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Characterization of the Histidine Residues in Alkaline Phosphatase by Carbon-13 Nuclear Magnetic Resonance†

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ABSTRACT: β,β-[γ-^{13}C]Dideuteriohistidine has been biosynthetically incorporated into alkaline phosphatase from *Escherichia coli* and utilized as a nonperturbing ^{13}C nuclear magnetic resonance (NMR) probe of the environments of the histidine residues in this zinc metalloenzyme. The ^{13}C NMR spectrum of the labeled enzyme exhibits 9 separate resonances arising from the 10 histidine residues located in each of the symmetrically disposed subunits of the dimer. The excellent resolution and large chemical shift range (14 ppm) displayed by these signals are direct consequences of the sensitivity of the histidine γ-carbon chemical shift to the ionization state and tautomeric form of the imidazole side chains and the coordination of several of these to metal ion. The environments of the individual histidine residues were inferred by investigating the chemical shift responses of their ^{13}C resonances to enzyme metal composition, pH, and inhibitor binding. Additional information concerning their motional freedom was obtained from spin relaxation measurements which were analyzed in terms of the contributions expected from intramolecular ^{13}C–^{1}H and ^{13}C–^{14}N dipolar relaxation and chemical shift anisotropy. The combined results indicate that 4 of the 10 histidines, the only ones that titrate with pH, are surface residues located relatively remote from the active site. Of the six nontitrating residues, one appears to be buried in a solvent-inaccessible region of the protein. Three others are almost certainly involved in metal ion ligation to active-site metal ion(s), two via their N^{π} nitrogen atoms and the other via N^{τ}. The spectral characteristics of the remaining two histidine residues strongly suggest they are also located at or near the active site. One or both may also participate in metal ion coordination, although the current evidence for this is inconclusive.

Escherichia coli alkaline phosphatase is a dimeric zinc metalloenzyme that has the ability to hydrolyze a broad spectrum of phosphate monoesters with little variation in rate. As the result of numerous investigations utilizing a wide variety of techniques, the mechanism by which this broad specificity is achieved is now well understood (Reid & Wilson, 1971; Coleman & Chlebowski, 1979). At the same time, however, conflicting reports have appeared concerning such questions as metal ion stoichiometry, the catalytic roles of the metal ions, and the existence of subunit interactions (negative cooperativity).

Much of the confusion regarding these issues stems from a lack of knowledge concerning the structural features of the enzyme involved in substrate and metal ion binding. At the present time, a high-resolution X-ray structure of alkaline phosphatase is unavailable. Previous work has established the existence of an active-site serine residue, whose covalent phosphorylation constitutes a key reaction intermediate (Schwartz & Lipmann, 1961; Levine et al., 1969), and other data have suggested the involvement of an arginine residue (Daemen & Riordan, 1974) and one or more histidine residues (Tait & Vallee, 1966; Taylor & Coleman, 1972; Csopak & Falk, 1974) in substrate and metal ion binding, respectively. Spectroscopic methods that have been applied to the study of alkaline phosphatase structure have virtually all relied on the replacement of the native group 2B metal ion Zn^{2+}, which is intrinsically a poor spectroscopic probe because of its filled d shell, with transition metal ions such as Co^{2+}, Mn^{2+}, or Cu^{2+} (Coleman & Chlebowski, 1979). These transition-metal probes have provided valuable structural information, but they suffer somewhat from their intrinsically perturbing nature, which arises from differences in their ionic radii, preferred

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0006-2960/80/0419-4011$01.00/0

coordination geometry, and susceptibility to ligand field-induced distortions. These perturbations are presumably responsible for the fact that enzymatic activity is greatly reduced or more commonly completely abolished upon substitution of nonnative metal ions in the enzyme.

In order to provide a sensitive, but nonperturbing, method with which to study the structural and functional properties of the native Zn^{2+} enzyme, we have undertaken a ^{13}C NMR[1] study of alkaline phosphatase labeled in vivo with histidine enriched in ^{13}C at the quaternary (γ) carbon. The advantages in terms of sensitivity and resolution of choosing a quaternary carbon enriched amino acid to serve as a nonperturbing probe of protein structure have been previously discussed (Browne et al., 1976). Histidine was chosen as the ^{13}C label in this study because of its probable participation as a zinc ligand at the metal binding site(s) in alkaline phosphatase (Tait & Vallee, 1966; Taylor & Coleman, 1972; Csopak & Falk, 1974). In this paper we show that the ^{13}C NMR spectrum of the [γ-^{13}C]histidine enzyme contains resolved resonances arising from most of the 10 histidine residues per subunit in the symmetric dimer. By examining the relaxation properties of these resonances and the influence of pH, metal ion, and inhibitor binding on their chemical shifts, it has been possible to draw conclusions regarding the environments of the individual histidine residues and the participation of several of these in metal ion coordination.

Materials and Methods

Materials. DL-[γ-^{13}C]Histidine and its deuterium analogue, β,β-[γ-^{13}C]dideuterio-DL-histidine, synthesized from 90% ^{13}C-enriched barium carbonate (Earl, 1978), were the generous gifts of Elaine M. Earl. Incorporation of [^{13}C]histidine into alkaline phosphatase was accomplished as previously described (Browne et al., 1976). Enzyme concentrations were determined spectrophotometrically at 280 nm at pH 7.4 using $E_{1\,cm}^{0.1\%} = 0.77$ (Browne & Otvos, 1976). For molar calculations a molecular weight of 95 000 was used.[2] This value is about 10% higher than had been assumed at the time the NMR measurements were performed. As a consequence, the stoichiometries in the inhibitor titrations are nonintegral, reflecting the slight overestimation of molar protein concentration in these samples.

D_2O was 99.8% from Bio-Rad. Ultrapure grade zinc chloride and magnesium nitrate were obtained from Alfa Ventron Corp. *p*-Aminobenzylphosphonate was prepared by acid hydrolysis of diethyl *p*-aminobenzylphosphonate (Aldrich Chemical Corp.), and the final product was recrystallized from water. All other chemicals were reagent grade. Buffers were prepared with distilled, deionized water and when necessary were rendered metal-free by treatment with Chelex 100 (200–400 mesh). Nalgene labware was used when possible, and all glassware was acid-washed to avoid phosphate or metal ion contamination.

Enzyme Preparations. The tightly bound "endogenous" phosphate present in native enzyme can be efficiently removed only by removal of the zinc ions (Bloch & Schlesinger, 1973). This was routinely accomplished by dialysis (1/500) of the protein at 4 °C against three changes of 0.01 M Tris and 0.02 M EDTA, pH ~6.5, for 2 days followed by dialysis for 3 days against six changes of metal-free 0.01 M Tris, pH 7.4, to remove the chelating agent. The resulting inactive apoenzyme was then reconstituted by dialysis against two changes of 0.01 M Tris, pH 7.4, containing equimolar amounts (1 mM) of both $ZnCl_2$ and $Mg(NO_3)_2$ followed by dialysis against metal-free Tris buffer. Phosphate analysis (Chappelet-Tordo et al., 1974) confirmed that the 1.5 to 2.0 mol of phosphate per dimer present in the native enzyme was totally removed by this procedure. The purged enzyme routinely gave specific activities of 2800 to 3200 units (micromoles of *p*-nitrophenyl phosphate hydrolyzed per hour per milligram of protein in 1 M Tris-HCl, pH 8, 22 °C).

Concentration of Enzyme Samples. Enzyme solutions (1–5 mL) were concentrated to 0.5–0.8 mL for NMR experiments by repeated ultrafiltration in the appropriate D_2O buffer using an Amicon PM-10 membrane. Following centrifugation to remove any precipitate, the enzyme solution was transferred to an 8-mm NMR tube, and specific activity and protein concentration were measured on aliquots suitably diluted into 0.1 M Tris, pH 7.4.

pH and Inhibitor Titrations. pH titrations of the [^{13}C]-histidine enzyme (in 0.01 M Tris (D_2O) and 0.1 M NaCl) were accomplished by addition of 1–3-μL aliquots of 0.2 M NaOD or DCl directly into the NMR tube followed by rapid manual mixing. Using this method, only slight denaturation (<5%) was observed during the 48 h required to titrate the enzyme between pH 9.5 and 4.5. At pH values less than 5.5, 2 equiv of Zn^{2+} was added to assure occupancy of the zinc binding sites, since it has been shown that, without added Zn^{2+}, metal begins to dissociate below this pH (Applebury & Coleman, 1969). pH measurements were made at 30 °C both before and after each spectrum with a Sargent Welch Model NX pH meter using a long, thin Ingold microcombination electrode, which was inserted directly into the 8-mm NMR tube. Reported pH values are uncorrected for the deuterium isotope effect at the glass electrode. Histidine pK_a values using uncorrected pH readings are reported to be the same in both H_2O and D_2O since the isotope effect on pK values is equal and opposite to the effect of deuterium ion at the glass electrode (Sachs et al., 1971).

Histidine pK_a values were determined by the dependence of observed chemical shift (δ_{obsd}) on pH. A nonlinear least-squares program was used to fit the data according to the following equation, assuming a single simple titration equilibrium (Markley, 1973):

$$(\delta_{obsd} - \delta_{H^+})/(\delta_{H^0} - \delta_{H^+}) = K_a/(K_a + [D^+])$$

where K_a is the dissociation constant of the histidine, $[D^+]$ is the deuterium ion concentration calculated from the pH measurements, and δ_{H^+} and δ_{H^0} are the chemical shifts of the protonated and neutral forms of histidine, respectively.

NMR Methods. ^{13}C NMR spectra were obtained at 25.14 MHz on a Nicolet TT-23 spectrometer using 8-mm tubes. All spectra were recorded under conditions of full proton decoupling at an ambient probe temperature of 31 ± 2 °C. The spectrometer was internally locked on the D_2O resonance of the solvent. The following parameters were used: spectral width, 5000 Hz; digital resolution, 1.25 Hz; acquisition time, 0.4 s; pulse repetition rate, 1.5 s; pulse angle, 70°; digital broadening, 4 Hz. Typical spectra required 4 to 8 h for enzyme concentrations ranging from 1.0 to 1.3 mM. Chemical shifts are reported in parts per million downfield from Me_4Si and were measured relative to dioxane (67.86 ppm downfield from external Me_4Si), which was added as an internal standard. Spin–lattice relaxation times (T_1) were obtained by the progressive saturation method (Freeman et al., 1972).

[1] Abbreviations used: NMR, nuclear magnetic resonance; $^{13}C(H)$ AP, [γ-^{13}C]histidine alkaline phosphatase; $^{13}C(D)$ AP, β,β-[γ-^{13}C]dideuteriohistidine alkaline phosphatase.

[2] 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).

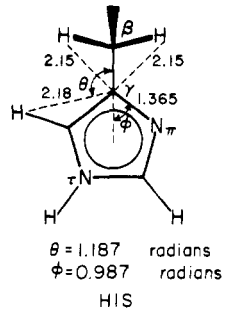

FIGURE 1: Internuclear distances (angstroms) and internal rotation angles for the histidine side chain. The values were computed from the known bond distances and angles in histamine (Bonnet & Ibers, 1973).

Data was analyzed using a nonlinear least-squares fit to a single exponential decay. Nuclear Overhauser enhancement (NOE) measurements were made using the gated decoupling technique (Opella et al., 1974).

Results and Discussion

Spectrum of [^{13}C]Histidine Alkaline Phosphatase. Natural abundance ^{13}C NMR has been shown to provide a valuable probe of the environments of the aromatic residues in several proteins (Oldfield et al., 1975; Ugurbil et al., 1977; Wilbur & Allerhand, 1977). Unfortunately, only relatively small proteins (mol wt <25 000) available in large quantities and exhibiting high solubility (>5 mM) give rise to interpretable spectra, and even then the assignment of resolved resonances to individual amino acid residues is a formidable problem due to the large number of signals present. In order to extend the molecular weight range of proteins accessible to NMR investigation, it is clear that incorporation of specifically ^{13}C-enriched amino acids is necessary. Besides providing the increased sensitivity needed to distinguish the labeled residues from the natural abundance background, a suitable label should exhibit sufficiently narrow signals and sensitivity to protein environment so that resolution of overlapping resonances is possible. The quaternary (nonprotonated) carbons of the aromatic amino acids hold the most promise of satisfying these criteria (Oldfield et al., 1975). For this reason, histidine enriched in ^{13}C at the γ position (Figure 1) was synthesized (Earl, 1978) and incorporated in vivo into alkaline phosphatase. Histidine was chosen because of its potential as an active-site probe, since histidine residues have been implicated in the coordination of the catalytically essential zinc ions (Tait & Vallee, 1966; Taylor & Coleman, 1972; Csopak & Falk, 1974).

The spectrum of ^{13}C(D) AP[1] is shown in Figure 2. The 7 well-resolved resonances between 126 and 140 ppm arise from the γ carbons of the 10 labeled histidine residues present in each subunit.[3] Without specific ^{13}C labeling, these histidine signals would be indistinguishable from the noise, as are the other aromatic carbons between 110 and 160 ppm. The other signals in the spectrum arising from ^{13}C nuclei at natural abundance can be assigned by comparison with other ^{13}C-labeled amino acid and protein spectra (Gurd & Keim, 1973). The broad envelopes at about 170–176, 50–65, and 15–35 ppm come from carbonyl carbons, α carbons, and aliphatic side-chain carbons, respectively. The relatively narrow peaks at 158.5 and 40.0 ppm arise from the 26ζ carbons of arginine and the 56ϵ carbons of lysine in the dimer, respectively. The signal at 67.9 ppm is from the internal dioxane standard and the peak at 61.0 ppm arises from the Tris buffer.

[3] Recent sequence data indicates the presence of 10 histidine residues per monomer (R. A. Bradshaw, personal communication). At a higher magnetic field, all 10 of these residues give rise to a resolved resonance (Otvos & Armitage, 1980).

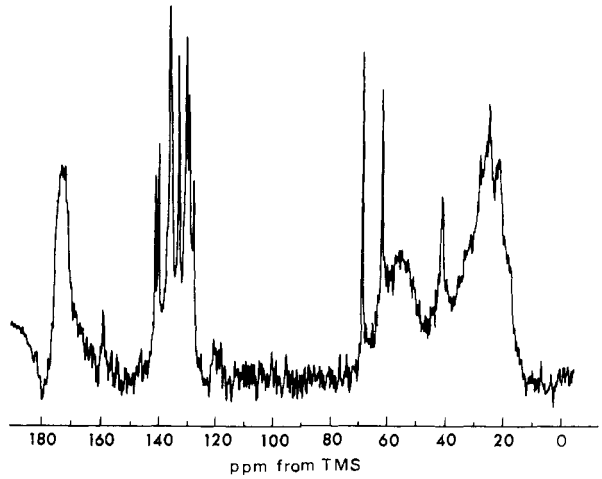

FIGURE 2: ^{13}C NMR spectrum (15 000 transients) of purged β,β-[γ-^{13}C]dideuteriohistidine alkaline phosphatase at 25.1 mHz. The enzyme concentration was 1.42 mM in 0.01 M Tris (D_2O) and 1.0 M NaCl, pH 7.4. Specific activity was 3000 units. Digital broadening = 4 Hz, pulse angle = 90°, pulse repetition rate = 4.0 s.

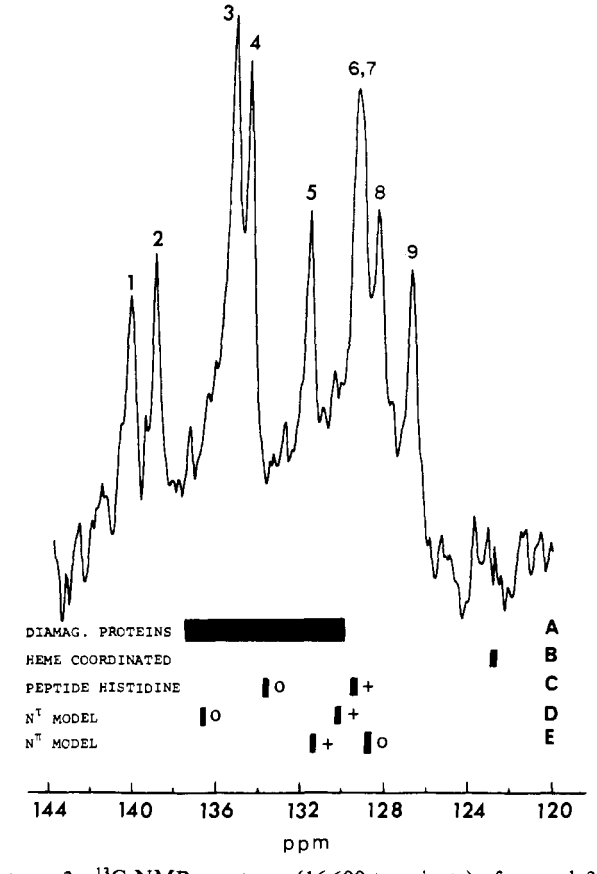

FIGURE 3: ^{13}C NMR spectrum (16 600 transients) of purged β,β-[γ-^{13}C]dideuteriohistidine alkaline phosphatase at 25.1 MHz and some literature histidine Cγ chemical shift values. The enzyme concentration was 1.20 mM in 0.1 M Tris (D_2O), pH 7.5. Specific activity was 2950 units. (A) Observed chemical shift range of histidine Cγ carbons in several diamagnetic proteins (Oldfield et al., 1975). (B) Chemical shift of the heme-coordinated His-18 in horse heart ferrocytochrome *c* (Oldfield et al., 1975). (C) Chemical shifts of the protonated and neutral forms of histidine in the small peptides angiotensin II and thyrotropin-releasing factor (TRF) (Deslauriers et al., 1974, 1975). (D) Chemical shifts of the protonated and neutral forms of N^{τ}-methylimidazole TRF (Deslauriers et al., 1974). (E) Chemical shifts of the protonated and neutral forms of N^{π}-methylimidazole TRF (Deslauriers et al., 1974). The protonated and neutral forms of histidine are denoted + and 0, respectively.

Chemical Shift Information. Another spectrum of the $^{13}C(D)$ enzyme is shown in Figure 3. Under these conditions, 8 resonances are clearly resolved corresponding to the 10 histidines per monomer in the symmetric dimer. The signal at 129.2 ppm is shown below to represent two histidine residues. At a higher magnetic field strength, the signal from the tenth histidine is found to be located under peaks 3 and 4 (Otvos & Armitage, 1980). The spectrum (not shown) of $^{13}C(H)$ AP is very similar to the spectrum of the deuterated histidine enzyme, although several pairs of resonances are not as well resolved. The reason for this is the approximately 30% reduction in line width when the β hydrogens are replaced by deuteriums, thereby eliminating a large portion of the intramolecular dipolar relaxation (see relaxation section below).

The most striking feature of the spectrum besides the large number of resolved resonances is the large chemical shift range (14 ppm) exhibited by the individual histidine residues in the folded enzyme structure. This is approximately double the range reported for the histidine γ carbons in several smaller proteins (Figure 3A) and in aspartate transcarbamylase (Moore, 1976) and the β subunit of tryptophan synthetase (Earl, 1978) studied in this laboratory. Several factors can contribute to the observed histidine Cγ chemical shifts in alkaline phosphatase: (1) the tautomeric form of the neutral imidazole ring; (2) the proton dissociation state of the imidazole ring; (3) the ligation of metal ion (Zn^{2+} and/or Mg^{2+}) to the histidine ring; and (4) the local environment of the histidine side chain in the tertiary and quaternary structures of the folded protein.

In basic solution the neutral histidine ring can exist in two tautomeric forms, the N^{τ} tautomer shown in Figure 1 and the N^{π} tautomer in which the ring proton is attached to the nitrogen atom adjacent to Cγ. ^{13}C NMR can be used to distinguish between these structures since, unlike the ^{1}H NMR shifts of the ring protons, the ^{13}C NMR chemical shift of Cγ is sensitive to tautomeric form. This is illustrated in Figures 3D and E for N^{τ}-methyl- and N^{π}-methylhistidine, which are model compounds for the N^{τ} and N^{π} tautomers and which have shifts of 136.4 and 128.7 ppm, respectively, in the tripeptide TRF at alkaline pH (Deslauriers et al., 1974). Furthermore, protonation of the two tautomeric forms causes Cγ to shift in opposite directions: upfield for the N^{τ} tautomer and downfield for the N^{π} form. By exploiting these ^{13}C chemical shift differences, it has been determined that the N^{τ} tautomer predominates for free L-histidine in basic solution (Reynolds et al., 1973). This is also the case when histidine is incorporated into synthetic or natural peptides of varying length or whenever the imidazole ring is free to interact with an aqueous environment (Reynolds et al., 1973; Deslauriers et al., 1975). From these considerations, it should be a simple matter to determine the tautomeric forms of the histidine residues in alkaline phosphatase from the direction of their chemical shift changes upon protonation. However, as will be discussed below, only the histidines giving rise to signals 3, 4, and 5 titrate between pH 4.5 and 9.5. From the upfield variation of their shifts as the pH is lowered, all four of these histidines are predominantly in the N^{τ} tautomeric form at alkaline pH. The other six histidine residues do not titrate, either because they are buried within a region of the protein structure that is inaccessible to solvent or because they are coordinated to a metal ion. In either case, their tautomeric forms can only be inferred from a comparison of their absolute chemical shift values to those of model compounds.

X-ray structures of a number of metalloproteins have shown that both tautomeric forms of histidine participate in metal ion coordination. For example, the two liganded histidines in carboxypeptidase A are N^{τ} tautomers (Quiocho & Lipscomb, 1971), while both histidines in the closely related thermolysin molecule are in the N^{π} tautomeric form (Colman et al., 1972). Carbonic anhydrase contains three histidine Zn^{2+} ligands: two N^{π} tautomers and one N^{τ} tautomer (Kannan et al., 1975). Apparently, the tautomeric form adopted by the coordinated histidine is determined solely by its ability to be stabilized or optimally oriented by the surrounding protein structure. In all metalloenzymes thus far examined, the imidazole nitrogen atom not participating in metal ion coordination is involved in hydrogen bond formation with a neighboring protein residue.

Histidine residues appear to be involved in metal ion coordination in alkaline phosphatase, although the exact number serving as ligands is not known. The best evidence suggests at least two histidine ligands, although three or more cannot be ruled out (Taylor & Coleman, 1972; Csopak & Falk, 1974). Of the six nontitrating histidines, the most likely candidates for zinc ligands are His-1 and -2, which lie well outside the chemical shift range normally observed for histidine residues in proteins (Figure 3A). From the downfield position of their chemical shifts it can be assumed that these histidines exist in the N^{τ} tautomeric form. The additional 2–4-ppm downfield shift of His-1 and -2 from the position of the model N^{τ} tautomer (136.4 ppm) is presumably the result of coordination of the diamagnetic Zn^{2+} ion to N^{π}. Unfortunately, there is no experimental information available regarding the direction or magnitude of the Cγ chemical shift induced by coordination of diamagnetic metals to histidine. A deshielding effect might be predicted on the basis of the expected reduction of electron density at Cγ, although changes in excitation energy have been shown capable of overshadowing this type of contribution (Reynolds et al., 1973; Quirt et al., 1974). Since it has been shown by X-ray diffraction that a 2:1 L-histidine–Zn^{2+} complex forms in solution with the metal ion coordinated to N^{π} (Freeman, 1967), a ^{13}C chemical shift determination was made at natural abundance to ascertain the effect of zinc addition to L-histidine at alkaline pH. It was found that Cγ shifts downfield by over 2 ppm at a histidine:Zn^{2+} ratio of more than 2:1. Thus, the assignment of peaks 1 and 2 to histidine residues liganded to Zn^{2+} at the N^{π} position appears reasonable.

Assignment of the upfield, nontitrating resonances 6, 7, 8, and 9 is far less straightforward. From their chemical shifts, these peaks could represent protonated histidines buried in a solvent-inaccessible region of the protein, neutral histidine constrained by interresidue hydrogen bonding to exist in the N^{π} tautomeric form, or histidine coordinated to metal ion at N^{τ}.

Effect of pH. The quaternary carbon of free histidine exhibits a much greater chemical shift change upon protonation (ca. 4.6 ppm) than do the other imidazole carbon atoms. As such, Cγ provides the most sensitive probe of the ionization behavior of histidine in a protein. This is illustrated by the pH titration profile of alkaline phosphatase shown in Figure 4. Of the nine resonances, only resonances 3, 4, and 5 titrate in a characteristic manner. From the downfield movement of their shifts with increasing pH, all four histidines giving rise to these signals must exist as N^{τ} tautomers in their neutral states. No detectable differences were observed when the titration was carried out in the presence and absence of inorganic phosphate, suggesting that signals 3, 4, and 5 may arise from histidines that are relatively remote from the active-site region. Furthermore, the experimental points give a good fit to the theoretical curve based on a single simple titration equilibrium, indicating that there is little or no interaction with

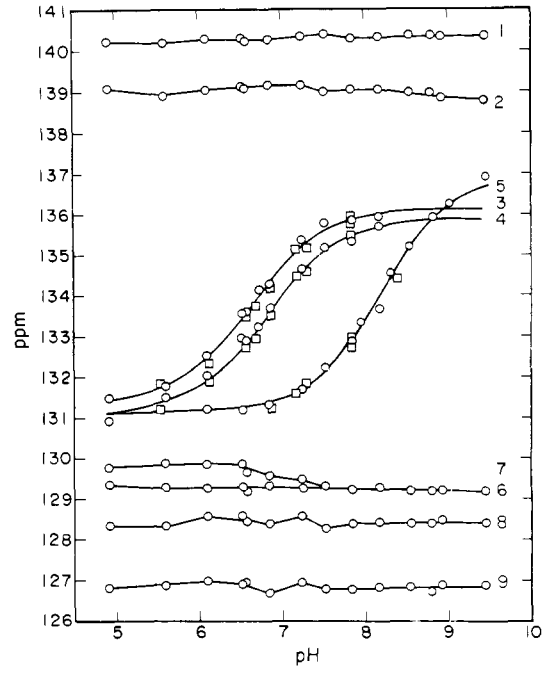

FIGURE 4: Effect of pH on the chemical shifts of the histidine residues in alkaline phosphatase. The data were combined from several experiments using both $^{13}C(H)$ and $^{13}C(D)$ alkaline phosphatase in 0.01 M Tris (D_2O) and 0.1 M NaCl. The labels 1 to 9 correspond to the numbered resonances in Figure 3. The squares and circles represent titrations of enzyme in the presence and absence of inorganic phosphate, respectively.

neighboring ionizable residues.

pK_a values for the histidines giving rise to signals 3 and 4 were 6.65 and 6.80, respectively, with both resonances exhibiting a normal chemical shift change upon ionization of 4.7 ppm. These pK_a values are very similar to those observed for histidine in small peptides, suggesting that resonances 3 and 4 represent exposed histidines on the enzyme surface that have little interaction with neighboring protein residues. His-5 exhibits a pK_a of 8.2, considerably higher than is normally observed for histidine residues in proteins. Factors that could be responsible for this abnormal pK_a include the presence of one or more nearby negatively charged groups or a stabilization of the imidazolium form by hydrogen bonding.

An additional effect of pH on the [^{13}C]histidine enzyme spectrum is the downfield movement of His-7 with decreasing pH, which allows it to be resolved from His-6 below pH 7.3. Assuming this chemical shift change reflects the protonation of His-7, the direction of the titration shift would indicate that the neutral imidazole ring exists as the N^{π} tautomer. Titration shift magnitudes of about 2 ppm have previously been observed for titratable histidine residues in the N^{π} tautomeric form (Deslauriers et al., 1974; Ugurbil et al., 1977). By contrast, the total titration shift of histidine-7 is only 0.5 ppm. It is therefore uncertain whether the imidazole ring is actually undergoing protonation or is instead monitoring an alteration in its environment brought about by titration of a neighboring group or a pH-dependent local conformational change. Evidence to be presented below and in an accompanying paper (Otvos & Armitage, 1980) suggests that His-7 is located in the active-site region and that the pH dependence of its chemical shift is influenced both by the total metal content of the enzyme and the presence of inorganic phosphate. These considerations, plus the dependence of the rate of phosphate monoester hydrolysis on an unknown process that has an apparent pK of 7.1 (Lazdunski & Lazdunski, 1969), suggest that His-7 may play an important role in the catalytic mechanism.

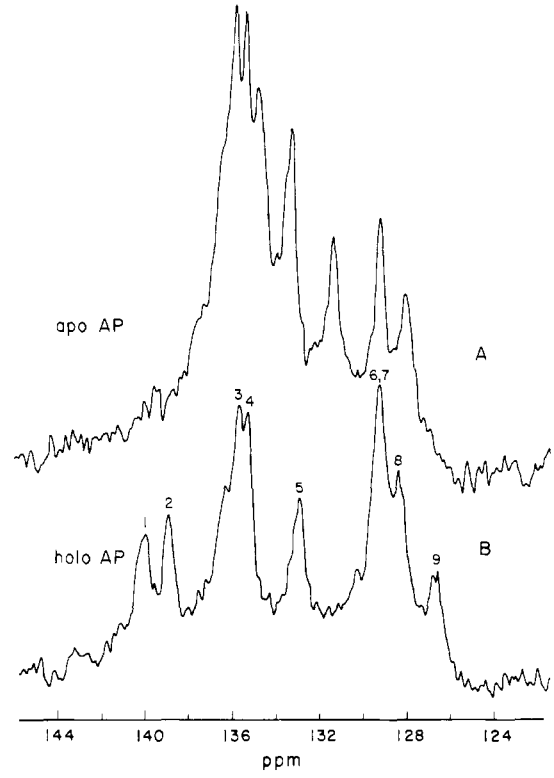

FIGURE 5: Effect of metal ion removal on the ^{13}C NMR spectrum of [^{13}C]histidine alkaline phosphatase. (A) $^{13}C(D)$ apoenzyme (42000 transients) prepared by dialysis against EDTA followed by extensive dialysis against metal-free buffer. Enzyme concentration was 1.98 mM in 0.01 M Tris and 0.1 M NaCl, pH 7.8. Specific activity was 6 units. (B) $^{13}C(D)$ holoenzyme (42000 transients) reconstituted with Zn^{2+} and Mg^{2+}. The enzyme concentration was 1.98 mM in 0.01 M Tris and 0.1 M NaCl, pH 7.8. The specific activity was 2800 units.

The nature of its possible participation in the catalytic process is currently under detailed investigation.

Effect of Metal Ion. Removal of metal ion from alkaline phosphatase is known to induce conformational changes (Knox & Wyckoff, 1973; Hull & Sykes, 1976), although the native dimeric structure is largely retained. Since the large chemical shift range observed for the [^{13}C]histidine enzyme has been attributed in part to metal ion coordination, it was of interest to determine the effects of metal ion removal on the spectrum. These are shown in Figure 5 for apoenzyme prepared by dialysis against EDTA followed by extensive dialysis against buffer to remove the chelator. Clearly, as was observed for the apofluorotyrosine enzyme (Hull & Sykes, 1976), there are dramatic spectral changes induced by removal of metal. The most noticeable of these is the contraction of the chemical shift range from both the upfield and downfield directions so that the apoenzyme shift range now closely resembles those observed in nonmetalloproteins (Figure 3A). Although assignment of resonances perturbed in the apoenzyme to histidines involved in metal ion ligation cannot be made with certainty due to the accompanying conformational change, several observations tend to support the tentative assignments that have previously been made.

The most obvious spectral change occurs in the downfield region, with His-1 and -2 shifting upfield at least 3 ppm. This provides strong supporting evidence for the involvement of these histidines as zinc ligands, since an upfield shift would be expected upon removal of the electron-withdrawing metal ion. Furthermore, His-1 and -2 in the apoenzyme may no longer be constrained to exclusively adopt the N^{τ} tautomeric form, which would further contribute to their upfield shift

(Figure 3, C and D). As might be expected, the solvent-exposed histidines that produce resonances 3, 4, and 5 are little affected by metal ion removal. The small 0.4-ppm downfield shift of His-5 in the apoenzyme is probably less due to metal ion removal than to a slight pH difference between the samples, since the chemical shift of this resonance is extremely sensitive to pH in this pH region (see Figure 4). Furthermore, metal ion does not appear responsible for the abnormally high pK_a of His-5, since it exhibits very similar titration behavior in the apoenzyme (results not shown). In the upfield region, the chemical shift of His-6 is identical in the holo- and apoenzymes, suggesting it is probably not a metal ligand. The participation of His-7 and -8 in metal ion coordination cannot be assessed from the spectra in Figure 5 because of uncertainty as to which resonances in the apoenzyme spectrum correspond to these two residues. His-9, on the other hand, is clearly missing from the upfield region of the apoenzyme spectrum, suggesting that it may be a third metal ligand with the metal coordinated to N^{τ}. The 2-ppm upfield shift of resonance 9 from the position of the model N^{π} tautomer (Figure 3E) also suggests the influence of metal ligation. Unfortunately, the effect of diamagnetic metal-ion coordination to this tautomeric form is unknown. The large upfield shift observed for the metal ion-coordinated N^{τ} tautomeric His-18 in horse heart ferrocytochrome *c* (Figure 3B) cannot be used for comparison, since most of its upfield shift is due to the ring current effect of the heme group (Oldfield et al., 1975).

The probable participation of His-1, -2, and -9 in metal ion coordination indicates the potential value of the [^{13}C]histidine enzyme as an active-site probe. However, since the holoenzyme used in these studies presumably contains its full complement of four Zn^{2+} and two Mg^{2+} (Bosron et al., 1975), it is not possible to state whether the three histidine residues are bound to the same or different metal ions or whether these metal(s) are the "catalytic" or "structural" Zn^{2+} ions or even Mg^{2+}. Detailed metal ion titrations of apoenzyme with both diamagnetic and paramagnetic metals are presented in an accompanying paper (Otvos & Armitage, 1980) in an attempt to answer these questions as well as to determine the stoichiometries and relative locations of the enzyme-bound metal ions.

Relaxation Studies. The ability to observe resolved ^{13}C resonances corresponding to individual histidine residues in alkaline phosphatase offers the possibility of extracting valuable motional information from the spin relaxation behavior of each signal. Several studies have demonstrated the utility of ^{13}C NMR for obtaining such information, particularly when the ^{13}C nucleus is directly bonded to one or more protons (Deslauriers et al., 1975; Torchia et al., 1975; Bleich et al., 1976). In these cases, relaxation is dominated by the ^{13}C–^{1}H dipolar interaction, and interpretation of relaxation times in terms of molecular motion is relatively unambiguous. In the case of the Cγ carbons of histidine, however, where no protons are attached to the ^{13}C nucleus, relaxation due to intramolecular dipolar relaxation becomes much less efficient because of the inverse sixth power dependence of this mechanism on the ^{13}C–^{1}H distance. Consequently, other less efficient relaxation mechanisms that would not normally compete with intramolecular ^{13}C–^{1}H dipolar relaxation need to be considered. These include spin rotation, scalar relaxation, ^{13}C–^{14}N dipolar relaxation, chemical shift anisotropy (CSA), intermolecular ^{13}C–^{1}H interactions from protons on neighboring residues, and dipolar relaxation due to paramagnetic species (impurities and dissolved oxygen). Spin rotation can confidently be neglected in a protein as large as alkaline phosphatase (Gurd & Keim, 1973). Scalar relaxation contributions from the bonded nitrogen and the nonbonded deuteriums (in ^{13}C(D) enzyme) are also expected to be small (Browne et al., 1973). Dipolar ^{13}C–^{14}N relaxation must be considered, since it has been shown to be significant for a quaternary carbon directly bonded to nitrogen (Oldfield et al., 1975). Likewise, chemical shift anisotropy can also measurably contribute to relaxation of a nonprotonated aromatic carbon, even at 25 MHz (Norton et al., 1977). Therefore, in our analysis of the relaxation parameters of the alkaline phosphatase histidine resonances both CSA and ^{13}C–^{14}N dipolar interactions will be taken into account along with the intramolecular ^{13}C–^{1}H dipolar relaxation mechanism. Additional dipolar relaxation contributed by protons on nearby residues and paramagnetic impurities may complicate the quantitative treatment of the data, but is not expected to seriously affect the qualitative conclusions that are made concerning the relative rotational freedom of the histidine residues.

Theoretical calculations of the contributions of C–H and C–N intramolecular dipolar relaxation to line width, T_1, and NOE values were carried out assuming isotropic diffusion of the enzyme molecule as a whole coupled with one degree of internal rotation about the C_β–C_γ bond (Doddrell et al., 1972). Four principal relaxation vectors were considered for the Cγ carbon of histidine, three with the hydrogens two bonds removed and one with the directly bonded nitrogen. Figure 1 shows the lengths and internal rotation angles of these vectors, which were used in the calculations. For ^{13}C(D) histidine, the contribution to relaxation from the two β deuteriums was ignored, since deuterium is only about 6% as effective compared to hydrogen in inducing dipolar relaxation. Similarly, interaction with a proton attached to the adjacent nitrogen, either in the N^{π} tautomer or in the imidazolium form, was ignored, since exchange at this position with deuterium from the solvent (D_2O) is rapid. This assumption may not be valid, however, for buried histidine residues that are inaccessible to solvent. A molecular correlation time τ_c of 70 ns for alkaline phosphatase was used in the calculations. This value was assigned on the basis of the τ_c = 67 ns obtained by extrapolation from fluorescence data (Yguerabide et al., 1970) and the τ_c = 76 ns calculated from fluorotyrosine NMR relaxation data (Hull & Sykes, 1975).

A calculation of the contribution to relaxation from the field-dependent mechanism of chemical shift anisotropy was carried out using a computer program (Moore, 1976) based on equations derived for the case of axially symmetric (symmetric top) diffusion (Hull & Sykes, 1975). The calculations were made possible by the determination of reasonably accurate values for the histidine Cγ chemical shift tensor and its orientation relative to the principal diffusion axes (Moore, 1976). These were acquired by an analysis of the powder spectrum of [γ-^{13}C]histidine by proton-enhanced nuclear induction spectroscopy. The following parameters, as defined by Hull & Sykes (1975), were used: δ_z = −78 ppm, η = −0.79, β = 90°, γ = 38°.

Figure 6 shows the calculated values of T_1, line width ($1/\pi T_2$), and NOE for the ^{13}C(H) and ^{13}C(D) histidine residues in alkaline phosphatase at 25 MHz as a function of the correlation time, τ_R, for rotation about the C_β–C_γ bond. All three relaxation parameters are virtually insensitive to internal rotation slower than the rate of overall molecular tumbling (70 ns). For less restricted motion, T_1 becomes quite sensitive to τ_R, decreasing for intermediate rates of motion ($\tau_R \simeq 10^{-7}$ to 10^{-9}) and increasing for faster motion. The calculations also predict that substitution of the histidine's β hydrogens with

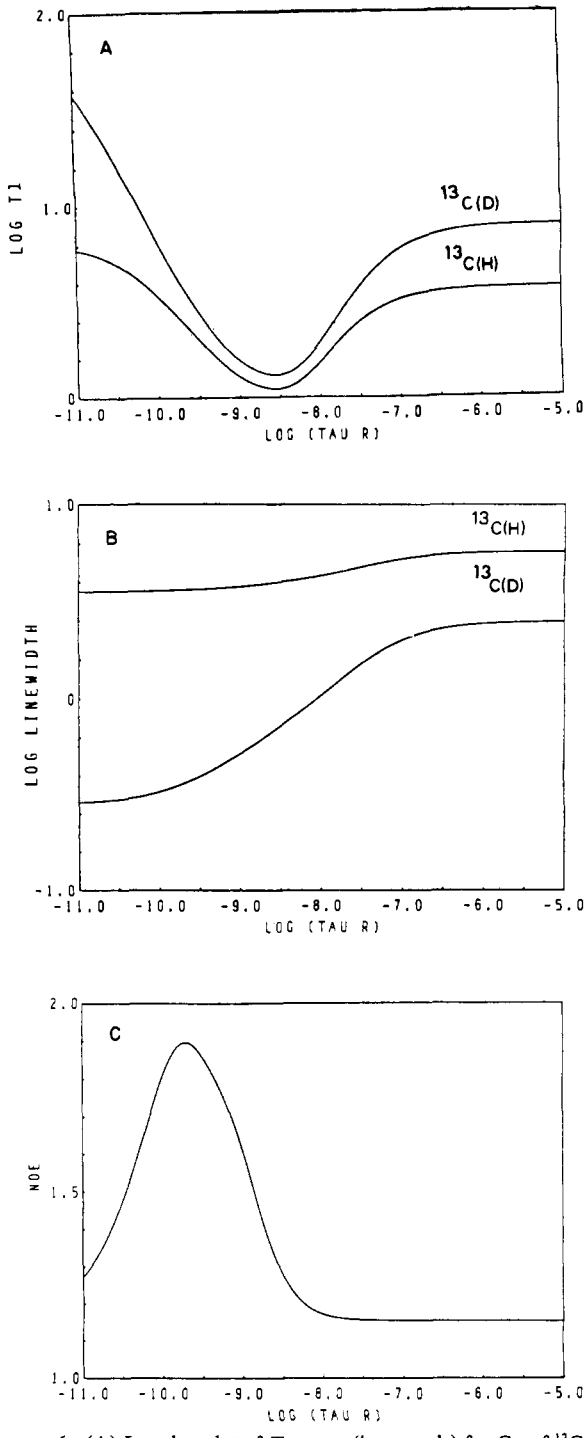

FIGURE 6: (A) Log–log plot of T_1 vs. τ_R (in seconds) for Cγ of ^{13}C(H) histidine and ^{13}C(D) histidine at 25 MHz with the overall molecular correlation time τ_C fixed at 70 ns. (B) Log–log plot of line width (in hertz) vs. τ_R (in seconds) for Cγ of ^{13}C(H) histidine and ^{13}C(D) histidine at 25 MHz with the overall molecular correlation time τ_C fixed at 70 ns. (C) Semilog plot of NOE vs. τ_R (in seconds) of Cγ of ^{13}C(H) histidine at 25 MHz with the overall molecular correlation time τ_C fixed at 70 ns.

deuteriums will result in ~4 Hz narrower line widths and T_1 values that are more sensitive to internal rotation. The NOE of ^{13}C(H) histidine is a very sensitive function of internal motion for $\tau_R < 5$ ns, increasing to a maximum of ca. 1.9 and then decreasing again for extremely fast internal rotation correlation times. Since the NOE will be reduced when relaxation mechanisms other than dipolar (e.g., CSA) become important, calculations were not done for ^{13}C(D) histidine, which has even fewer efficient intramolecular dipolar interactions. For ^{13}C(H) histidine, CSA is calculated to contribute about 10% to T_1 at 25 MHz, which will somewhat decrease the magnitude of the NOE shown in Figure 6C but will not affect its dependence upon τ_R.

A summary of the chemical shifts and relaxation parameters for each of the eight resolved histidine resonances in the [γ-^{13}C]histidine enzyme spectrum is shown in Table I. Several conclusions concerning the relative rotational freedom of the histidine residues in alkaline phosphatase can be drawn from this data using the theoretical curves in Figure 6. Since a given value of T_1 in Figure 6A corresponds to two values of τ_R, it is necessary to use another relaxation parameter to unambiguously determine the rotational correlation time. In this case, the NOE can conveniently be used for this purpose, since only for τ_R values less than about 3 ns can a measurable enhancement be observed. Table I shows that the NOE for each of the eight histidine peaks is within experimental error of the minimum value of 1.15. Therefore, all histidine residues are subject to the constraint that $\tau_R \geq 3 \times 10^{-9}$ s; hence, the right-hand side of the T_1 curve in Figure 6A is applicable.

The T_1 values in Table I appear to fall into three distinct groups. His-1 and -2 have relatively long T_1s, the histidines producing signals 3, 4, and 5 have short T_1s near the theoretical minimum, and His-6, -7, and -8 have intermediate T_1 values. This trend is shown more clearly by the data for the ^{13}C(D) enzyme, which was predicted to be a more sensitive probe of internal motion than the ^{13}C(H) enzyme (Figure 6A). By correlating the experimental T_1 data with the calculated values in Figure 6A, a qualitative picture of the mobilities of the histidine residues can be obtained that is consistent with their tentative assignments made in previous sections. His-1 and -2 are clearly more immobilized relative to the other histidines, giving τ_R values greater than about 50 ns. This conclusion provides additional evidence for their involvement in metal ion coordination, since ligation of metal to N^{π} and probable concomitant hydrogen bonding to N^{τ} should firmly lock these residues in the protein matrix. The histidines corresponding to signals 3, 4, and 5, on the other hand, appear to be relatively free to rotate, as might be expected of surface residues that rotate freely and are exposed to solvent. Apparent τ_R values for these residues are in the range of 3–5 ns. His-6, -7, and -8 have calculated rotational correlation times on the order of 10–20 ns, characteristic of residues experiencing restricted mobility. Chemical shift evidence has indicated that these histidines are almost certainly either buried protonated residues or N^{π} tautomers. Since the same factors that constrain these residues to exist in these forms might also limit solvent accessibility, it is not at all certain that deuterium exchange has occurred on the N^{π} nitrogen. If this is the case, additional intramolecular relaxation not accounted for in the calculations could exist, causing the calculated τ_R values for these residues to be too low. Thus, His-9, which has been suggested to be a third metal ligand, may be just as immobilized as His-1 and -2, although this fact is not reflected in the experimental T_1 data.

It should be emphasized here that the τ_R values obtained above for the histidine residues in alkaline phosphatase are very approximate due to the likelihood of significant contributions to relaxation from unknown sources. It is unfortunate that the same lack of efficient intramolecular dipolar relaxation which makes the Cγ carbon of histidine an excellent ^{13}C NMR probe in large proteins due to its narrow resonances is also the factor that makes interpretation of its relaxation parameters so difficult. That relaxation mechanisms other than those treated in the calculations are operating in alkaline phosphatase

Table I: Chemical Shift and Relaxation Parameters for the [γ-^{13}C]Histidine Resonances in Alkaline Phosphatase at 25 MHz

	resonance							
	1	2	3	4	5	6,7	8	9
chemical shift[a]	140.20	138.95	135.70	135.15	132.20	129.20	128.30	126.75
^{13}C(H) T_1 (s)[b]	1.9 ± 0.2	1.5 ± 0.2	1.1 ± 0.1[g]	1.1 ± 0.1[g]	1.1 ± .02	1.7 ± 0.2	1.3 ± 0.2	1.1 ± 0.4
^{13}C(D) T_1 (s)[c]	8.5 ± 1.0	6.4 ± 0.7	1.1 ± 0.2[g]	1.1 ± 0.2[g]	1.0 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	2.4 ± 0.4
^{13}C(H) NOE[d]	1.3	1.0	1.1[g]	1.1[g]	1.1	1.2	1.2	1.3
^{13}C(H) line width (Hz)[e]	15 ± 2	16 ± 2	17 ± 3	17 ± 3	18 ± 2	22 ± 3	19 ± 3	17 ± 2
^{13}C(D) line width (Hz)[f]	10 ± 2	10 ± 2	13 ± 2	13 ± 2	13 ± 2	18 ± 3	14 ± 3	11 ± 2

[a] Chemical shifts in parts per million downfield from Me_4Si are average values for purged enzyme at pH 7.6. Estimated accuracy is ±0.1 ppm. [b] T_1 values determined for purged ^{13}C(H) enzyme in 0.1 M Tris (D_2O) and 0.9 M NaCl, pH 7.3. [c] T_1 values determined for purged ^{13}C(D) enzyme in 0.01 M Tris (D_2O) and 1.0 M NaCl, pH 7.4. [d] NOE values (±0.2) were determined for native, phosphate-containing ^{13}C(H) enzyme in 0.1 M Tris (D_2O), pH 7.8. [e] Average line widths for several spectra of ^{13}C(H) enzyme corrected for digital broadening. [f] Average line widths for several spectra of ^{13}C(D) enzyme corrected for digital broadening. [g] Combined values for peaks 3 and 4 which were unresolved under these conditions.

Table II: Changes in [γ-^{13}C]Histidine Chemical Shifts upon Inhibitor Binding[a]

		resonance								
inhibitor	conditions	1	2	3	4	5	6	7	8	9
phosphate	0.1 M Tris, pH 7.8	0.0	0.0	0.0	0.0	0.0	+0.1	+0.5	+0.5	+0.1
phosphate[b]	0.1 M Tris, pH 7.25	0.0	0.0	0.0	0.0	0.0	0.0	+0.5	+0.6	+0.1
phosphate	0.05 M NaOAc–0.95 M NaCl, pH 4.6[c]	0.0	+0.2	0.0	0.0	0.0	0.0	0.0	~+0.6	~0.6
arsenate	0.1 M Tris, pH 7.3	+0.3	+0.1	0.0	0.0	0.0	0.0	+1.4	+0.6	+0.8
p-aminobenzylphosphonate	0.1 M Tris, pH 7.7	0.0	0.0	0.0	0.0	+0.3	0.0	0.0	+0.2	~0.6

[a] Chemical shifts were measured relative to Me_4Si with an accuracy of ±0.1 ppm. Chemical-shift changes are the ^{13}C chemical shifts for enzyme saturated with inhibitor minus the shifts for purged enzyme. A positive shift is upfield. [b] In this experiment, phosphate was generated by adding the substrate *p*-nitrophenyl phosphate to the enzyme. [c] Excess Zn^{2+} (3 equiv) was present to assure occupancy of the metal binding sites at the low pH.

is clear from a consideration of the line widths. While the theoretical calculations accurately predict the observed 4–5 Hz reduction in line width accompanying replacement of the β hydrogens with deuteriums, the absolute values of the observed line widths are about 10 Hz greater than calculated. This could partially be due to T_2 relaxation contributions which would also affect T_1 values, such as dipolar relaxation from protons on nearby residues or from paramagnetic impurities. A large portion of the observed line widths, however, probably arises from factors that would not affect the internal motion analysis, such as instrumental broadening, exchange broadening due to tautomerization or ionization equilibria, and perhaps small unresolved chemical shift differences between residues in the two subunits. In conclusion, the good correlation between the calculated rotational mobilities of the alkaline phosphatase histidines, in particular residues 1–5, and the information about their local environments inferred previously from chemical shift, pH, and metal ion studies offers confirmatory evidence that the relaxation analysis is at least qualitatively valid.

Binding of Inorganic Phosphate. Addition of inorganic phosphate to purged (phosphate-free) alkaline phosphatase results in the formation of the tight noncovalent complex (E·P) whose dissociation constitutes the rate-determining step in enzyme turnover at alkaline pH (Hull et al., 1976). The ^{13}C spectral perturbations brought about by formation of E·P are shown in the phosphate titration experiment in Figure 7. His-7 and -8 undergo significant chemical shift changes, both shifting 0.5 ppm upfield (Table II). Approximately two phosphates per dimer are required to complete the spectral change; additional phosphate up to 20 equiv does not alter the spectrum shown in Figure 7D. The simultaneous observation of His-8 in both purged (8a) and bound (8b) states at intermediate levels of phosphate saturation (Figure 7, B and C) indicates that phosphate binding is a slow exchange process, as would be expected from the known stability of the E·P complex ($K_D \simeq 10^{-6}$ M). In the presence of phosphate, His-7 is always easily resolved on the upfield side of His-6 (Figure 7, C and D). Its chemical shift is found to be invarient between pH 6 and 8, in marked contrast to its apparent titration in this pH region in the phosphate-free enzyme (see Figure 4).

It is interesting to note that His-1, -2, and -9 are affected little, if at all, by formation of the E·P complex. As discussed earlier, these three histidines have been implicated as probable metal ion ligands, presumably located at the active site. Their failure to monitor the presence of phosphate may reflect their relatively distant spatial orientations with respect to the noncovalently bound phosphate. On the other hand, the chemical-shift perturbations of His-7 and -8, whose involvement in metal coordination is uncertain based on previous data, suggest that these residues are located in close proximity to the phosphate binding site. The possibility cannot be excluded, however, that these chemical shift changes may be induced indirectly by the conformational change brought about by E·P formation (Hull & Sykes, 1976).

Besides E·P, the other major intermediate in the hydrolysis of phosphate monoesters by alkaline phosphatase is the covalent E–P complex resulting from phosphorylation of an active-site serine residue. The effects of E–P formation on the [^{13}C]histidine enzyme spectrum are not conveniently measured because high equilibrium concentrations of E–P are generated only at low pH values where Zn^{2+} begins to dissociate from the enzyme (Chlebowski et al., 1976). Nonetheless, one phosphate binding experiment was conducted at pH 4.6 where ≳75% of the bound phosphate exists as E–P. The results are presented in Table II. Two significant differences are detected compared to the spectral changes brought about by noncovalent phosphate binding: (1) His-7 does not undergo a chemical shift change and (2) His-9, one of the suspected metal ligands, shifts downfield by 0.6 ppm.

Binding of Arsenate and Phosphonate. Orthoarsenate is a potent inhibitor of alkaline phosphatase, exhibiting a K_I value

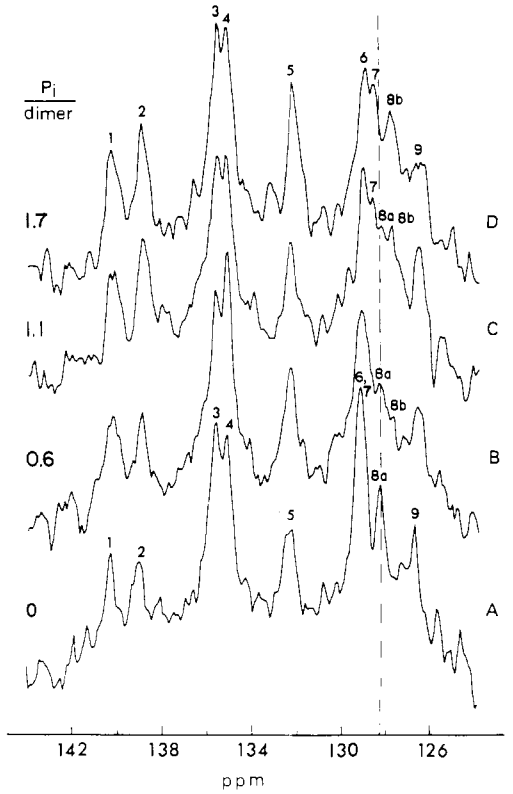

FIGURE 7: ^{13}C NMR titration of purged [γ-^{13}C]histidine alkaline phosphatase with inorganic phosphate at alkaline pH. Spectrum A is of 1.16 mM purged $^{13}C(D)$ alkaline phosphatase in 0.1 M Tris (D_2O), pH 7.8. Specific activity was 2900 units. Spectra B, C, and D were obtained following addition of aliquots of 78 mM phosphate to sample A to give the indicated stoichiometries. The spectra required 12000 to 15000 transients (pulse repetition rate = 1.5 s). Resonances labeled a and b represent histidines whose chemical shifts are markedly different in A and D.

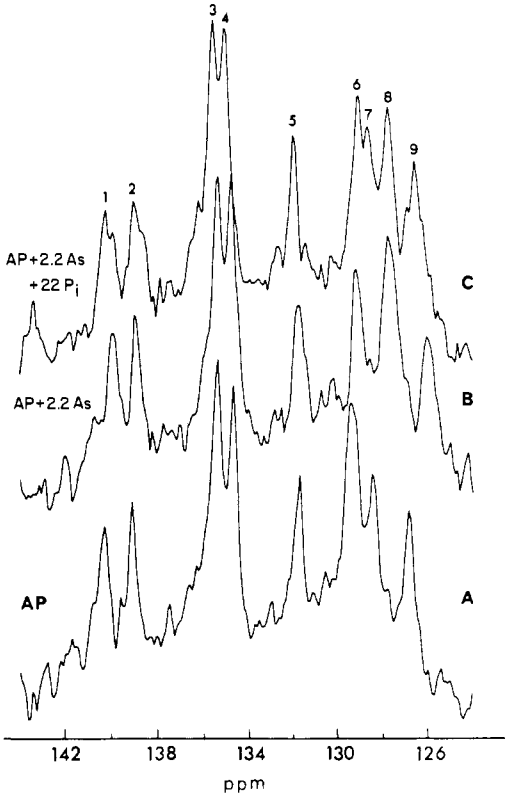

FIGURE 8: Comparison of ^{13}C NMR spectra of purged [γ-^{13}C]histidine alkaline phosphatase in the presence and absence of phosphate (P_i) and arsenate (As) at alkaline pH. Spectrum A is of 1.20 mM purged $^{13}C(D)$ enzyme in 0.1 M Tris (D_2O), pH 7.3. Specific activity was 2900 units. Spectrum B was obtained following the addition of 2.2 mol of arsenate/mol of dimer to the purged enzyme. Spectrum C resulted from the addition of 22 mol of P_i/mol of dimer to sample B. The spectra required 15000 to 17000 transients.

similar to phosphate ($K_I \simeq 10^{-6}$ M) (Gottesman et al., 1969). The effects of arsenate addition to the purged [^{13}C]histidine enzyme are shown in Figure 8B and Table II. To aid comparison between the arsenate and phosphate-containing enzymes, Figure 8C shows a spectrum generated by displacing the arsenate with a large excess of P_i. Like phosphate, arsenate induces large upfield shifts of His-7 and -8. Unlike phosphate, however, arsenate binding causes significant perturbations to the chemical shifts of His-1 and -9. These residues have been tentatively assigned to active-site metal ligands. Their altered environments in the presence of arsenate presumably reflect local differences in active-site conformation induced by the greater size of the arsenate anion compared to phosphate. Similar differences in environment surrounding the metal binding site(s) have been inferred from the circular dichroism changes induced by arsenate complexation to the Co^{2+} enzyme, which were opposite to those caused by P_i binding (Taylor et al., 1973).

Phosphonates are also competitive inhibitors of alkaline phosphatase, although they bind less tightly than P_i or arsenate at alkaline pH. The addition of a fivefold excess of *p*-aminobenzylphosphonate ($K_I \simeq 2 \times 10^{-4}$ M) to the [^{13}C]-histidine enzyme leads to minor changes compared to those induced by phosphate and arsenate (Figure 9 and Table II). Only His-9 undergoes a major perturbation, shifting downfield 0.6 ppm (the 0.3-ppm shift of His-5 may result from phosphonate interaction, but is more likely due to a small pH difference between the purged and phosphonate-bound enzyme samples). It is interesting to note the similarity between the effects of noncovalent phosphonate binding and covalent E–P

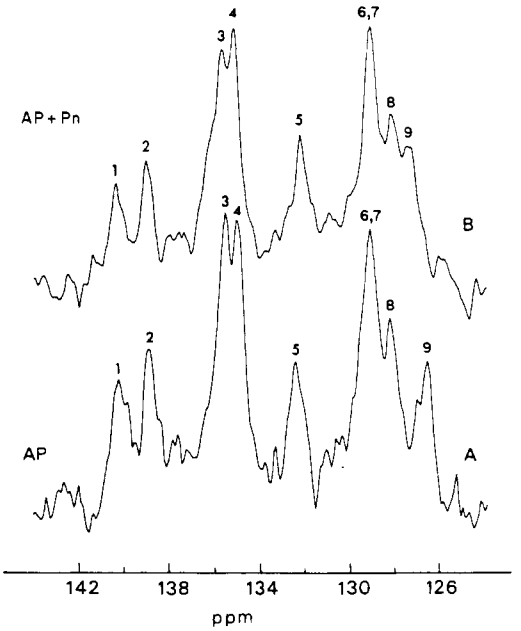

FIGURE 9: Comparison of ^{13}C NMR spectra of purged [γ-^{13}C]histidine alkaline phosphatase in the presence and absence of *p*-aminobenzylphosphonate (Pn) at alkaline pH. Spectrum A (18500) transients) is of 1.20 mM purged $^{13}C(D)$ enzyme in 0.1 M Tris (D_2O), pH 7.7. Specific activity was 2800 units. Spectrum B (18000 transients) was obtained following addition of 5.5 mol of *p*-aminobenzylphosphonate/mol of dimer to the purged enzyme.

formation at low pH (see Table II); only in these species is His-7 unperturbed and His-9 shifted downfield. The possible

mechanistic significance of this observation must await additional information regarding the locations of these histidine residues in the enzyme structure.

Summary

The results presented in this report demonstrate the unique suitability of [γ-^{13}C]histidine as a probe of protein structure. The usefulness of this ^{13}C NMR label stems directly from the sensitivity of the histidine γ-carbon chemical shift to a variety of structural features of the imidazole ring and to perturbations in its environment contributed by the surrounding protein structure. The resulting chemical shift dispersion offers the likelihood that resolved single histidine carbon resonances will be observed even from large proteins containing numerous histidine residues. The situation is expected to be especially favorable for study of metalloproteins, which, like alkaline phosphatase, employ histidine residues as metal ion ligands. Since metal coordination freezes the imidazole ring in either the N^{τ} or N^{π} tautomeric states, those histidines participating as metal ligands will exhibit pH-independent ^{13}C resonances >8 ppm apart in the downfield and upfield regions of the spectrum, respectively. Resonances from the remaining histidine residues in the protein will generally have pH-dependent chemical shifts located in the region between these two extremes.

^{13}C resonances arising from 9 of the 10 histidine residues in each of the symmetrically disposed subunits of the alkaline phosphatase dimer have been resolved in the present study (the tenth is resolved at a higher magnetic field (Otvos & Armitage, 1980). An analysis of their spin relaxation properties and chemical shift responses to pH, metal content, and inhibitor binding has revealed many details of the histidine environments in this enzyme which have heretofore been unknown. These are summarized as follows:

(1) His-1 and -2 are almost certainly metal ion ligands coordinated via their N^{τ} nitrogens. This is suggested by their abnormally large downfield chemical shifts, which are unperturbed by changes in pH but which are markedly upfield-shifted upon formation of apoenzyme. Their long T_1 values suggest they are immobilized in the protein structure ($\tau_R > 50$ ns) as would be expected for residues anchored in place by metal ligation and hydrogen bonding. Coordination to active-site metal ion(s) is suggested by the chemical shift perturbation brought about by arsenate binding.

(2) The four histidines giving rise to signals 3, 4, and 5 are surface residues exposed to solvent that have little or no interaction with neighboring protein residues or enzyme ligands. All exist predominantly in the N^{τ} tautomeric form and have resonances that titrate freely with pK_a values of 6.65, 6.80, and 8.20, respectively. Relaxation studies indicate that all four residues have relatively great rotational freedom with τ_R values of ca. 2–5 ns.

(3) His-6 appears to be a buried residue whose chemical shift indicates that it must be either protonated and involved in a very stable salt linkage or constrained by hydrogen bonding to exist in the N^{π} tautomeric state. The total insensitivity of its chemical shift to metal or ligand binding suggests that its location is probably remote from the active site.

(4) His-7 and -8, from their sensitivity to ligand binding, appear to be located in or near the active site. Their chemical shifts indicate that they exist in the N^{π} tautomeric form. Their failure to titrate normally with pH suggests that they may participate in active-site metal ion coordination, although the present evidence for this is not conclusive. In the absence of inhibitor, His-7 undergoes a pH-dependent chemical shift change, the magnitude of which suggests that it is less likely due to ionization of the imidazole ring than to a pH-induced alteration in its microenvironment.

(5) His-9 is almost certainly an active-site metal ion ligand, coordinated via its N^{τ} nitrogen atom. This is suggested by its abnormal upfield chemical shift and its perturbation in apoenzyme and ligand-bound enzyme. From the current data it cannot be stated whether His-9 is coordinated to the same metal ion as His-1 or -2.

Acknowledgments

We are grateful to Dr. Elaine Earl for her kind gift of [^{13}C]histidine, to Dr. Anne Moore for use of her computer program, to Dr. Oleg Jardetzky and Dr. Tom James for their generous gifts of spectrometer time, to Dr. Willy Shih and Dr. Steve Patt for experimental help, and to Dr. Jack Kirsch and Dr. Ian Armitage for helpful discussions.

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

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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.

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0006-2960/80/0419-4021$01.00/0