³ This value is about 10% higher than had

been assumed at the time most of the reported NMR titrations were performed. As a consequence, many of the stoichiometries are nonintegral, reflecting the slight overestimation of molar protein concentration in these samples. Apophosphatase was prepared by dialysis of the Zn^{2+} enzyme at 4°C against four changes of 5×10^{-3} M α -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 eight changes of the same buffer, pH 6.5, for 4 to 6 days. 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 (spectrograde from Johnson Matthey Chemicals, Ltd., London) and $^{113}\text{CdCl}_2$ or $^{111}\text{CdCl}_2$ (prepared from the 96% isotopically enriched oxides from Oak Ridge Laboratory) to the apoenzyme. All enzyme solutions were in 0.01 M Tris-HCl, 0.01 M NaOAc, and 0.1 M NaCl at the pH values indicated.

NMR Methods. ^{113}Cd NMR spectra at 19.96 MHz were recorded on an extensively modified Bruker HFX-90 spectrometer (Armitage et al., 1978). ^{113}Cd and ^{111}Cd NMR spectra at 44.37 and 42.41 MHz, respectively, were obtained using a Bruker CXP-200 spectrometer equipped with a broad-band tunable probe. Measurements at the low and high frequencies were made at 25°C on 1- and 2-mL samples, respectively, contained in 10-mm NMR tubes fitted with Vortex plugs. Except for the NOE experiments, proton decoupling was not employed. D_2O present in a 3-mm coaxial capillary insert was used as a field-frequency lock. Chemical shifts are reported relative to the resonance position of 0.1 M $\text{Cd}(\text{ClO}_4)_2$. Digital broadening (35 Hz) was employed to enhance the S/N of all spectra. Spin-lattice relaxation times (T_1 s) were measured using the progressive saturation method (Freeman et al., 1972). NOEs are expressed as the integrated intensity of a signal in the presence of continuous ^1H decoupling divided by its intensity in ^1H coupled or gated ^1H decoupled spectra.

Results

^{113}Cd NMR of $^{113}\text{Cd}^{2+}$ Alkaline Phosphatase. Titration of the apoenzyme with $^{113}\text{Cd}^{2+}$ at pH 6.2 gives rise to the

² The number of metal ions added to apoalkaline phosphatase is indicated by the subscript number following the metal symbol; e.g., Cd_2^{2+} identifies apoenzyme to which 2 g-atoms of Cd^{2+} /mol of enzyme has been added.

³ This molecular weight value was calculated from recent sequence data which indicates the presence of about 450 amino acid residues per monomer (R. A. Bradshaw, personal communication).

Table I: ^{113}Cd Chemical Shifts and Relaxation Parameters

figure no.	sample composition ^a	chemical shift (ppm)	rel $^{113}\text{Cd}^{2+}$ content ^b	T_1 (s) ^c	NOE
2A	2.2 $^{113}\text{Cd}^{2+}$	168.8	2.2	5.2	0.0
2B	2.2 $^{113}\text{Cd}^{2+}$, 2.2 P_i	141.4	1.1	6.4	
		55.8	0.9	6.2	
2C	2.2 $^{113}\text{Cd}^{2+}$, 2.2 P_i , 2.2 Mg^{2+}	156.3	0.9	7.8	
		75.1	1.1	7.8	
2D	2.2 $^{113}\text{Cd}^{2+}$, 2.2 P_i , 2.2 Mg^{2+} , 2.2 $^{113}\text{Cd}^{2+}$	158.7	2.0	5.6	
		76.0	1.3	6.1	
		70.1	0.6	5.8	
		2.0	0.5		
7C	4.4 ^{113}Cd , 3.3 P_i , 2.2 Mg^{2+}	158.5		3.6 (5.9) ^d	0.4
		75.8		5.6 (6.0) ^d	0.7
		69.6		(3.9) ^d	1.1
		2.0			<0.5

^a Metal ions and phosphate are listed in the order to which they were added to apoenzyme. ^b The relative amount of $^{113}\text{Cd}^{2+}$ represented by each resonance was calculated by scaling the integrated peak areas (after correcting for differential attenuation resulting from different T_1 values (Gupta, 1977)) to the total peak area of Figure 2D, which was assumed to represent all 4.4 $^{113}\text{Cd}^{2+}$ ions in the sample. For this calculation, the T_1 value of the 2.0-ppm resonance was assumed to be 6.0 s, since its low intensity precluded a T_1 measurement. ^c T_1 values at 19.96 MHz were obtained by progressive saturation using three to five pulse delay times. Estimated error is $\pm 25\%$. ^d Values in parentheses are T_1 values at 44.37 MHz obtained by progressive saturation using four pulse delay times. Estimated error is $\pm 25\%$.

spectra shown in Figure 1. To enable intensity comparisons to be made within this series, all spectra were acquired under identical conditions. Addition of 1.1 equiv of $^{113}\text{Cd}^{2+}$ to the dimer gives rise to a single resonance at 169 ppm (Figure 1A), the intensity of which approximately doubles when 2.2 equiv of $^{113}\text{Cd}^{2+}$ is present (Figure 1B). The absolute chemical shift of this resonance has previously been found to be sensitive to ionic strength and the composition of the medium, varying from about 120 to 180 ppm as a function of halide concentration, although under all conditions only a single resonance is detected (Coleman et al., 1979). This result indicates that the first two Cd^{2+} ions added to the apoenzyme dimer are bound to identical sites, consistent with a parallel ^{13}C NMR experiment conducted under identical conditions which demonstrated selective occupancy of the pair of symmetrically disposed A metal binding sites in Cd_2^{2+} enzyme (Otvos & Armitage, 1980). The effects on the ^{113}Cd NMR spectrum of additional $^{113}\text{Cd}^{2+}$ bound to the enzyme are shown in Figures 1C and 1D. As shown by the ^{13}C NMR results in the preceding paper (Otvos & Armitage, 1980), these additional Cd^{2+} ions apportion themselves approximately equally between the competing B and C sites until both sites become fully occupied in the Cd_6^{2+} enzyme. In view of this, it is interesting that no new resonances are detected in Figures 1C and 1D corresponding to $^{113}\text{Cd}^{2+}$ bound at the B and C sites. Furthermore, the resonance at 169 ppm assigned to the A-site $^{113}\text{Cd}^{2+}$ ions decreases in intensity as the B and C sites become populated. When 4.5 equiv of $^{113}\text{Cd}^{2+}$ has been added (Figure 1D), the intensity of the A-site resonance has decreased by about 80% compared to Figure 1B and a barely detectable broad resonance is observed at about 200 ppm. In spectra of the $^{113}\text{Cd}_6^{2+}$ enzyme, where the A, B, and C sites are fully occupied, no resonance can be detected (see Figure 3A). Only when $^{113}\text{Cd}^{2+}$ in excess of 6 equiv is present is a resonance with a chemical shift corresponding to free $^{113}\text{Cd}^{2+}$ ion observed (unpublished result).

One possible explanation for the inability to detect B- and C-site resonances is that the spin-lattice relaxation times, T_1 , for these resonances are much longer than for the A-site $^{113}\text{Cd}^{2+}$. Similarly, the progressive decrease in the area of the A-site resonance upon occupation of the B and C sites might indicate signal attenuation due to an increase in T_1 for those A-site $^{113}\text{Cd}^{2+}$ ions in subunits containing an occupied B or C site. A signal attenuation of this magnitude would require

that the T_1 value increase by at least a factor of 4 to a value of >20 s. From a comparison with the T_1 values for a variety of $^{113}\text{Cd}^{2+}$ alkaline phosphatase species (Table I) and from theoretical considerations (see relaxation section), a T_1 value of >20 s is considered highly unlikely.

A more reasonable explanation for the intensity modulation of the A-site resonance as well as for the failure to observe a B- or C-site resonance is chemical exchange broadening, which renders the resonances too broad to detect under our experimental conditions. Several such instances of severe exchange broadening have been reported for $^{113}\text{Cd}^{2+}$ in small coordination complexes as well as in proteins and are a direct consequence of the extreme sensitivity of the ^{113}Cd chemical shift to changes in the geometry and identity of the ligands in its coordination sphere (Kostelnik & Bothner-By, 1974; Cardin et al., 1975; Haberkorn et al., 1976; Armitage et al., 1978). The nature of the chemical exchange mechanism(s) operating in alkaline phosphatase is not presently known. Since the exchange rates required to induce excess broadening depend upon the chemical shift differences between exchanging species, and these are unknown because no resonances are observed, the modulating rates can only be estimated. For chemical shift differences of from about 10 to 100 ppm, excess broadening might be expected to occur with exchange rates on the order of 10^2 to 10^4 s⁻¹. The facts that ^{13}C NMR shows Cd^{2+} to be in slow exchange with the B and C sites (Otvos & Armitage, 1980) and that addition of $^{113}\text{Cd}^{2+}$ in excess of 2 equiv to the enzyme does not broaden the residual 169-ppm A-site resonance (Figures 1B, 1C, and 1D) indicate that exchange of B- or C-site Cd^{2+} with free Cd^{2+} is too slow to provide the required chemical exchange modulation. Instead, binding of Cd^{2+} to the B or C sites may induce a local conformational fluctuation or ligand exchange process which creates an intermediate exchange situation for the A-site Cd^{2+} ion. Whether or not the same modulation that is responsible for the loss of the A-site resonance is also responsible for the inability to detect a B- or C-site resonance is not known. The likelihood that the A and B sites are located very close to one another in each monomer (H. W. Wyckoff, personal communication) certainly suggests this might be the case for B-site $^{113}\text{Cd}^{2+}$, since all that is required to link two metal ions to the same exchange process is that both their chemical shifts be modulated to a similar extent by the same event.

^{113}Cd NMR of Phosphoryl $^{113}\text{Cd}^{2+}$ Alkaline Phosphatase.

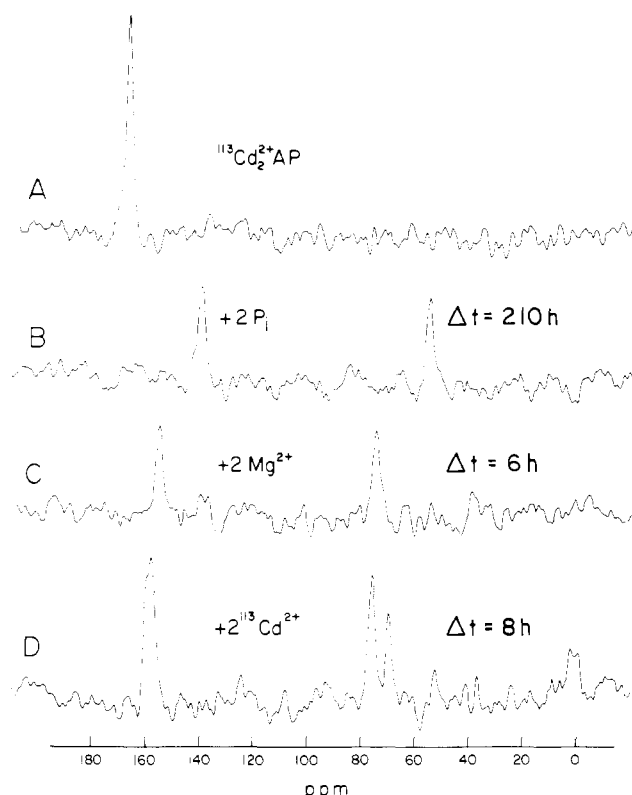


FIGURE 2: ^{113}Cd NMR spectra at 19.96 MHz of phosphoryl $^{113}\text{Cd}^{2+}$ alkaline phosphatase at pH 6.3. Each spectrum was acquired using a pulse angle of 90° , recycle time of 8.0 s, and 10 000 transients (22.2 h): (A) 2.8 mM apophosphatase reconstituted with 2.2 equiv of $^{113}\text{Cd}^{2+}$; (B) A plus 2.2 equiv of P_i ; (C) B plus 2.2 equiv of Mg^{2+} ; (D) C plus 2.2 equiv of $^{113}\text{Cd}^{2+}$. The Δt values refer to the time interval between metal or P_i addition and the midpoint of spectral acquisition.

Addition of inorganic phosphate to alkaline phosphatase results in the formation of a noncovalent complex, E-P, and a covalent complex, E-P, arising from phosphorylation of a specific serine residue at the active site (Applebury et al., 1970). The distribution of enzyme-bound phosphate between these forms is governed by both pH and the nature of the metal ion bound to the protein. In the Cd^{2+} enzyme at pH values below 6.5, nearly 100% of the bound phosphate exists as E-P under equilibrium conditions. ^{31}P NMR and ^{32}P radiolabeling experiments show that only half the enzyme active sites can be phosphorylated in the Cd_2^{2+} enzyme (Otvos et al., 1979b). The "half-of-the-sites" phosphorylation exhibited by Cd_2^{2+} enzyme has been suggested to result from ligand-induced conformational changes communicated across the subunit-subunit interface, which abolishes the affinity of the second active site for phosphate. Experimental evidence consistent with this hypothesis was provided by preliminary ^{113}Cd NMR results (Chlebowski et al., 1977), which showed that the single resonance at 169 ppm in the $^{113}\text{Cd}_2^{2+}$ enzyme (Figure 2A) was converted into two resonances at 141 and 56 ppm upon phosphorylation of one of the two subunits of the dimer (Figure 2B). The fact that both resonances in the monophosphoryl enzyme had chemical shift values that differed from the position of the original resonance in the unliganded enzyme suggested that the environments of *both* A-site $^{113}\text{Cd}^{2+}$ ions had been altered by phosphorylation at only one of these sites, presumably as the result of a conformational change.

By adding an additional pair of Cd^{2+} ions and 2 equiv of Mg^{2+} to the Cd_2^{2+} monophosphoryl enzyme, it was demonstrated by ^{31}P NMR that both active sites on the dimer can be phosphorylated to give an apparently symmetrical di-

phosphorylated species (Otvos et al., 1979b). On the basis of the previous argument, one might anticipate that the two A-site ^{113}Cd resonances in the asymmetric monophosphoryl $^{113}\text{Cd}_2^{2+}$ enzyme would again coalesce into a single peak upon conversion to the symmetric diphosphoryl $^{113}\text{Cd}_4^{2+}, \text{Mg}_2^{2+}$ enzyme. In fact, as shown in Figure 2, this result was not observed. Addition of 2 Mg^{2+} to the $^{113}\text{Cd}_2^{2+}$ monophosphoryl enzyme (Figure 2C) resulted in a deshielding of both resonances. Subsequent binding of a second pair of $^{113}\text{Cd}^{2+}$ ions to the enzyme (Figure 2D), which induces diphosphorylation of the dimer, gave rise to a doubling in the area of the downfield resonance and to the appearance of three resonances at 76, 70, and 2 ppm in the upfield region.

Several aspects of the spectrum in Figure 2D are noteworthy. First, integration of the spectrum indicates that, within experimental error, twice the resonance intensity is observed compared to that in Figures 2A, 2B, and 2C (Table I). In other words, all $^{113}\text{Cd}^{2+}$ ions in the diphosphorylated enzyme give rise to their expected resonance intensities. This is in striking contrast to the almost total absence of resonance intensity in the ^{113}Cd spectrum of the unphosphorylated enzyme in Figure 1D and suggests that phosphorylation slows down or abolishes the exchange process(es) responsible for the chemical exchange broadening observed in the unliganded enzyme.

Since the resonances in Figure 2D represent all the $^{113}\text{Cd}^{2+}$ ions bound to the diphosphorylated protein, it is of obvious interest to be able to assign them to specific classes of metal binding sites. In Figure 2D, as well as in numerous other spectra of the phosphorylated enzyme taken under a variety of conditions, it is reproducibly observed that resonance intensity is equally divided between the downfield (130 to 160 ppm) and upfield (56 to 76 ppm) regions of the spectrum. This distribution strongly suggests that resonances located in these two regions correspond to $^{113}\text{Cd}^{2+}$ occupying either A or B metal binding sites (reasons will be given below for not attributing these resonances to C-site $^{113}\text{Cd}^{2+}$). From the known dependence of ^{113}Cd chemical shift on the nature of the ligands within its first coordination sphere and the current knowledge of the identity of the protein ligands to the metal ions at the A and B binding sites (Otvos & Armitage, 1980), we were able to tentatively assign the downfield and upfield resonances to the A- and B-site $^{113}\text{Cd}^{2+}$ ions, respectively (see Discussion). Unambiguous confirmation of this assignment was subsequently provided by a ^{13}C NMR experiment (Figure 4 in Otvos & Armitage, 1980) based on the differential susceptibility of A- and B-site Cd^{2+} to isotope exchange (see below).

The assignment of the downfield and upfield resonances in Figure 2D to $^{113}\text{Cd}^{2+}$ coordinated to different classes of metal binding sites necessitates a reevaluation of the previous assignment of the 141- and 56-ppm peaks in the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme spectrum in Figure 2B to A-site $^{113}\text{Cd}^{2+}$ ions (Chlebowski et al., 1977). As previously mentioned, the large chemical shift difference between these peaks was originally attributed to conformational differences between the subunits induced by the negatively cooperative interaction of phosphate with the enzyme. In view of the above data on enzyme containing its full complement of metal ion, it is more reasonable to assign these two resonances to $^{113}\text{Cd}^{2+}$ bound to an A and B site rather than to the two A sites on the dimer. Thus, although both $^{113}\text{Cd}^{2+}$ ions in the unliganded $^{113}\text{Cd}_2^{2+}$ enzyme originally occupy the relatively "tight" A binding sites, there is an apparent migration of metal ion that accompanies phosphorylation such that at equilibrium the most stable species is one which contains $^{113}\text{Cd}^{2+}$ bound in equal amounts

to an A and B binding site. This arrangement then leaves the A site on the unphosphorylated subunit of the dimer vacant. Since metal ion occupancy of the A site is an absolute requirement for interaction of enzyme with phosphate (Applebury et al., 1970), there can be no phosphorylation of the other subunit, thereby accounting for the half-of-the-sites reactivity displayed by the Cd_2^{2+} enzyme (see Discussion). An additional pair of $^{113}\text{Cd}^{2+}$ ions added to the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme selectivity binds to the vacant A and B sites, thereby allowing rapid phosphorylation by a second equivalent of phosphate. This in turn leads to the expected observation of twice the resonance intensity in both the upfield and downfield regions of the ^{113}Cd NMR spectrum (Figure 2D).

It must be emphasized here that the ^{113}Cd NMR spectrum in Figure 2B monitors the distribution of the metal ions in the enzyme under equilibrium conditions. Establishment of equilibrium is apparently a very slow process, since the spectrum in Figure 2B was obtained only after waiting 10 days following addition of phosphate to the enzyme. Spectra taken prior to the attainment of equilibrium exhibited very poor signal-to-noise characteristics with resonances detected primarily in the downfield region. Confirmation that phosphate addition to the Cd_2^{2+} enzyme brings about a slow migration of half the Cd^{2+} from the A sites to the B sites was provided by ^{13}C NMR data in the accompanying paper (Figure 5 in Otvos & Armitage, 1980). The time course for covalent phosphorylation of the Cd_2^{2+} enzyme as monitored by ^{31}P NMR is also found to be very slow (results not shown). Establishment of equilibrium (1 equiv of phosphate bound per dimer) is achieved only after several days.

An additional aspect of the spectrum in Figure 2D that requires comment is that the upfield region, which we have assigned as the region containing resonances arising from B-site $^{113}\text{Cd}^{2+}$ ions, contains two resonances at 76 and 70 ppm instead of the single resonance one would anticipate for a pair of metal ions bound to identical sites on a symmetric dimer. As we show below, the origin of the two B-site ^{113}Cd resonances is heterogeneity in the protein sample created by competition between Mg^{2+} and excess $^{113}\text{Cd}^{2+}$ for the available C sites on the enzyme. When full occupancy of the C sites by Mg^{2+} is achieved, which can be accomplished by taking care not to exceed a stoichiometry of 4.0 equiv of Cd^{2+} when preparing the $^{113}\text{Cd}_4^{2+}, \text{Mg}_2^{2+}$ diphosphoryl-enzyme as in Figure 2D, a spectrum characteristic of a homogeneous species is observed which exhibits single A- and B-site resonances at 159 and 76 ppm, respectively (unpublished results). Alternatively, in the absence of Mg^{2+} and in the presence of 6 equiv of $^{113}\text{Cd}^{2+}$, a homogeneous diphosphoryl-enzyme with the C sites occupied by $^{113}\text{Cd}^{2+}$ can be prepared which exhibits single A- and B-site resonances at 160 and 70 ppm, respectively (Figure 3B).

The generation of the $^{113}\text{Cd}_6^{2+}$ diphosphoryl-enzyme species is depicted in the spectral series in Figure 3. The starting point in this experiment is apoenzyme to which 6.0 equiv of $^{113}\text{Cd}^{2+}$ has been added (Figure 3A). As discussed in the previous section, the A, B, and C sites are fully occupied by Cd^{2+} under these conditions, yet no resonances are detected due to the presence of excessive chemical exchange broadening. Following addition of 2 equiv of inorganic phosphate, however, three resonances are observed at 160, 70, and 2 ppm (Figure 3B). Unlike the slow approach to equilibrium observed for the generation of the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme spectrum (Figure 2B), the spectrum in Figure 3B was acquired immediately following phosphate addition. A ^{31}P NMR spectrum of this sample showed that the enzyme was diphosphorylated. The most interesting feature of this spectrum,

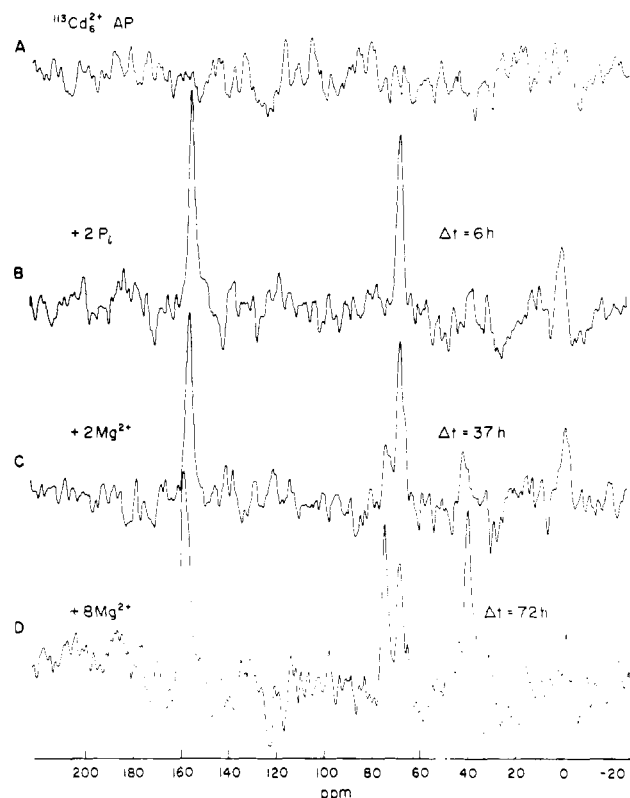


FIGURE 3: The effect of Mg^{2+} on phosphoryl $^{113}\text{Cd}^{2+}$ alkaline phosphatase at pH 6.3. Each ^{113}Cd NMR spectrum was recorded at 19.96 MHz using a pulse angle of 45° , recycle time of 2.0 s, and 20000 transients (11.1 h): (A) 1.43 mM apophosphatase reconstituted with 6.0 equiv $^{113}\text{Cd}^{2+}$; (B) A plus 2.0 equiv of P_i ; (C) B plus 2.0 equiv of Mg^{2+} ; (D) C plus 8.0 equiv of Mg^{2+} . The Δt values refer to the time interval between metal or P_i addition and the midpoint of spectral acquisition.

besides the homogeneous nature of the A- and B-site resonances, is the presence of the relatively broad resonance at 2 ppm. A resonance with the same chemical shift was also observed in the spectrum in Figure 2D. From its high-field chemical shift, it can be inferred that the ligands to this $^{113}\text{Cd}^{2+}$ ion are predominantly, or exclusively, oxygen atoms (Haberkorn et al., 1976). Furthermore, the resonance does not arise from Cd^{2+} ion free in solution, since the chemical shift of free $^{113}\text{Cd}^{2+}$ under these conditions (0.1 M NaCl, pH 6.3) is located at about 40 ppm. Therefore, it is most likely that the 2-ppm resonance corresponds to $^{113}\text{Cd}^{2+}$ located at the pair of C sites on the dimer. Confirmation of this assignment is provided by the ability of Mg^{2+} to compete with $^{113}\text{Cd}^{2+}$ for this site. The spectral changes seen in Figure 3C are the result of the addition of 2 equiv of Mg^{2+} to the sample. Based on the relative intensities of the 76- and 70-ppm resonances, and assuming that the chemical shift of the B-site $^{113}\text{Cd}^{2+}$ ions will be 76 ppm when the C sites are occupied by Mg^{2+} and 70 ppm when occupied by Cd^{2+} , only about 10–15% of the C sites contain Mg^{2+} after 37 h of incubation at 22°C . This suggests that either the C site favors Cd^{2+} over Mg^{2+} or that equilibrium has not been achieved after 37 h due to the very slow dissociation of Cd^{2+} from this site. To distinguish between these alternatives, an excess of Mg^{2+} (an additional 8 equiv) was added to the sample. As shown in Figure 3D, the intensity of the 76-ppm resonance has increased at the expense of the signal at 70 ppm, such that after 72-h incubation the two species are present in approximately equal amounts. The resulting displacement of some of the $^{113}\text{Cd}^{2+}$ from the C sites by Mg^{2+} is confirmed by the observation in Figures 3C and 3D of increasing intensity in the 42-ppm resonance, which

arises from free $^{113}\text{Cd}^{2+}$ ion no longer bound to the protein. That the 72-h spectrum in Figure 3D indeed represents an equilibrium situation, which reflects the fact that the C sites have a greater affinity for Cd^{2+} than for Mg^{2+} , is indicated by the fact that this spectrum is identical with several spectra acquired in the intervening time interval. Establishment of equilibrium does appear to require about 10 h, however, since the first spectrum acquired immediately following the addition of the 2- and 8-equiv aliquots of Mg^{2+} differed from later spectra such as those in Figures 3C and 3D, respectively (results not shown). From this information, it is possible to estimate an off-rate of Cd^{2+} from the C sites of $\approx 10^{-4} \text{ s}^{-1}$. In addition, assuming about half the C sites in the enzyme sample used in Figure 3D are occupied by Cd^{2+} and half by Mg^{2+} , it can be calculated that the equilibrium binding constant for Mg^{2+} to these sites is about an order of magnitude lower than for Cd^{2+} .

^{113}Cd and ^{111}Cd NMR of Hybrid ^{113}Cd – ^{111}Cd Phosphoryl Alkaline Phosphatase. The spectra in Figure 3 demonstrated that excess Mg^{2+} can compete with Cd^{2+} for the C sites, but not for the A and B sites. In order to obtain information concerning the relative affinities of Cd^{2+} for the A and B sites, advantage was taken of the ability to observe Cd NMR signals from another spin $1/2$ isotope of cadmium, ^{111}Cd . ^{111}Cd and ^{113}Cd possess nearly identical NMR properties and both are commercially available at >95% isotopic enrichment. At the magnetic field strength employed to obtain the spectra below (47 kG), the two isotopes have resonance frequencies differing by approximately 2 MHz, allowing each to be detected separately by tuning the spectrometer to the appropriate frequency. By adding an excess of, for instance, $^{111}\text{Cd}^{2+}$ to the $^{113}\text{Cd}^{2+}$ phosphoryl enzyme, it is possible to monitor the kinetics of the displacement of $^{113}\text{Cd}^{2+}$ from the A and B sites by observing either the loss of ^{113}Cd NMR signal from the 160- and 70-ppm resonances or the increase in ^{111}Cd NMR resonance intensity at these two positions.

A kinetic experiment employing both ^{113}Cd and ^{111}Cd NMR to monitor the stabilities of the A- and B-site Cd^{2+} ions is shown in Figure 4. The starting point in this series of spectra is apoenzyme, to which 2 equiv each of $^{113}\text{Cd}^{2+}$ and $^{111}\text{Cd}^{2+}$ has been added, followed by an excess (3 equiv) of phosphate. The ^{111}Cd spectrum of this sample is shown in Figure 4A. An identical spectrum was obtained by tuning the spectrometer to observe ^{113}Cd (spectrum not shown). Thus, the $^{113}\text{Cd}^{2+}$ and $^{111}\text{Cd}^{2+}$ ions are uniformly distributed among the metal binding sites and give rise to four resonances at 156, 141, 70, and 56 ppm. The origin of the two pairs of resonances is again, as in Figure 2D, heterogeneity in the protein sample caused by the strong affinity of Cd^{2+} for the C sites. One pair, the 141- and 56-ppm resonances, arises from Cd^{2+} bound to the A and B sites, respectively, in subunits containing a vacant C site, while the 156- and 70-ppm pair arises from A- and B-site Cd^{2+} in subunits containing Cd^{2+} at the C site (see Discussion). Incubation of the sample for several days does not appreciably alter the spectrum, in keeping with the strong affinity of Cd^{2+} for the C sites demonstrated in Figure 3 (the 2-ppm resonance corresponding to C-site Cd^{2+} is not observed in the spectra in Figure 4 because the frequency range employed did not extend far enough upfield). The ^{113}Cd NMR spectrum acquired immediately following the addition of 6 equiv of $^{111}\text{Cd}^{2+}$ to the sample is shown in Figure 4B. As expected, only two resonances are observed at 160 and 70 ppm, corresponding to the presence of a single equiv of $^{113}\text{Cd}^{2+}$ at the A and B sites, respectively. No evidence of the heterogeneity present in Figure 4A is observed because the excess $^{111}\text{Cd}^{2+}$ has fully

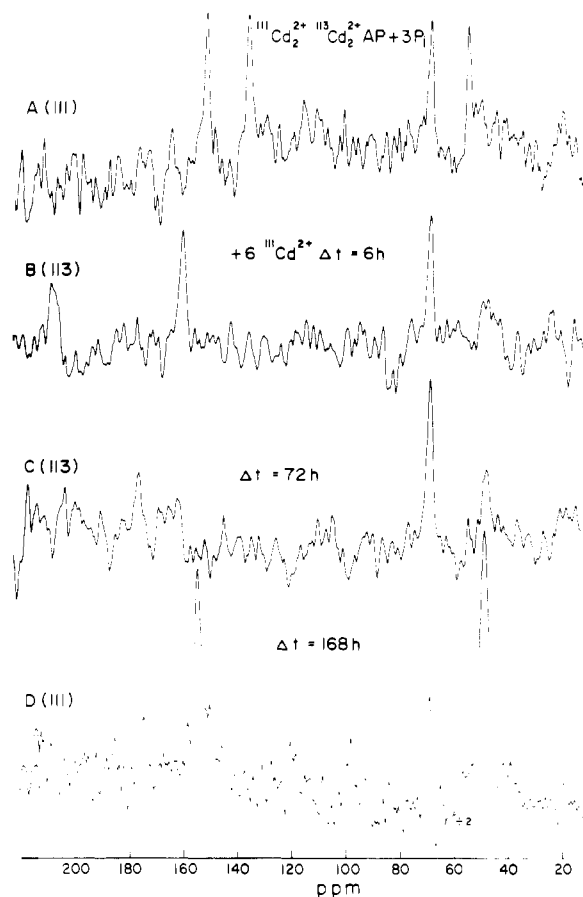


FIGURE 4: ^{113}Cd and ^{111}Cd NMR spectra at 44.37 and 42.41 MHz, respectively, of hybrid ^{113}Cd – ^{111}Cd phosphoryl alkaline phosphatase at pH 6.3. ^{113}Cd was monitored in spectra A and D, ^{111}Cd in spectra B and C. Spectrum A was acquired using a pulse angle of 50° , recycle time of 5.0 s, and 6000 transients (8.3 h). Spectra B, C, and D were acquired using a pulse angle of 40° , recycle time of 2.0 s, and 20 000 to 30 000 transients (11.1 to 16.7 h): (A) 2.03 mM apophosphatase reconstituted with 2.2 equiv of $^{113}\text{Cd}^{2+}$, 2.2 equiv of $^{111}\text{Cd}^{2+}$, and 3.3 equiv of P_i ; (B, C, and D) A plus 6.6 equiv of $^{111}\text{Cd}^{2+}$. The Δt values refer to the time interval between the addition of $^{111}\text{Cd}^{2+}$ and the midpoint of spectral acquisition.

populated the pair of C sites on the dimer. At this stage, there has been no significant replacement of either A- or B-site $^{113}\text{Cd}^{2+}$ by the excess $^{111}\text{Cd}^{2+}$ in the sample. However, after allowing the sample to equilibrate for 3 days, the situation is quite different. As shown by the ^{113}Cd NMR spectrum in Figure 4C, no detectable A-site resonance at 160 ppm is observed, while the B-site resonance at 70 ppm remains unaltered. Replacement of the large majority of the original 1 equiv of $^{113}\text{Cd}^{2+}$ located at the A site by $^{111}\text{Cd}^{2+}$ is confirmed both by the observation of a free $^{113}\text{Cd}^{2+}$ resonance at 48 ppm in Figure 4C and by the ^{111}Cd NMR spectrum in Figure 4D, which from the relative intensities of the 160- and 70-ppm peaks indicates that there is now approximately double the amount of $^{111}\text{Cd}^{2+}$ in the A sites compared to the B sites (the large peak at 48 ppm arises from the excess free $^{111}\text{Cd}^{2+}$ in the sample). From this and similar data, the off-rate of Cd^{2+} from the A site is estimated to be about 10^{-4} – 10^{-5} s^{-1} . The most surprising finding provided by the data in Figure 4, however, is the extremely strong affinity of Cd^{2+} for the B sites in the phosphorylated enzyme. No exchange of B-site Cd^{2+} with excess metal ion was detected in this sample and in similar experiments even after incubation for several weeks. The off-rate of Cd^{2+} from the B sites must therefore be at least two orders of magnitude slower than from the A sites, or $<10^{-7} \text{ s}^{-1}$. This result demonstrates that there is a dramatic increase

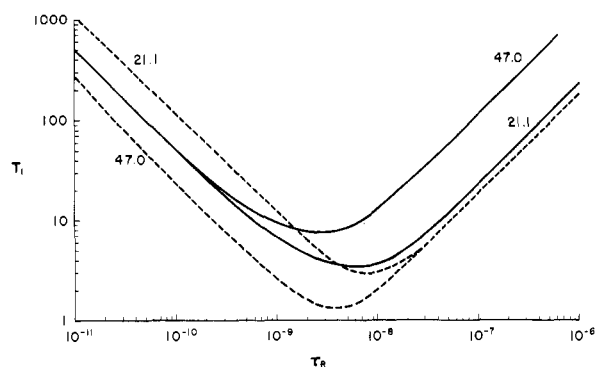


FIGURE 5: Theoretical plots of T_1 vs. correlation time, τ_R (both in seconds), for ^{113}Cd relaxed by the dipolar (solid line) or chemical shift anisotropy (dashed line) mechanisms at two magnetic field strengths (21.1 and 47.0 kG). The calculations were made using the isotropic rigid rotor model (Doddrell et al., 1972; Norton et al., 1977) with the parameters described in the text.

in the affinity of Cd^{2+} for the B sites that accompanies phosphorylation of the enzyme. Such a process clearly provides sufficient driving force to induce a redistribution of metal ion in enzyme which is exposed to phosphate (see Discussion).

Spin-Lattice Relaxation Properties. To date, very little information is available concerning the contributions from different relaxation mechanisms to ^{113}Cd spin-lattice relaxation in macromolecular systems. A quantitative analysis of the relaxation properties of $^{113}\text{Cd}^{2+}$ bound to a protein can, in principle, provide detailed information concerning molecular motion at the metal binding site. Our interest in this information has prompted us to report here a preliminary analysis of ^{113}Cd relaxation in alkaline phosphatase that utilizes existing theory and follows an approach that parallels that applied in the early stages of ^{13}C NMR investigations on macromolecules (Doddrell et al., 1972).

As a starting point, we considered only motion arising from overall isotropic molecular reorientation. This assumption allows theoretical calculations of T_1 and NOE to be made using the standard isotropic rigid rotor expressions (Doddrell et al., 1972; Norton et al., 1977). In the absence of a high-resolution crystal structure, additional assumptions about the environments of the bound $^{113}\text{Cd}^{2+}$ ions are necessary in order to calculate the contribution to relaxation from dipolar interactions with nuclei within the coordination spheres of the metal ions. Since the ligands to the A-site Cd^{2+} ions have been best characterized (four histidine residues, two in the N^+ tautomeric form and two in the N^* form), we have included in the calculations a dipolar contribution from 10 histidyl protons at an average distance of 3.7 Å and four nitrogens at 2.3 Å. In addition, two protons at 2.9 Å are included from a coordinated water molecule, which is believed to complete the coordination sphere of the A-site metal ion. Because of the relatively long distances involved for these dipolar interactions, contributions from other relaxation mechanisms are likely to be important. From relaxation studies on several small $^{113}\text{Cd}^{2+}$ complexes (our unpublished results) a relatively large contribution from chemical shift anisotropy (CSA) is anticipated. Since values for the anisotropy of the chemical shift ($\Delta\sigma$) for ^{113}Cd in suitable model complexes have not as yet been measured, we have assumed a value for $\Delta\sigma = 200$ ppm in the calculations.

The theoretical T_1 curves for the dipolar and CSA mechanisms at two magnetic field strengths are shown in Figure 5. The correlation time for alkaline phosphatase has previously been determined to be about 7×10^{-8} s (Hull & Sykes, 1975). From the theoretical plots, it is seen that at this

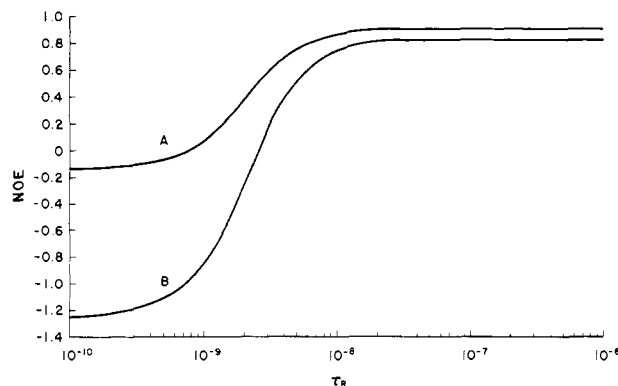


FIGURE 6: Theoretical plots of NOE vs. correlation time, τ_R (in seconds), for ^{113}Cd at 21.1 kG assuming that the contribution to overall relaxation from proton dipolar relaxation is 50% (curve A) or 100% (curve B). The calculations were made using the isotropic rigid rotor model (Doddrell et al., 1972) with the parameters described in the text.

correlation time the dipolar contribution exhibits a large field dependence while the CSA mechanism is field independent. Therefore, if relaxation occurs exclusively via the dipolar mechanism one would expect approximately a factor of four increase in T_1 when going from 21.1 to 47.0 kG. As shown in Table I, the experimental T_1 values are similar at the two fields. Furthermore, the absolute dipolar T_1 values of ~16 and 80 s at 21.1 and 47.0 kG, respectively, which are predicted by this simple model for $\tau_R = 7 \times 10^{-8}$ s, are much longer than the experimental values. Chemical shift anisotropy must therefore make a major contribution to overall relaxation, particularly at the high magnetic field strength. Summation of the contributions from both dipolar and CSA mechanisms at $\tau_R = 7 \times 10^{-8}$ s predicts T_1 values of 7 and 11 s at 21.1 and 47.0 kG, respectively, in reasonable agreement with the experimental values.

Whereas the T_1 data can be accounted for reasonably well by the isotropic rigid rotor model, the NOE data clearly show that this model is oversimplified. Theoretical NOE curves at 21.1 kG are shown in Figure 6 for the isotropic model assuming a 50 and 100% dipolar contribution to relaxation. The maximum $^{113}\text{Cd}\{-^1\text{H}\}$ NOE is negative because of the negative value of the nuclear magnetic moment for ^{113}Cd . As defined, an NOE value of zero would result in the complete loss of a ^{113}Cd resonance. Both curves exhibit an identical dependence of the NOE on τ_R but differ significantly in the maximum absolute values. From the considerations discussed above for the T_1 data, curve A is more likely to be representative of the magnitude of the dipolar contribution to relaxation in alkaline phosphatase. Despite the uncertainty in the degree to which protons contribute to the overall relaxation, at the correlation time of 7×10^{-8} s a minimum isotropic NOE of ~0.9 is predicted from this plot. As shown by the data in Figure 7 and Table I, a much larger NOE is in fact observed for ^{113}Cd in both the unliganded and phosphorylated enzyme. In the unliganded enzyme, no resonance is observed under conditions of continuous proton decoupling in Figure 7B, corresponding to a zero NOE. Significantly different results are obtained for the diphosphorylated enzyme shown in Figures 7C and 7D. With continuous proton decoupling, the three resonances at 159, 76, and 70 ppm are observed, although the absolute value of the NOE (Table I) differs for each resonance, reflecting differences in their respective protein environments.

The observation of a significant NOE for $^{113}\text{Cd}^{2+}$ bound to alkaline phosphatase demonstrates that the isotropic rigid rotor model does not provide an adequate description of the dipolar

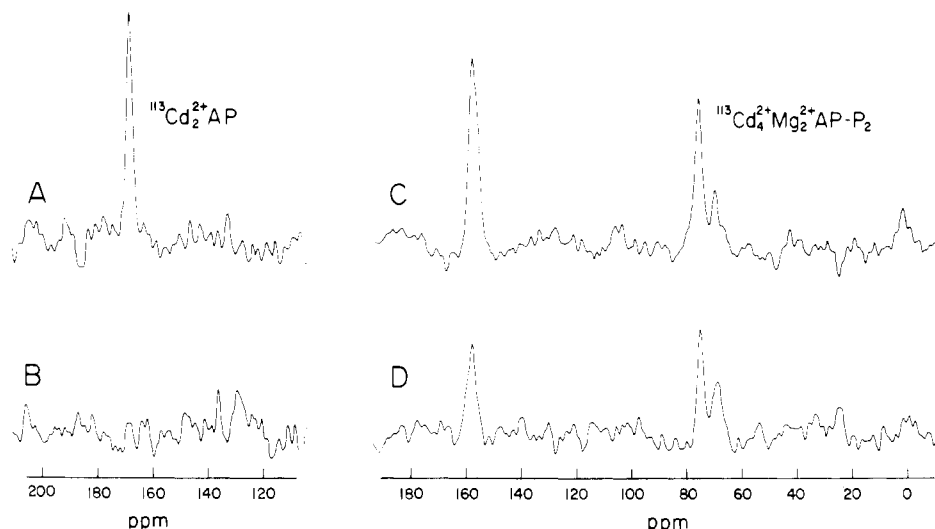


FIGURE 7: ^{113}Cd - ^1H NOE determinations at 21.1 kG on ^{113}Cd alkaline phosphatase at pH 6.3. Spectra A and B are of apophosphatase reconstituted with 2.2 equiv of $^{113}\text{Cd}^{2+}$ acquired with a pulse angle of 90° , recycle time of 20.0 s, and 2100 transients. A was gated proton decoupled and B was continuously proton decoupled. Spectra C and D are of apophosphatase reconstituted with 4.4 equiv of ^{113}Cd , 2.2 equiv of Mg^{2+} , and 2.2 equiv of P_i acquired with a pulse angle of 90° , recycle time of 8.0 s, and 10 000 transients. C was proton coupled and D was continuously proton decoupled.

relaxation in this system. Clearly, a dipolar modulation process at a more effective relaxation frequency than overall protein reorientation is required. Internal rotation of the protein's metal ion ligands could provide the necessary modulation; however, the degree of motion that is required is considered to be extremely unlikely due to strict steric constraints resulting from hydrogen bonding interactions that are usually present on metal ion ligands in metalloproteins. A more reasonable modulation process might be provided by the protons on one or more metal ion coordinated water molecules. This internal motion might take the form of rotation about the metal-oxygen bond with an effective correlation time of $\sim 10^{-9}$ s or exchange of water on and off the metal ion with effective exchange rates of $\sim 10^8$ – 10^9 s^{-1} (Cotton & Wilkinson, 1967). Solvent exchange rates of this magnitude have been reported in Mn^{2+} carbonic anhydrase (Hsi & Bryant, 1977). NOE and T_1 determinations on enzyme in D_2O rather than H_2O should provide a means of quantitating the contribution to the overall relaxation from solvent protons and these experiments are currently in progress.

Discussion

Despite the essential requirement for four Zn^{2+} and two Mg^{2+} ions to provide maximal catalytic activity, structural stabilization, and phosphate binding stoichiometry in the alkaline phosphatase dimer (Bosron et al., 1977; Chlebowski & Mabrey, 1977; Otvos et al., 1979b), very little is known concerning the direct or indirect roles played by these metal ions in the catalytic mechanism of the enzyme. The primary reason for this situation is a lack of knowledge regarding the structural characteristics of the multiple metal binding sites in the protein, i.e., the identity of the ligands to the various metal ions, the relative affinities of the sites for different metal ions, and the relative locations of the metal binding sites, both with respect to one another and to the catalytic center. In the absence of this information it has proved difficult to obtain accurate structure-function relationships by employing methods involving metal ion titration of the enzyme because of uncertainty as to which of the six available metal binding sites in the dimer are occupied under a given set of conditions. Spectroscopic techniques relying on the substitution of chromophoric and/or paramagnetic metal ions for the native zinc

(absorption, CD, MCD, EPR) have been used as probes of metal ion location, but are in general limited by their inability to unambiguously resolve spectral contributions from metals bound to more than one class of binding site. In contrast, the results presented in this paper demonstrate that it is possible using ^{113}Cd NMR to simultaneously monitor the distribution of metal ion among all three classes of binding sites in the protein. This capability not only allows the affinities of Cd^{2+} for the multiple sites to be measured, but provides the first opportunity to directly correlate a functional property of the enzyme (phosphorylation) with specific structural requirements. The ^{113}Cd NMR data also suggest answers to several questions concerning properties of the native enzyme that have been the subject of considerable past uncertainty.

The most striking result provided by the ^{113}Cd NMR data is the effect that formation of the covalent phosphoryl-enzyme intermediate has on the properties of the multiple metal binding sites on the enzyme. Before discussing these results, it will be useful to briefly summarize what is known about the properties of the metal binding sites in enzyme containing no bound phosphate. The most well-characterized binding sites are the A sites, previously referred to as the catalytic sites. They are located approximately 32 Å apart in the dimer (Knox & Wyckoff, 1973), have distorted tetrahedral or penta-coordinate geometry (Applebury & Coleman, 1969; Anderson et al., 1976), and contain four histidyl nitrogen metal ligands (Otvos & Armitage, 1980). Two other symmetrically disposed metal binding sites that are located in close proximity (~ 5 Å) to the A sites (H. W. Wyckoff, personal communication) are most likely those which we have designated as the B sites (Otvos & Armitage, 1980). The coordination geometry about the B sites appears to be octahedral-like (Anderson et al., 1976) with one histidine residue participating as a metal ligand (Otvos & Armitage, 1980). The location of the third pair of metal binding sites in the enzyme is presently unknown. The coordination geometry about these C sites also appears to be octahedral-like (Anderson et al., 1976), although nothing is known concerning the identity of its protein ligands other than that histidine is apparently not involved (Otvos & Armitage, 1980).

Reliable binding constants for metal ion interaction at the A, B, and C sites in alkaline phosphatase are not available

because of the difficulties associated with their experimental determination. Measurements employing either equilibrium dialysis (Csopak, 1969) or fractional enzymatic activity (Lazdunski et al., 1969; Cohen & Wilson, 1966) to monitor metal binding give ambiguous results because of uncertainty in the former case as to which binding sites in the protein are interacting with metal and in the latter case as to what relationship exists between catalytic activity and metal ion occupancy. An assessment of the *relative* binding strengths of the A, B, and C sites is, by contrast, somewhat easier. Care must be taken, however, to avoid making generalizations based on results obtained with a particular metal ion. Different metals can, and do, distribute themselves differentially among the competing binding sites based on their preferences for particular coordination geometries or ligands. Experimental variables such as pH, temperature, and ionic strength can likewise alter the distribution of metal among the multiple sites (Angelici, 1973). Bearing these considerations in mind, the bulk of accumulated evidence suggests that the A sites have the highest affinity for metal ion ($K_B \gg 10^6 \text{ M}^{-1}$). In spectral titrations of apoenzyme with Mn^{2+} at pH 8, the first two equivalents of metal bind selectively to the A sites (Weiner et al., 1979). The same behavior is observed with Cd^{2+} at pH 6.2 monitored both by ^{13}C NMR (Otvos & Armitage, 1980) and ^{113}Cd NMR (Figure 1). Thus, in these cases binding to the A sites must be stronger by more than an order of magnitude than to the B and C sites. In contrast, both Zn^{2+} (Otvos & Armitage, 1980) and Co^{2+} (Anderson et al., 1976) appear to be considerably less selective, since a substantial fraction of A sites remain unoccupied following addition of the first pair of these metal ions to the apoenzyme. The affinity for the B sites relative to the C sites also depends on the nature of the metal ion under consideration. Zn^{2+} appears to strongly favor B-site binding at pH 8 (Otvos & Armitage, 1980; Bosron et al., 1977), while Co^{2+} has roughly equal affinities for the two classes of sites (Anderson et al., 1976). The ^{13}C NMR data in the accompanying paper showed that at pH 6.3 Cd^{2+} also has nearly identical affinities for the B and C sites (Otvos & Armitage, 1980). Unfortunately, ^{113}Cd NMR cannot be employed to directly explore the binding characteristics of Cd^{2+} to these sites because of our inability to detect B- and C-site resonances in unphosphorylated enzyme (see Figure 1). We believe the origin of this problem is excessive line broadening brought about by one or more chemical exchange processes, but at present we are unable to define these events at the molecular level.

By contrast, exchange broadening in the phosphorylated enzyme is not a problem since the total amount of $^{113}\text{Cd}^{2+}$ in the sample is always found to be directly proportional to the integrated areas of the observed ^{113}Cd resonances (Figure 2 and Table I). Phosphorylation of the active-site serine residue must therefore slow down or abolish whatever exchange process(es) is responsible for modulating the chemical shifts of the A-, B-, and C-site ^{113}Cd resonances in $^{113}\text{Cd}_6^{2+}$ enzyme. Although the mechanism by which this occurs is not known, the apparent close proximity of the A and B sites to one another (H. W. Wyckoff, personal communication) and to the active site (Otvos et al., 1979a) suggests the likelihood that a local conformational stabilization or steric restriction is involved, which is brought about by direct interaction with the phosphoryl group. The fact that enzyme phosphorylation is also required to detect a C-site ^{113}Cd resonance may indicate that the C site is also located relatively close to the active-site region, although there is currently no direct evidence for this.

The ability to resolve separate resonances corresponding to $^{113}\text{Cd}^{2+}$ located at each of the three classes of metal binding sites in the phosphoryl-enzyme allows the properties of these sites to be characterized under various experimental conditions once each resonance has been assigned to its respective binding site. In order to make these assignments, we relied initially on the relationship that exists between the identity of the ligands to a $^{113}\text{Cd}^{2+}$ ion and its chemical shift. From chemical-shift determinations of $^{113}\text{Cd}^{2+}$ in a number of small coordination complexes as well as in proteins whose binding sites have been characterized by X-ray crystallography, there has emerged a consistent correlation between degree of deshielding and the number of halide or nitrogen ligands in the first coordination sphere (where the remaining ligands are oxygen atoms) (Kostelnik & Bothner-By, 1974; Cardin et al., 1975; Haberkorn et al., 1976; Armitage et al., 1978; our unpublished results). At the present time, this correlation remains more qualitative than quantitative because an insufficient number of well-defined complexes have been examined and because there is as yet an incomplete understanding of the influence that coordination geometry has on chemical shift. Nonetheless, we considered the correlation to be sufficiently well-characterized to enable its use for tentative assignment purposes in the present case, particularly since all the resonances are separated by more than 50 ppm. Information regarding the ligands to the multiple metal binding sites in alkaline phosphatase was provided by the ^{13}C NMR studies reported in the previous paper, which demonstrated the participation of four, one, and zero histidyl nitrogen ligands at the A, B, and C sites, respectively (Otvos & Armitage, 1980). On this basis, we have assigned the most deshielded resonances (141–160 ppm) to A-site $^{113}\text{Cd}^{2+}$, the 55–76-ppm resonances to B-site $^{113}\text{Cd}^{2+}$, and the least deshielded resonance at 2 ppm to C-site $^{113}\text{Cd}^{2+}$. Absolute confirmation of these assignments has been achieved by demonstrating a direct correlation between the ^{13}C NMR spectral properties of the histidyl ligands at the A and B sites with the metal distribution among the multiple sites as monitored by ^{113}Cd NMR (Otvos & Armitage, 1980). The properties of $^{113}\text{Cd}^{2+}$ bound to the three classes of binding sites in phosphorylated enzyme are reviewed below.

A Site. The chemical shift of A-site $^{113}\text{Cd}^{2+}$ is sensitive to the total metal composition of the enzyme, ranging from 141 ppm in the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme (Figure 2B) to 160 ppm in the $^{113}\text{Cd}_6^{2+}$ diphosphoryl-enzyme (Figure 3B). These chemical-shift values are close to the 169-ppm shift observed for A-site $^{113}\text{Cd}^{2+}$ in the unphosphorylated $^{113}\text{Cd}_2^{2+}$ enzyme (Figure 2A). The chemical shift of A-site $^{113}\text{Cd}^{2+}$ is sensitive to the ionic composition of the medium in both phosphorylated and unphosphorylated enzyme. As previously reported, the A-site resonance in unphosphorylated enzyme shifts from about 120 to 180 ppm with increasing Cl^- concentration in a manner that is consistent with fast Cl^- exchange at the A-site metal (Coleman et al., 1979). The same behavior has been noted in the $^{113}\text{Cd}_4^{2+}, \text{Mg}_2^{2+}$ diphosphoryl-enzyme, where the chemical shift varies from 130 ppm in the absence of Cl^- to 159 ppm in the presence of 0.1 M Cl^- (our unpublished results). These results suggest that A-site metal contains one or more coordination sites that have access to solvent. Since the ^{13}C NMR data identifies four histidyl ligands at this site, A-site metal must be at least 5-coordinate. The affinity of Cd^{2+} for the A sites is relatively high. From isotope exchange experiments such as the one presented in Figure 4, k_{off} for A-site Cd^{2+} is estimated to be $\sim 10^{-4}$ – 10^{-5} s^{-1} . In an analogous isotope exchange study, it was unambiguously demonstrated that the downfield ^{113}Cd resonance we have

attributed to A-site $^{113}\text{Cd}^{2+}$ indeed arises from metal ion bound to the site containing four histidyl ligands (Figure 4 in Otvos & Armitage, 1980).

B Site. The chemical shift of B-site $^{113}\text{Cd}^{2+}$ in the phosphorylated enzyme is either 56, 70, or 76 ppm depending upon the occupancy of the other metal binding sites in the enzyme. These chemical-shift values are totally insensitive to the ionic composition of the medium (our unpublished results). Thus, unlike metal bound to the A site, B-site Cd^{2+} does not apparently have coordination sites that are accessible to the solvent. In the phosphorylated enzyme, the B sites have a much greater affinity for Cd^{2+} than either the A or C sites. Isotope exchange experiments indicate that Cd^{2+} binds at least two orders of magnitude more tightly to the B sites than to the A sites ($k_{\text{off}} < 10^{-7} \text{ s}^{-1}$). This is in marked contrast to the situation in unphosphorylated enzyme, where binding to the B sites is more than an order of magnitude weaker than to the A sites. Phosphorylation of the active-site serine therefore stabilizes the complex of Cd^{2+} with the B sites by $>10^3$, suggesting that the phosphoryl group may physically impair access of the B site to the medium.

C Site. The chemical shift of the resonance assigned to C-site $^{113}\text{Cd}^{2+}$ (2 ppm) is in the region of the spectrum that one would predict for a metal ion coordinated exclusively to oxygen ligands (Haberkorn et al., 1976). The chemical shift dependence of this resonance on the halide composition of the medium has not been determined, so it is presently not known whether the C-site metal is accessible to solvent. Mg^{2+} competes with Cd^{2+} for the C sites, but has a binding constant that is approximately an order of magnitude lower (Figure 3). From the rate of replacement of $^{113}\text{Cd}^{2+}$ by Mg^{2+} , k_{off} for C-site Cd^{2+} is estimated to be $\sim 10^{-4} \text{ s}^{-1}$ in the phosphoryl-enzyme. Occupation of the C sites by either Mg^{2+} or Cd^{2+} causes both the A- and B-site resonances to shift >14 ppm downfield (see below). This observation provides additional support for the notion that the C sites may be relatively close to the A and B sites, although we cannot exclude the possibility that the chemical shift changes may be induced by a conformational change transmitted through some distance.

The information supplied by ^{113}Cd NMR concerning the effects of phosphate on the properties of the multiple metal binding sites in alkaline phosphatase provides considerable insight into the origin of the half-of-the-sites reactivity exhibited by the enzyme. Alkaline phosphatase has been generally considered to represent a classic example of a negatively cooperative enzyme, i.e., one which displays an exclusive or preferential ability to interact with substrate or ligand at only one of the two subunits. Evidence in support of anticooperativity has been supplied by numerous rapid-flow kinetic, spectroscopic, and equilibrium binding studies (for a review, see Coleman & Chlebowski, 1979). Nonetheless, several reports have appeared that suggest that the two subunits are completely independent in their interactions with ligand (Simpson & Vallee, 1968; Hull & Sykes, 1976; Hull et al., 1976; Bloch & Schlesinger, 1973; Bloch & Bickar, 1978). The discrepancies in these results might plausibly be explained by recent ^{31}P NMR experiments, which demonstrated that the expression of anticooperativity is directly related to the metal ion stoichiometry of the enzyme; apoenzyme reconstituted with 2 equiv of metal ion binds only 1 mol of phosphate/dimer, while enzyme containing its full complement of six metal ions binds phosphate equally well at both subunits (Otvos et al., 1979b).

The inability of Me_2^{2+} enzyme (containing either Cd^{2+} , Zn^{2+} , Co^{2+} , or Mn^{2+}) to interact with more than a single mole of

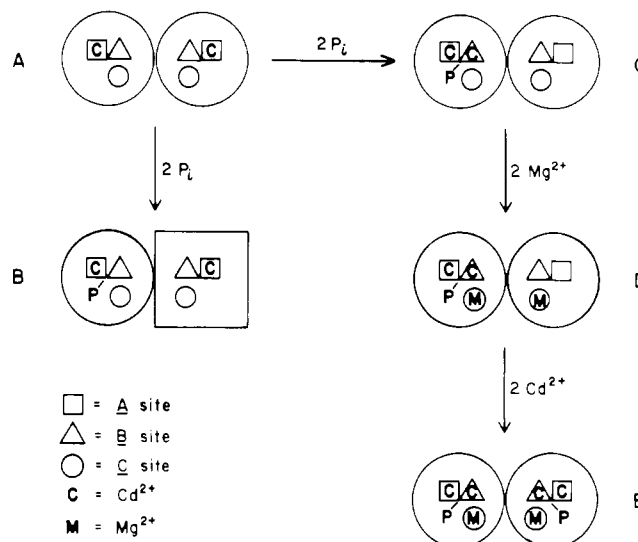


FIGURE 8: A schematic representation of the metal ion distribution in the alkaline phosphatase dimer brought about by covalent phosphorylation of the enzyme. See text for details.

phosphate/mole of enzyme dimer has generally been attributed to ligand-induced conformational changes propagated across the subunit-subunit interface which diminishes (or abolishes) the ligand binding capacity at the second active site. This explanation appeared to be substantiated by preliminary ^{113}Cd NMR results, which showed the single A-site resonance of $^{113}\text{Cd}_2^{2+}$ enzyme to be converted into two resonances upon the covalent phosphorylation of one of the two subunits of the dimer (Chlebowski et al., 1977). Since there was no reason to suspect that the Cd^{2+} bound tightly to the A sites in the unphosphorylated enzyme should be located elsewhere in the phosphorylated protein, the existence of the two resonances was taken as evidence for a conformational change induced by phosphorylation at one active site which altered the environments of the A sites in both subunits. This interpretation is represented schematically in Figures 8A and 8B.

From the spectral changes brought about by addition of $^{113}\text{Cd}^{2+}$, Mg^{2+} , and phosphate to the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme (Figure 2 and Table I) it is clear that this original interpretation is incorrect. The doubling of resonance intensity in the downfield and upfield regions of the spectrum brought about by formation of the $^{113}\text{Cd}_2^{2+}, \text{Mg}_2^{2+}$ diphosphoryl-enzyme (Figure 2D) forces the conclusion that the 141- and 56-ppm resonances in $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme (Figure 2B) arise from $^{113}\text{Cd}^{2+}$ bound to A and B sites. Phosphorylation of Cd_2^{2+} enzyme must therefore be accompanied by a migration of A-site metal as depicted in Figure 8C. The existence of such a migration was unambiguously confirmed in the accompanying paper by using ^{13}C NMR to monitor the release of Cd^{2+} from about half the A-site histidyl ligands in Cd_2^{2+} monophosphoryl-enzyme (Otvos & Armitage, 1980).

The key to understanding the driving force for this metal ion redistribution is provided by the fact that the time dependence of metal migration is similar to that of phosphorylation of the Cd_2^{2+} enzyme. As monitored by both the incubation time required to generate a clean ^{113}Cd NMR spectrum containing only the two A and B resonances (Figure 2B) and by the time course of the A-site [^{13}C]histidyl ligand coordination changes brought about by phosphorylation (Otvos & Armitage, 1980), several days are required to complete the metal ion redistribution. The amount of time required to phosphorylate Cd_2^{2+} enzyme to the equilibrium stoichiometry

of 1 mol/mol of dimer is of approximately equal duration. This has been shown by the observation that the ^{31}P resonance corresponding to the covalent phosphoryl-enzyme complex only reaches its maximum intensity several days after addition of phosphate to Cd_2^{2+} enzyme (our unpublished result). Direct measurement of the amount of $^{32}\text{P}_i$ incorporated into Cd_2^{2+} enzyme as a function of time also indicates very slow phosphorylation characterized by a rate constant of $\sim 1.5 \times 10^{-5} \text{ s}^{-1}$ (reaction half-time $\sim 800 \text{ min}$) (Chlebowski & Coleman, 1979). Significantly, these experiments also show that enzyme containing its full complement of Cd^{2+} phosphorylates much more rapidly (half-time $\sim 1 \text{ min}$). From the above evidence, it is clear that metal ion migration *accompanies*, rather than follows, enzyme phosphorylation. The simplest explanation for this behavior is that enzyme phosphorylation is catalyzed *only* when subunits contain Cd^{2+} bound to both the A and B sites. In Cd_2^{2+} enzyme only a very small fraction of subunits satisfies this condition, since the equilibrium state greatly favors enzyme containing Cd^{2+} located solely at the A sites. As phosphorylation of this minority of subunits proceeds, however, the equilibrium will be gradually displaced in favor of the species represented in Figure 8C, owing to the large increase in metal ion stability that accompanies phosphorylation. The slow rate of phosphorylation of Cd_2^{2+} enzyme does not therefore correspond to the actual phosphorylation process, but is instead a reflection primarily of the off-rate of Cd^{2+} from the A sites. The "absolute" anticooperativity exhibited by Cd_2^{2+} enzyme follows as a direct consequence of the above model. Only one active site per dimer can be phosphorylated simply because insufficient metal ion is present to satisfy the requirement for A- and B-site occupancy in the remaining subunits. If phosphorylation were to require only the presence of A-site Cd^{2+} , it would be difficult to adequately explain the clear-cut half-sites binding stoichiometry as well as the correspondence between the rates of metal ion migration and enzyme phosphorylation.

The metal ion distribution that we propose gives rise to the ^{113}Cd NMR spectra in Figures 2C and 2D is represented schematically in Figures 8D and 8E, respectively. Mg^{2+} addition to the $^{113}\text{Cd}_2^{2+}$ monophosphoryl-enzyme causes the A- and B-site resonances at 141 and 56 ppm to shift downfield by about 15 and 19 ppm, respectively (Figure 2C). C-site occupation by Mg^{2+} therefore alters the electronic environments of both the A and B sites, reflecting the probable proximity of all three sites in the phosphorylated subunit. For simplicity, the second equivalent of Mg^{2+} is depicted in Figure 8D as occupying the C site in the unphosphorylated subunit, though in fact it is probably distributed between the B and C sites (Otvos & Armitage, 1980). Once the requirement for A- and B-site occupancy is satisfied by the binding of an additional 2 equiv of $^{113}\text{Cd}^{2+}$ to the enzyme, phosphorylation off the second subunit proceeds rapidly (Figure 8E). This is evidenced by the fact that no incubation period was required before the ^{113}Cd spectrum in Figure 2D could be generated. Had the sample at this stage contained exactly 4.0 equiv of $^{113}\text{Cd}^{2+}$, a spectrum would have been observed consisting of two resonances at 159 and 76 ppm, each arising from a pair of $^{113}\text{Cd}^{2+}$ ions located at identical binding sites in the symmetric dimer depicted in Figure 8E. Instead, because of an underestimation of the enzyme molecular weight (see Materials and Methods), excess $^{113}\text{Cd}^{2+}$ was present. Since Cd^{2+} has a higher affinity than Mg^{2+} for the C sites (Figure 3), the excess $^{113}\text{Cd}^{2+}$ displaces some Mg^{2+} . The fraction of subunits containing ^{113}Cd rather than Mg^{2+} at the C sites not only exhibits a 2-ppm C-site resonance, but also gives rise to A-

or B-site chemical shifts (160 and 70 ppm), which are slightly different than those observed when the C sites are occupied by Mg^{2+} (159 and 76 ppm).

An important point regarding all of the above ^{113}Cd resonance assignments is that it has been possible to fully account for the experimental results solely on the basis of the metal ion occupancies of the three classes of metal binding sites in the individual subunits. There has been no necessity to invoke the existence of subunit interactions that, via conformational changes, might link the NMR parameters of the metal ions at one subunit of the dimer to events occurring at the opposite subunit. In view of numerous previous reports suggesting the existence of functional subunit interactions in alkaline phosphatases reconstituted with various metals besides Cd^{2+} , it is of obvious interest to assess to what extent the properties of the Cd^{2+} enzyme may be extrapolated to the other metallophosphatases, particularly the native Zn^{2+} enzyme.

Substitution of Cd^{2+} for Zn^{2+} in alkaline phosphatase is known to decrease by orders of magnitude the rates of enzyme phosphorylation and dephosphorylation as well as shift the pH dependence of the equilibrium between the covalent (E-P) and noncovalent (E·P) enzyme-phosphate complexes (Chlebowski & Coleman, 1976; Otvos et al., 1979a). At the pH used in the ^{113}Cd NMR experiments (pH ~ 6.3), virtually 100% of enzyme-bound phosphate exists as E-P in the Cd^{2+} enzyme, while E-P predominates under the same conditions in the Zn^{2+} enzyme. In attempting to relate the ^{113}Cd NMR results to the Zn^{2+} enzyme, the question therefore arises as to whether noncovalently bound phosphate provides a similar degree of metal ion stabilization as that shown to be conferred by covalent phosphorylation. Evidence in favor of this has been supplied by the observation that noncovalently bound phosphate greatly inhibits the rate of removal by chelators of zinc from the enzyme (Lazdunski et al., 1969; Simpson & Vallee, 1968). Somewhat more indirect, but at least as persuasive, is the fact that all Me_2^{2+} enzymes that have been examined (Zn_2^{2+} , Cd_2^{2+} , Co_2^{2+} , Mn_2^{2+} , Cu_2^{2+}) exhibit absolute half-of-the-sites phosphate binding irrespective of whether the phosphate is bound covalently or noncovalently (Chlebowski et al., 1976, 1977; Csopak & Falk, 1974; Applebury et al., 1970). Since it is reasonable to assume that a common mechanism gives rise to this behavior, the situation shown to exist in Cd_2^{2+} monophosphoryl-enzyme (Figure 8C) is likely to represent the equilibrium metal ion distribution in the other phosphate-containing Me_2^{2+} enzymes as well. If so, the implication is that (1) covalently and noncovalently bound phosphates confer similar degrees of stabilization on the metal ions in liganded subunits and (2) A- and B-site occupancy may be a minimum requirement for tight noncovalent phosphate binding as well as for covalent phosphorylation.

A reevaluation of a recent ^{31}P NMR study also strongly supports the idea that the metal ion distribution brought about by noncovalent phosphate binding to the Zn^{2+} enzyme corresponds closely to that which is induced by covalent phosphorylation of the Cd^{2+} enzyme. In phosphate titrations of apoenzyme reconstituted with varying amounts of Zn^{2+} , a single E-P resonance at 5.0 ppm was observed in Zn_2^{2+} enzyme and two resonances of approximately equal intensity at 5.0 and 3.7 ppm were generated in Zn_4^{2+} enzyme, while the $\text{Zn}_4^{2+}, \text{Mg}_2^{2+}$ enzyme gave rise to a single peak at 2.9 ppm (Otvos et al., 1979b). At the time of the experiment, the different chemical shifts of these resonances could only be ascribed to conformers of E-P of unknown origin. From a consideration of the ^{113}Cd NMR data in this paper, it is now reasonable to assume that the ^{31}P chemical shifts of E-P are

in fact reflecting the metal ion composition of the subunits to which the phosphate is bound. By analogy to the species generated by phosphorylation of the corresponding $^{113}\text{Cd}^{2+}$ enzymes, the resonances at 5.0, 3.7, and 2.9 ppm can now be suggested to arise from E·P in subunits containing Zn^{2+} at the A and B sites with the C site either vacant, occupied by Zn^{2+} , or occupied by Mg^{2+} , respectively. It is particularly significant that the ^{31}P NMR evidence for the existence of two distinct classes of subunits generated by phosphate binding to the Zn_4^{2+} enzyme has its exact parallel in the heterogeneous metal distribution observed following phosphorylation of the Cd_4^{2+} enzyme (Figure 4A). These ^{31}P NMR results, in conjunction with the apparent universality of the half-site reactivity of Me_2^{2+} enzyme, suggest that the ^{113}Cd NMR data regarding the relative affinities of metal ion for the A, B, and C sites in phosphorylated enzyme may indeed be extrapolated with some confidence to the phosphate-containing Zn^{2+} enzyme, as well as to other metallophosphatases.

The necessity for considering the possible participation of all three classes of metal ions in the catalytic mechanism of alkaline phosphatase has not always been appreciated. The reason for this is that previous metal-activity titrations of the apoenzyme have generally yielded conflicting results. Full catalytic activity has been variously reported to require either two Zn^{2+} ions (Cohen & Wilson, 1966; Simpson & Vallee, 1968; Csopak & Szajn, 1973), four Zn^{2+} ions (Reynolds & Schlesinger, 1969; Petittclerc et al., 1970; Trotman & Greenwood, 1971), or four Zn^{2+} and two Mg^{2+} ions (Bosron et al., 1977). Most of these discrepancies can now be rationalized in terms of (1) variations in the methods used to purify, prepare, and reconstitute apoenzyme, (2) overestimation of protein concentrations resulting from the use of low enzyme molecular weight values (see Materials and Methods), and (3) failure to recognize the importance of Mg^{2+} . In our hands, we consistently find that maximal catalytic activity can be generated only by adding sufficient metal ion to populate all three pairs of metal binding sites on the enzyme dimer. Beyond this it is not, unfortunately, possible simply on the basis of the partial enzymatic activities generated by submaximal metal contents to make any firm conclusions regarding the functions of the different classes of metal ions. The problem is that Zn^{2+} does not, as previously believed, exhibit sufficiently different affinities for the multiple sites to ensure a defined metal distribution under a given set of conditions (Otvos & Armitage, 1980). Thus, it is difficult to unambiguously distinguish between cases where partial activity may be due to full activity in a fraction of subunits, to reduced catalytic efficiency in all subunits, or to both.

As demonstrated by the results in this and the preceding paper (Otvos & Armitage, 1980), a study of the ^{113}Cd -substituted enzyme would seem to offer significant advantages over the Zn^{2+} enzyme in terms of providing insight into how the A-, B-, and C-site metal ions may participate in the several distinct steps that comprise the overall catalytic mechanism of alkaline phosphatase (i.e., phosphorylation and dephosphorylation of the active-site serine residue and dissociation of product (P_i) from the enzyme). The main advantage derives from the ability to characterize the metal ion distribution of the subunits in far greater detail for the $^{113}\text{Cd}^{2+}$ enzyme than for any of the other metallophosphatases. This can be accomplished not only by direct observation of individual A-, B-, and C-site ^{113}Cd NMR resonances but also by monitoring the characteristic chemical shifts and spin-spin couplings that $^{113}\text{Cd}^{2+}$ ions bound to the different binding sites induce in the resonances of nearby ^{13}C and ^{31}P nuclei (Otvos & Armitage,

1980; Otvos et al., 1979a). The other key property of the $^{113}\text{Cd}^{2+}$ enzyme is the differential affinity that metal ions display for the A, B, and C sites in the phosphoryl-enzyme. This makes it possible to selectively replace $^{113}\text{Cd}^{2+}$ at a particular binding site with either another Cd isotope or with a different metal ion (e.g., Mg^{2+} or Zn^{2+}). By characterizing the structural and kinetic properties of a variety of such stable, well-defined hybrid metallophosphatases, it should in principle be possible to clarify the role played by each of the three classes of metal ion in the catalytic mechanism.

Of particular current interest are questions relating to the function of the A- and B-site metal ions. The present ^{113}Cd NMR data has shown that enzyme phosphorylation is catalyzed only by subunits containing metal ion bound to both the A and B sites. The tight noncovalent binding of phosphate to the enzyme, whose dissociation is the rate-limiting step in enzyme turnover at alkaline pH, may also require A- and B-site metal occupancy. Since recent X-ray data suggests that the A- and B-site metal ions are located only about 5 Å apart (H. W. Wyckoff, personal communication), it is important to determine their relative dispositions with respect to other constituents of the active site. We have recently demonstrated by the observation of ^{31}P - ^{113}Cd spin coupling in the E·P complex of the $^{113}\text{Cd}^{2+}$ enzyme that a phosphate oxygen is directly coordinated to an active-site metal ion (Otvos et al., 1979a). By employing a nonspin cadmium isotope, ^{112}Cd , in place of ^{113}Cd , an isotope exchange experiment similar to that described in Figure 4 has been performed that indicates that the $^{113}\text{Cd}^{2+}$ ion involved in the spin coupling is the A, rather than B, metal ion (J. D. Otvos and I. M. Armitage, unpublished results). Attempts are currently underway to further uncouple the contributions of the A- and B-site metal ions to the catalytic process by measuring the kinetic properties of several Cd,Zn and Cd,Mg hybrid enzymes. Since the Cd^{2+} and Zn^{2+} enzymes exhibit markedly different, well-characterized rates of phosphorylation, dephosphorylation, and dissociation, it is hoped that an analysis of these rates in stable Cd,Zn hybrid species will enable an assessment to be made of the extent to which the A- and B-site metals function as indirect structural or direct catalytic participants in the enzyme mechanism. Similar experiments designed to explore the role of C-site metal ion and Mg^{2+} are also in progress.

Summary

The work presented in this report was undertaken with two objectives in mind: first, to understand the structural basis for the half-of-the-sites reactivity exhibited by all Me_2^{2+} alkaline phosphatases, and secondly to better characterize the properties of the three classes of metal binding sites in the protein with the hope of gaining insight into the nature of their participation in the catalytic process. We have demonstrated by ^{113}Cd NMR that reaction of only half the subunits in the $^{113}\text{Cd}_2^{2+}$ enzyme with phosphate is the result of migration of half the A-site metal ions to B sites. The driving force for this migration, which leaves half the subunits devoid of metal and hence incapable of interaction with ligand, is the greatly increased stabilization of the B-site Cd^{2+} complex that accompanies subunit phosphorylation. The lengthy time course observed for both phosphorylation and metal redistribution in Cd_2^{2+} enzyme has been interpreted as evidence that occupation of both A and B sites, rather than the A site alone, constitutes the minimal requirement for substrate turnover.

The ability to make the above conclusions has primarily depended on a key property of Cd_2^{2+} phosphatase that is not shared by the Zn_2^{2+} enzyme, i.e., the selective binding of metal ion to the pair of A sites in the dimer (Otvos & Armitage,

1980). By our use of the Cd_2^{2+} enzyme in the NMR studies we have not meant to suggest that the half-sites reactivity and metal migration exhibited by this species are representative of the in vivo state of the enzyme, which most likely contains 6 equiv, rather than 2 equiv, of bound metal ion. Using enzyme containing its full complement of metal ion, none of our ^{113}Cd , ^{13}C , or ^{31}P NMR studies have provided any evidence for the existence of subunit interactions; both subunits appear to interact independently with phosphate and other ligands.

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