Fluorine-19 Nuclear Magnetic Resonance Study of Fluorotyrosine Alkaline Phosphatase: The Influence of Zinc on Protein Structure and a Conformational Change Induced by Phosphate Binding†

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ABSTRACT: ^{19}F nuclear magnetic resonance (NMR) spectroscopy has been used to study a fully active *E. coli* fluorotyrosine alkaline phosphatase. The fluorotyrosine resonances provide sensitive probes of the conformational states of the protein. They were used to follow the addition of zinc or cobalt to the apoprotein, and the titration of the protein with inorganic phosphate or the inhibitor 2-hydroxy-5-nitrobenzylphosphonate. The results indicate that 2 molecules of inorganic phosphate per dimer of alkaline phosphatase are required to complete a general conformational change in the protein involving perturbations to the environment of several tyrosines. Spectra of the cobalt enzyme indicate that one specific tyrosine per subunit may be near the metal site. The ^{19}F NMR results, combined with the ^{31}P NMR results in the accompanying paper, lead directly to the conclusion that dissociation of noncovalently bound inorganic phosphate from the enzyme is the rate-limiting process in enzyme catalysis at high pH. The local environment of the individual fluorotyrosines is also discussed.

Alkaline phosphatase (EC 3.1.3.1) from *E. coli* is a dimeric zinc metallo-protein (mol wt = 86 000) which catalyzes the hydrolysis of a wide variety of phosphate esters. This protein is currently the subject of considerable controversy in the literature concerning metal content, active site stoichiometry, and subunit interaction or cooperativity. The preparation and properties of the fully active fluorotyrosine alkaline phosphatase prepared by in vivo incorporation of *m*-fluorotyrosine have been previously described (Sykes et al., 1974). From measurements of ^{19}F nuclear spin relaxation times, nuclear Overhauser enhancements, and comparison of the ^{19}F spectra at 94 and 235 MHz, considerable information concerning the molecular dynamics of individual tyrosines has been obtained as well as a better understanding of the role of chemical shift anisotropy as a ^{19}F nuclear spin relaxation mechanism in proteins (Hull and Sykes, 1974, 1975a,b).

We would now like to turn our attention to the important biochemical questions concerning alkaline phosphatase. Presumably, the 11 tyrosines of each subunit of alkaline phosphatase are more or less uniformly distributed throughout the protein structure. We were therefore hopeful that the fluorotyrosines would provide a sensitive probe of both the local and general conformational properties of the protein. We have, in fact, been able to resolve all 11 individual fluorotyrosines of the two apparently identical subunits. Distinct changes in protein structure were observed upon removal of zinc, and a conformational change upon binding of inorganic phosphate to phosphate-free enzyme was found to require 2 P_i/dimer for completion. This conformational change is also induced by the turnover of 2 mol of substrate per dimer but *not* by a large excess of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate. The question of metal stoichiometry remained unresolved. Apoenzyme prepared either by dialysis against nitrilotriacetic acid (NTA) or chelex treatment regained much of the native structure with 2 Zn/dimer, but the NTA-treated protein required 4 Zn/dimer to restore complete structure and activity. Significant perturbations of ^{19}F chemical shifts were observed when Co(II) was introduced due to the presence of paramagnetic centers. Finally, we will discuss an apparent correlation between ^{19}F chemical shift, dipolar relaxation, and solvent isotope shift (the difference between chemical shifts in H_2O and D_2O) with regards to the local environment of individual tyrosines. In the accompanying paper (Hull et al., 1976), we described ^{31}P NMR experiments involving the binding of inorganic phosphate to wild-type alkaline phosphatase. Combining the ^{31}P NMR data with the ^{19}F results leads directly to the conclusion that dissociation of *noncovalently* bound phosphate *coupled* with an enzyme conformational change is the rate-limiting process in enzyme catalysis at alkaline pH.

Experimental Procedure

Materials. Fluorotyrosine alkaline phosphatase was the generous gift of Dr. H. I. Weingarten prepared in the laboratory of Dr. M. J. Schlesinger, Washington University, St. Louis, Missouri. The protein was isolated from a tyrosine auxotroph of *E. coli* W3747 (ATCC 27256) as previously described (Sykes et al., 1974). The extinction coefficient is $\epsilon_{280}^{0.1\%} = 0.90$. The labeled protein was indistinguishable from wild type in both transient and steady-state kinetics at pH 5.5 and 8.0.

Tris(hydroxymethyl)aminomethane (Tris[1]) was the Ultra Pure grade from Mann Research Laboratories or

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[1] Abbreviations used: NMR, nuclear magnetic resonance; P_i, inorganic phosphate; NTA, nitrilotriacetic acid; Tris, tris(hydroxymethyl)aminomethane; FID, free induction decay; SIS, solvent isotope shift.

Trizma from Sigma. Nitrilotriacetic acid (NTA) and *p*-nitrophenyl phosphate were from Sigma. Spectro grade cobalt nitrate was obtained from Johnson Matthey Chemicals Ltd., London. $ZnCl_2$ solutions were prepared from Fisher analytical grade metal. The inhibitor 2-hydroxy-5-nitrobenzylphosphonate was the generous gift of S. E. Halford. All other chemicals were reagent grade, and distilled water was supplied by Belmont. Buffers were made metal free by extraction with 0.001% dithizone in CCl_4 followed by extraction of dissolved dithizone with pure CCl_4 and aeration with nitrogen to remove CCl_4.

Enzyme Assays. All assays were performed in 1 mM *p*-nitrophenyl phosphate, 1 M Tris, pH 8, 22–25 °C. Absorption at 400 nm was followed on a Cary 15 spectrophotometer. The protein from NMR samples was usually diluted to 5 μl/ml in 1 M Tris, pH 8, and 5 μl of this pre-dilution was used in 1-ml assays. For assays of apoenzyme and partially reconstituted enzyme, pre-dilution and assay media contained 10^{-5}–10^{-3} M NTA as described in the text. Specific activity was calculated from the initial absorbance slopes using $\epsilon_{400}{}^{mM} = 16.2$ for *p*-nitrophenol and the extinction coefficient of the protein given above. The units of activity will be taken as micromoles of substrate cleaved per hour per milligram of protein. As received, fluorotyrosine alkaline phosphatase had a specific activity of 3000 ± 200 units compared with the value of 3250 units for pure crystalline wild-type enzyme (Malamy and Horecker, 1964). Enzyme which was carried through several experiments showed a gradual decrease in activity over a period of 1 year to 2000–2200 units, but there were no noticeable changes in the properties of the protein that were being studied by ^{19}F NMR.

Preparation of Apoenzyme. For metal titration experiments apofluorotyrosine alkaline phosphatase was prepared by dialysis against chelator. The lyophilized protein was dissolved in 0.30 ml of dialysis buffer, 10 mM NTA, 10 mM Tris, 0.1 N NaCl, pH ~6. After dialysis against 1 l. of this buffer (2 days with one change after 24 h), the buffer was changed to 0.03 mM NTA, 10 mM Tris, 0.1 N NaCl, pH 7.8, and dialysis continued for two more days. For these experiments chelator (about 10% of protein concentration) was *not* removed by further dialysis in order to ensure a completely metal-free protein. The protein sample was withdrawn from the dialysis tubing by syringe and transferred directly to an NMR tube. The apoenzyme, when assayed in a medium containing 1 mM NTA, showed less than 1% activity. Addition of apoenzyme to an assay medium containing 10^{-4} M Zn(II) resulted in immediate and complete reactivation. Apoenzyme when assayed directly in "metal-free" media without NTA present showed a time-dependent partial reactivation due to a very low level of contaminating Zn (~10^{-8} M). Reconstituted holoenzyme when pre-diluted and assayed immediately in media containing 10^{-5} M NTA showed normal activity. However, if the pre-dilution mixture is allowed to stand several minutes, the specific activity decreases, probably due to loss of Zn.

In a few cases Chelex 100 resin (BioRad, 200–400 mesh) was used to remove zinc from alkaline phosphatase. The resin was prepared by washing with 1 N HCl, distilled water, 1 N NaOH, and distilled water. Before use the Chelex was equilibrated with the appropriate buffer. While other groups have had good results with preparing apoenzyme by incubation with Chelex (Csopak, 1969; Taylor et al., 1973), our results at higher protein concentrations were less satisfactory.

A sample of native wild-type alkaline phosphatase (courtesy of S. E. Halford) was subjected to a Chelex treatment to remove zinc. At a protein concentration of 16 mg/ml in 1 M Tris, pH 8, the enzyme was only slowly deactivated by an equal volume of Chelex slurry (total volume 0.5 ml). The native protein activity was 2800. After 24 h of Chelex treatment at room temperature, the activity had dropped to 115 when assayed in metal-free medium, while the activity in medium containing 10^{-4} M Zn was only 1200. Apparently, prolonged exposure to Chelex resulted in some denaturation. Titration of the apoenzyme with zinc gave activities of 625 and 1200 for 1 Zn/dimer and 2 Zn/dimer, respectively.

Another sample of wild-type alkaline phosphatase was subjected to the NTA dialysis procedure discussed above with NTA beginning at 0.05 M and decreasing to 10^{-5} M. Pre-dilutions and assays were done in the presence of 10^{-5} M NTA. The apoprotein gave an activity of 52 in the NTA-supplemented medium and 2000 in 10^{-4} M Zn-supplemented medium. Titration of apoenzyme with Zn gave a *linear* increase in activity until the maximum activity of 2000 was reached at 4 Zn/dimer. Thus, it appears that NTA can influence metal stoichiometries in a manner analogous to EDTA (Csopak and Szajn, 1973).

Preparation of Purged Enzyme. In order to remove tightly bound endogenous phosphate from alkaline phosphatase, it is necessary to remove the zinc atoms. Thus, for experiments involving titration of purged enzyme (phosphate-free, reconstituted with Zn) the protein sample was dialyzed (1/500) against decreasing concentrations of NTA (20 to 1 mM) over a period of 2–3 days in Tris buffer at pH ~6.5, then against two changes of 1 mM $ZnSO_4$ in Tris buffer, pH ~8, and finally against metal-free Tris at the desired pH for two to three changes. This procedure generally resulted in no change in specific activity.

Concentrations of Protein Samples. When dialysis procedures were carried out on samples with volumes greater than 0.25–0.30 ml, protein was concentrated either by lyophilization or by ultrafiltration using the Amicon PM-10 membrane to obtain ~1-ml volumes which could then be reduced to 0.2 ml with a Minicon A-25 device. Samples were periodically passed through a millipore filter to remove precipitates and bacteria.

NMR Techniques. All ^{19}F NMR spectra were recorded at 94 MHz on a Varian XL-100-15 spectrometer equipped with a Varian 620-i 16K computer and a LINC magnetic tape unit from Computer Operations, Inc. Spectra were acquired by a modified Varian Block Mode procedure using a spectral width of 2500 Hz, a 90° pulse, and a pulse repetition time of 0.3–0.4 s. Most protein samples were 0.1–0.4 mM in dimer in 0.20–0.30 ml in 5-mm NMR tubes. The probe temperature was 26–28 °C. The solvent (H_2O or D_2O) was used as a field-frequency lock, and all chemical shifts are relative to a capillary of trifluoroacetic acid.

In order to circumvent to some degree the problems encountered in Fourier transform NMR with a finite computer word length, the Varian Block Mode Averaging procedure has been utilized in the following way. Three levels of accumulation are defined. First, a group of transients is acquired with each sufficiently scaled down so that their sum does not overflow the computer word length. This group is called a batch and, as subsequent batches are acquired, the total free induction decay (FID) is checked for overflow after each batch. When overflow is imminent, the total FID and all subsequent batches are scaled down by an additional

factor of 2. After a specified number of batches has been acquired, the FID is transformed to a double precision spectrum or block. The process is begun anew and subsequent blocks are added to the first using double precision arithmetic. The procedure extends the dynamic range of the spectrum as well as controlling the amount of scaling applied to the incoming signal. In general 128 transients/batch and 32 or 64 batches/block were used to acquire spectra over a period of 6–20 h. Using this procedure we have obtained useful spectra of 80 μM protein in 20 h. A sensitivity enhancement exponential filter function is necessary to improve the signal/noise ratio of these spectra. In general, multiplication of the FID by a decreasing exponential with time constant T_{SE} will lead to noise reduction with an increase in line width given by $1/\pi T_{SE}$. For systems where the desired signal in the FID decays very rapidly, the line-width effect of such digital filtering can be minimized if the starting point for the multiplication of the transient is delayed or shifted from $t = 0$ to $t \sim 2T_2^*$, where T_2^* is the decay time of the desired signal. This "delayed" sensitivity enhancement procedure can be incorporated easily into the automatic sequence of Block Mode Averaging. For the work presented here the delay time is 64 memory locati ons which equals 12.5 ms and T_{SE} = 0.025–0.035 s, parame ers found by trial and error to give the best results.

In the Results section we discuss the int :rpretation of the ^{19}F dipolar relaxation for the fluorotyrosine residues in terms of the interaction of these residues with the protein. In previous reports (Hull and Sykes, 1974, 1975b), the ^{19}F relaxation data have been presented and analyzed to yield an overall tumbling correlation time $\tau_c = 1/6D$ of 76 ns. Furthermore, the tyrosine residues were found to exhibit slow rotation about the C_α–C_β bond ($D_{int}(\alpha-\beta) < 10^6$ s^{-1}) and C_β–C_γ bond ($D_{int}(\beta-\gamma) < 10^8$ s^{-1}). Since dipolar relaxation depends on the total internuclear interaction Σr^{-6} between fluorine and neighboring protons, the term Σr^{-6} can be calculated from the observed relaxation data once the motional properties have been characterized. In this way, we have used relaxation formulas for isotropic motion to estimate Σr^{-6} for fluorine on each individual tyrosine, assuming the tumbling time mentioned above. For *m*-fluorotyrosine, the Σr^{-6} could be determined directly from molecular models. The relaxation data pertain to D_2O solutions; hence, the solvent plays no role in the relaxation or calculated Σr^{-6}. For the purposes of our analysis, an exchangeable tyrosine hydroxyl proton (e.g., in the free amino acid) is considered to be part of the solvent and does not contribute to Σr^{-6}, while a nonexchanging hydroxyl proton contributes to Σr^{-6} just as protons on neighboring residues might.

Results

The Effect of Metal Ions on Protein Structure. The role of metal ions in establishing and stabilizing the tertiary and quaternary structure of metallo-enzymes has been long appreciated. In particular, native alkaline phosphatase exists as a dimer containing 2–4 Zn atoms. While the zinc atoms can be removed at neutral pH leaving an intact dimer, uv difference spectra have indicated 2–3 tyrosines per subunit become more exposed to solvent, and there is a change in the circular dichroism (Reynolds and Schlesinger, 1969). The extreme sensitivity of ^{19}F to small changes in environment therefore allows us to examine local and long-range structural effects of metal binding. The question of metal stoichiometry is also of prime interest but as yet remains uncertain because alkaline phosphatase exhibits different metal stoichiometries, depending on the isolation procedures and methods for preparing apoenzyme. If apo- and holoenzyme have different ^{19}F NMR spectra, then titration of apoenzyme with metal ion should provide information on the question of stoichiometry.

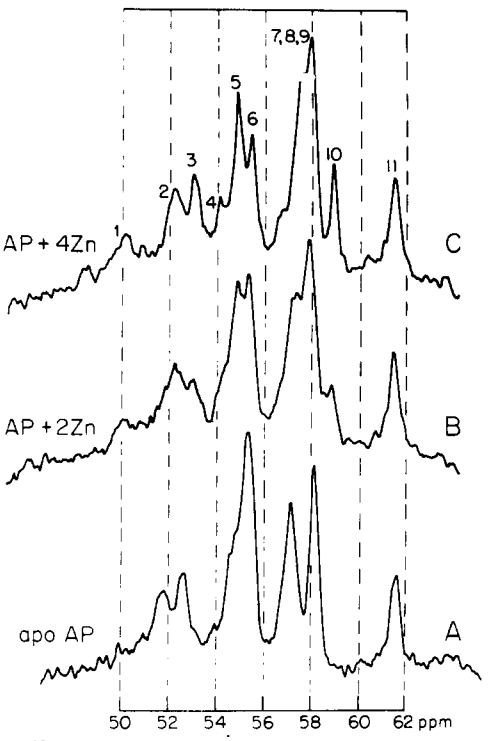

FIGURE 1: ^{19}F NMR spectra of fluorotyrosine alkaline phosphatase titrated with Zn(II). The protein was purged of metal and P_i by dialysis against NTA. Spectrum A is of 0.21 mM apoprotein in 10 mM Tris, 0.03 mM NTA, 0.1 N NaCl, H_2O, pH 7.8. Aliquots of 20 mM $ZnCl_2$ were added to give B and C. The maximum specific activity of the Zn(II) protein was 2800 units. Each spectrum required 82 000 transients (pulse interval 0.4 s), and the chemical shifts are relative to a capillary of trifluoroacetic acid.

We have prepared apofluorotyrosine alkaline phosphatase by dialysis against the chelator nitrilotriacetic acid (NTA). Although the binding of chelators to alkaline phosphatase has been documented (Csopak et al., 1972; Csopak and Falk, 1974), we did not attempt to remove the chelator in these experiments. In two separate experiments apoenzyme was titrated with increments of zinc or cobalt, and the activity of the enzyme was also monitored after each NMR spectrum. Figures 1 and 2 present ^{19}F NMR spectra for the zinc and cobalt titration, and Tables I and II present the corresponding activity data.

From Figure 1 is appears that the conformation of the apoprotein is considerably different than that of the reconstituted enzyme. The complete resolution of 11 tyrosines is not possible in the apo spectrum implying less differentiation of tyrosine environments or, in other words, a more open or looser structure. The addition of two zinc atoms restores most of the native structure but, under our conditions, four zincs were required to produce a clean spectrum of holoenzyme. No further spectral changes were observed up to 8 Zn/dimer. The activity data indicate that the first two zinc atoms which restore much of the structural integrity of alkaline phosphatase do *not* restore activity to a similar extent; alternatively, these zinc atoms are not stabilized in the protein structure and are rapidly lost under the conditions of the assay (see Experimental Procedure). The latter

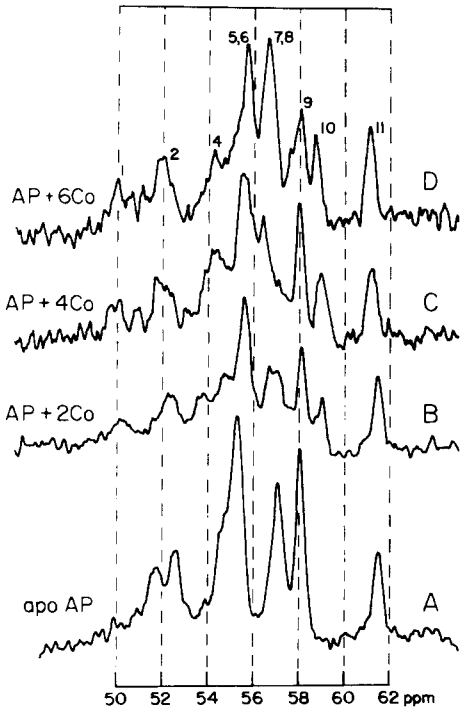

FIGURE 2: ^{19}F NMR spectra of fluorotyrosine alkaline phosphatase titrated with Co(II). The protein was purged of metal and P_i by dialysis against NTA. Spectrum A is of 0.16 mM apoprotein in 10 mM Tris, 0.03 mM NTA, 0.1 N NaCl, H_2O, pH 7.8. Aliquots of 20 mM $Co(NO_3)_2$ were added to B, C, and D, and additional cobalt gave no further spectral change. The maximum specific activity of the Co(II) enzyme was 200 units. The spectra required 70 000–100 000 transients (pulse interval 0.4 s), and chemical shifts are relative to a capillary of trifluoroacetic acid.

Table I: Specific Activity of Apoenzyme Titrated with Zinc.[a]

Zn/Dimer[b]	Pre-dilution[c] NTA (M)	Specific Activity: NTA Present[d]	Specific Activity: 10^{-4} M Zinc[e]
0	None	3-4	
	10^{-3}		2700
2	10^{-3}	100	2900
4	10^{-3}	42	2700
	10^{-5}	1400	
6	10^{-5}	3100	
8	10^{-5}	2800	

[a] Apo-fluorotyrosine alkaline phosphatase was prepared by NTA dialysis as described in Experimental Procedure. The initial sample was 0.21 mM dimer, 0.03 mM NTA, 10 mM Tris, 0.1 N NaCl, pH 7.8. Specific activity is in μmol/h per mg. The samples assayed are those whose spectra appear in Figure 1. [b] The sample was titrated with 20 mM $ZnCl_2$, and with the first addition of Zn sufficient extra Zn was added to titrate the expected free NTA present in the buffer (0.03 mM). [c] The protein was diluted to 5 μl into 1 ml of 1 M Tris pH 8 containing the indicated concentration of NTA. The entry "none" means no predilution. [d] The amount of NTA added to the assay medium is 10^{-3} M for 0, 2, and 4 Zn/dimer, 10^{-4} M for 6 Zn/dimer, 10^{-5} M for 8 Zn/dimer. [e] The assay medium is supplemented with 10^{-4} M $ZnSO_4$.

Table II: Specific Activity of Apoenzyme Titrated with Cobalt.[a]

Co(II)/Dimer	Specific Activity: 10^{-3} M NTA	Specific Activity: 10^{-4} M Zn
0	28	3200
2	35	1000
4	80	220
6	150–200	150–200
8	150	160

[a] The experiment is the same as in Table I. The titration is with 20 mM $Co(NO_3)_2$ and no pre-dilutions were made before assays, except for apoenzyme assayed in 10^{-4} M zinc. These samples gave the spectra as in Figure 2.

proposal is supported by the observation that the Zn_4 enzyme shows a considerably higher activity when pre-diluted in 10^{-5} M NTA rather than 10^{-3} M NTA. When excess Zn has been added (Zn_6AP), the specific activity is stabilized at the value for the native enzyme prior to the experiment, although a slow deactivation was observed when the enzyme, pre-diluted into NTA buffer, was allowed to stand for several minutes before the assay.

Figure 2 and Table II illustrate a similar experiment for the Co(II) enzyme. Again a major spectral change was observed when two cobalt atoms were added to apoprotein. Additional changes were observed after 4 and 6 cobalts had been added, but no further changes were observed with 8 cobalt atoms. The activity data are similar to the behavior observed with the zinc enzyme. The first two metal atoms do *not* give stable activity but significantly reduce the reactivation by zinc. At four cobalts the activity is about one-half the maximum possible for the cobalt enzyme and reactivation by zinc appears to be completely inhibited. Finally, with 6 or 8 cobalts the activity of the Co(II) enzyme is stabilized at 150–200 units.

The chemical shifts of fluorotyrosines in the Co(II) enzyme reflect not only the structural characteristics of the protein but also the influence of the unpaired electron spin on the cobalt ions (Zn(II) is diamagnetic). A direct comparison of the fully reconstituted zinc and cobalt enzymes can be made by comparing Figures 1C and 2D. The numbering scheme (*not* indicating the position in the amino acid sequence) for the zinc protein has been used in previous publications, and this scheme was transferred to the cobalt protein spectrum assuming, for simplicity, the closest possible correlation between individual tyrosines. Thus, it appears that tyrosines No. 1, 2, 4, 6, 9, 10, and 11 are relatively unperturbed and therefore distant from the metal ions while tyrosines No. 3, 5, 7, and 8 may be closer to the metal, which in the paramagnetic cobalt case can result in line broadening and/or perturbations in chemical shift. Interpretation of this effect is complicated by the presence of multiple metal sites. We have chosen to compare the Co_6 spectrum with that of Zn_4 primarily because these spectra are unchanged by excess metal and represent enzyme with stable activity. In view of the small difference between the Co_4 and Co_6 spectra (Figures 2C and 2D), it may be that the last two cobalts bind to surface sites which do not affect structure but only cause paramagnetic shifts of nearby residues. Until experiments can be done in the complete absence of chelator, it is simpler to compare enzymes that are "saturated" with metal, but this choice is not crucial to subsequent discussions.

Detailed structural studies of chelator-free enzyme have not yet been carried out. A sample of the fluoro-labeled enzyme was treated with Chelex but 10% activity remained after 24 h. This apoprotein could be fully reactivated by 2–3 Zn/dimer, and the reconstituted enzyme produced a ^{19}F spectrum identical with that in Figure 1C.

Active Site Stoichiometry and the Binding of Inorganic

Table III: Changes in Fluorotyrosine ^{19}F Chemical Shifts upon P_i Binding.[a]

Tyrosine	pH 7 Titrate with: P_i	pH 8 p-NPP[b]	pH 8 P_i
1	0.28	−0.1	+0.08
2	0.20	+0.03	0.36
3	0.63	+0.60	0.69
4	0.15	0.27	0.15
5	0.20	−0.14	0.0
6	−0.12	−0.10	−0.05
7	−0.48	−0.39	−0.29
8	≤±0.1	≤±0.1	≤±0.1
9	≤±0.1	≤±0.1	≤±0.1
10	0.0	0.0	0.05
11	−0.08	−0.15	0.01

[a] Absolute chemical shifts were measured relative to a capillary of trifluoroacetic acid with an accuracy of ±0.05 ppm for well-resolved resonances. The chemical-shift differences are the ^{19}F shift for protein saturated with P_i minus the shift for phosphate-free protein. A positive shift is upfield. [b] *p*-Nitrophenyl phosphate as a reactive substrate.

Phosphate. Alkaline phosphatase demonstrates the ability to bind inorganic phosphate (a potent inhibitor and a product of catalysis) both as a noncovalent complex (predominant at pH 8) and a covalent complex (predominant at pH 4). After the initial discovery of the covalent phosphoryl enzyme labeled at a serine residue (Schwartz and Lipmann, 1961; Engström, 1962), there have been several attempts to quantitate the binding of P_i, and thus the number of active sites, by isolation of the phosphoryl enzyme (Lazdunski et al., 1969b; Petitclerc et al., 1970; Reid et al., 1969). These experiments have shown the covalent binding of at least 1 P_i/dimer at pH 5 and as much as 2 P_i/dimer at pH 4. Equilibrium dialysis techniques have been used to study P_i binding at alkaline pH and the results have generally indicated 1 tight P_i binding site ($K_D \sim 10^{-6}$ to 10^{-5} M) and a second weaker site ($K_D \sim 10^{-4}$ to 10^{-3} M; Simpson and Vallee, 1970; Applebury et al., 1970). Titrations of the visible spectrum of Co(II) alkaline phosphatase have indicated both 1 P_i/dimer (Applebury and Coleman, 1969b) and 2 P_i/dimer (Simpson and Vallee, 1968).

We have investigated the binding of P_i to alkaline phosphatase by observing the effect of inorganic phosphate on the ^{19}F NMR spectrum of purged (phosphate-free) fluorotyrosine alkaline phosphatase. We were initially led to this study by the observation that experiments such as that illustrated by Figure 1, the reconstitution of the apoprotein with zinc, did not yield a spectrum identical with the original native enzyme (as received). Furthermore, the addition of a severalfold excess of P_i to the native protein (as isolated) had failed to alter its ^{19}F spectrum. On the other hand, it was found that the addition of excess P_i to purged protein reconstituted with zinc converted its spectrum (Figure 1C) to a spectrum identical with that of the original native enzyme. Consequently, titration experiments were performed in which aliquots of P_i or the substrate *p*-nitrophenyl phosphate were added to purged enzyme (see Experimental Procedure). Both P_i and substrate gave identical results since cleavage of substrate produces a stoichiometric amount of P_i. The ^{19}F spectra observed following hydrolysis of substrate at pH 8 are shown in Figure 3. Quite distinct and reproducible spectral changes associated with the interaction of P_i with enzyme were observed; in particular, tyrosines

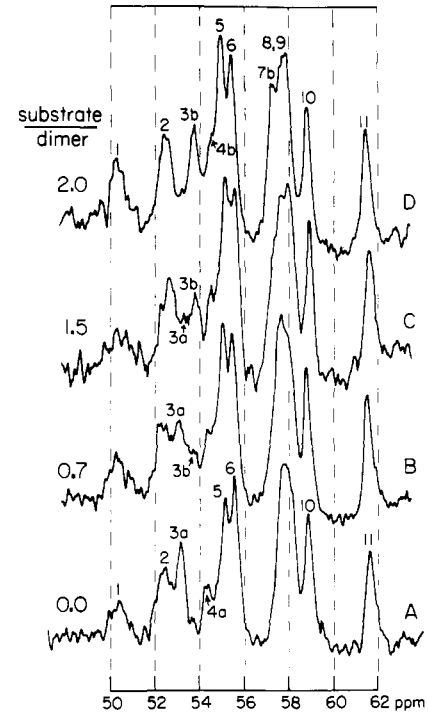

FIGURE 3: ^{19}F NMR spectra of phosphate-free Zn(II) fluorotyrosine alkaline phosphatase titrated with *p*-nitrophenyl phosphate. The protein was purged of metal and P_i by dialysis against NTA and reconstituted by dialysis against $ZnSO_4$. Spectrum A is of 0.26 mM Zn(II) dimer (specific activity = 2000 units) in 0.1 M Tris-Cl, H_2O, pH 8. Spectra B, C, and D were obtained by adding aliquots of 10 mM *p*-nitrophenyl phosphate (in Tris buffer) to sample A. The spectra required 50 000–100 000 transients (pulse interval 0.3 s), and the chemical shifts are relative to a capillary of trifluoroacetic acid. Tyrosine resonances labelled a and b (No. 3, 4, and 7) are those whose chemical shifts are distinctly different in A and D.

No. 3, 4, and 7 exhibited changes in chemical shift which were larger than the experimental uncertainty. Table III summarizes chemical-shift data for three different experiments. Tyrosine No. 3 provides us with the most clearly observable measure of the phosphate content of alkaline phosphatase. In Figures 3A and 3D only single resonances are observed for this tyrosine with chemical shifts differing by about 0.65 ppm. In Figures 3B and 3C partially resolved resonances were observed at both positions in both spectra, and the intensities of these resonances were consistent with the fractional saturation of phosphate sites based on a 2 P_i/dimer stoichiometry. The addition of excess P_i or substrate had no further effect on the protein spectrum. Simple dialysis of the phosphate saturated protein for a few days did not alter its spectrum. The purging procedure *always* produced protein in a state represented by Figure 3A while exposure to P_i or substrate by titration or dialysis always gave protein represented by Figure 3D, and the stoichiometry has always been 2 P_i/dimer. Since the observed spectral changes involve several tyrosines, it would appear that there are two distinct conformations of the protein which are interconverted at pH 8 by the binding of P_i or the turnover of substrate (which presumably results in P_i bound to the enzyme) and there is no difference in their specific activity.

From these experiments we have found no evidence that the two phosphate binding sites are distinguishable (i.e., nonidentical). Judging by the simple titration behavior shown in Figure 3 and the absence of further change for P_i/dimer > 2, it appears that less than 10% of the E·P_i complex is dissociated at the concentration levels of Figure 3D.

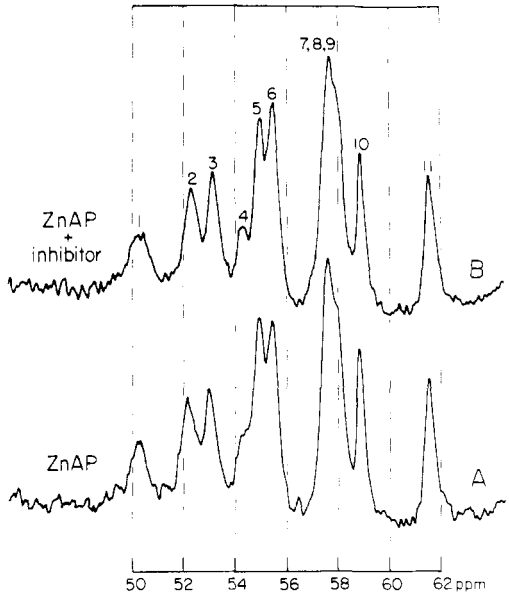

FIGURE 4: ^{19}F NMR comparison of phosphate-free fluorotyrosine alkaline phosphatase and protein saturated with 2-hydroxy-5-nitrobenzylphosphonate. The protein was purged of metal and P_i by dialysis against NTA, and reconstituted by dialysis against $ZnSO_4$. Spectrum A is of 0.24 mM P_i-free Zn(II) dimer (specific activity 2000 units) in 0.1 M Tris-Cl, H_2O, pH 8.0. In B, 5 mol of inhibitor/mole of dimer have been added. Each spectrum represents ~180 000 transients (pulse interval 0.3 s), and chemical shifts are relative to a capillary of trifluoroacetic acid.

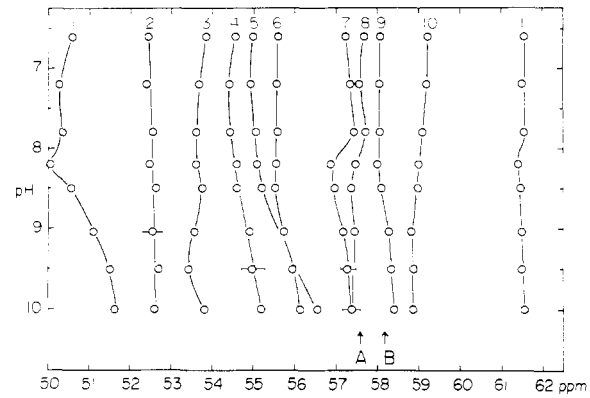

FIGURE 5: ^{19}F NMR chemical shifts of fluorotyrosine alkaline phosphatase as a function of pH. The chemical shifts relative to trifluoroacetic acid of the individual fluorotyrosine residues are plotted vs. pH for a sample containing 0.2–0.3 mM Zn protein (P_i bound). The buffer initially contained 10 mM Tris, 0.1 N NaCl in D_2O. For pH's above 8, methylamine was added (in 0.1 N NaCl, D_2O) to a final concentration of ~10 mM. The pH values are direct meter readings, and positions A and B mark the chemical shift of denatured protein in 6 M guanidine at pH 7.2 and 10.4, respectively.

Thus, an upper limit for the K_D for either phosphate site becomes $K_D \lesssim 0.01\ E_0$, where E_0 is the concentration of binding sites which in Figure 3 is 0.52 mM. Consequently, K_D is $\lesssim 5 \times 10^{-6}$ M, and the second phosphate must bind nearly as tightly as the first has generally been assumed to bind.

The above experiments have dealt with the noncovalent or Michaelis E·P_i complex which is stable in the pH range 7–8. Since the formation of a covalent E–P complex at pH $\lesssim 5$ has been well documented, experiments were performed to determine the conformational state of alkaline phosphatase in the presence and absence of phosphate at low pH. At pH 4.3 a twofold excess of Zn was added to prevent dissociation of the enzyme and the protein was stable at this pH for 2 days (activity 2400 units before the experiment, 2200 units after). It was found that the purged enzyme gave nearly identical ^{19}F spectra at pH 4.3 and 8.0, and that the addition of P_i at low pH caused very little perturbation. In the pH range 7–8, the ^{19}F chemical shift of tyrosine No. 3 is 53.05 ± 0.05 ppm in the purged enzyme and 53.70 ± 0.05 ppm in the E·$(P_i)_2$ complex. At pH 4.3 the chemical-shift values for this tyrosine are 53.2 and 53.4 ppm for purged E and E–P, respectively. Thus, while E and E·P_i appear to have different conformations, E and E–P differ to a lesser extent.

The Binding of Inhibitor. The binding of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase has been characterized by visible difference spectra, stopped-flow kinetics, and temperature-jump relaxation studies (Halford et al., 1969; Halford, 1972), and a dissociation constant for the binding of 1 inhibitor/dimer was found to be $K_D = 3 \times 10^{-5}$ M at pH 8. A slow transient phase in the temperature jump was identified with a slow unimolecular E·I ↔ E*·I conformational change coupled to the rapid bimolecular binding step. We have attempted to observe such a conformational change (proposed to be the rate-limiting step in catalysis at pH 8) using the ^{19}F NMR spectrum of fluorotyrosine alkaline phosphatase. However, as can be seen in Figure 4, there is no observable spectral change in the presence of excess (up to 100-fold) phosphonate.

In the presence of a small excess of phosphonate (Figure 4B), alkaline phosphatase could be titrated with either P_i or *p*-nitrophenyl phosphate giving spectral changes identical with those observed in Figure 3 with the same two-site stoichiometry. Evidence for a ternary complex I·E·P_i has been presented (Halford, 1972), but our data provide no further information on this point since no spectral change associated with E·I could be observed.

The binding of orthoarsenate to alkaline phosphatase has also been demonstrated (Petitclerc et al., 1970; Taylor et al., 1973; Holmquist et al., 1975). Arsenate acts as an inhibitor with a K_i similar to orthophosphate (Gottesman et al., 1969). The addition of 5 AsO_4/dimer to a sample of 0.08 mM purged fluorotyrosine alkaline phosphatase (specific activity = 2200) in 0.1 M Tris-Cl, pH 8, did not result in any resolvable spectral change. Subsequent addition of 10 mM P_i brought about the expected conformational change. It has been observed that the circular dichroism changes induced by arsenate binding to Co(II) alkaline phosphatase were opposite to those caused by P_i binding. Thus, there may be some difference between the interaction of arsenate and phosphate with the enzyme.

The Chemical Shifts and Environments of Individual Tyrosines. One of the most striking features of the ^{19}F spectra presented here is the range of chemical shifts observed (11.5 ppm). Furthermore, under conditions of higher ionic strength than those used for these studies, all 11 tyrosines can be resolved as distinct resonances (Hull and Sykes, 1974). Thus, the uniqueness of each tyrosine in the folded subunits of alkaline phosphatase is clearly expressed by the chemical shifts. There are several factors which may cause otherwise identical ^{19}F nuclei to exhibit different chemical shifts. Since tyrosine residues have an ionizable hydroxy group, the state of ionization or hydrogen bonding to this group will influence the ^{19}F chemical shift. Other effects such as neighboring charged groups, ring currents, and the van der Waals or hydrophobic interactions will influence the chemical shift.

Observation of the ^{19}F NMR signal of *m*-fluorotyrosine during a pH titration indicated a pK_A of 8.2 for the hydroxyl proton. Previous work with the native wild-type enzyme using uv spectroscopy has indicated that only 3 tyrosines/subunit are exposed to solvent and susceptible to titration (Reynolds and Schlesinger, 1967). We have examined the ^{19}F spectrum of fluorotyrosine alkaline phosphatase as a function of pH in the range 6.5–10 as shown in Figure 5. Up to pH 8 there is little perturbation of tyrosine chemical shifts, but above pH 8 definite changes occur. From the behavior of the chemical shifts it is clear that we are observing not only tyrosine ionizations but deprotonation of other basic groups and possible conformational changes as well. The arrows A and B in Figure 5 mark the chemical shift observed for completely denatured protein at the low and high pH extremes. Thus, it appears that the ionization state of a particular tyrosine can only account for a small fraction of its chemical shift relative to the denatured protein shift. The positions of tyrosines No. 7, 8, and 9 suggest that they are the three "exposed" tyrosines detected by uv spectra (Reynolds and Schlesinger, 1967), and the chemical shifts of these particular residues are susceptible to ionic strength effects.

Of all the chemical-shift mechanisms that might be influencing shifts in a folded protein structure, it is probably the van der Waals interaction that is most important for "buried" tyrosines. This mechanism involves distortion of an atom's electron cloud by close interaction with neighboring atoms (London dispersion forces) and theoretically exhibits a r^{-6} dependence, where r is the internuclear vector between interacting atoms (Emsley and Phillips, 1971; Bothner-By, 1960; Buckingham et al., 1960). ^{19}F has been found to be approximately 10–20 times more sensitive to this interaction than ^{1}H, due to the presence of 2p electrons and their inherent paramagnetic shift contribution σ_p (Howard et al., 1962; Rummens and Bernstein, 1965). Since dipolar relaxation of ^{19}F by protons also has a r^{-6} dependence, it is possible that a correlation between relaxation times and chemical shifts might exist. We have previously reported on the dipolar relaxation of ^{19}F in fluorotyrosine alkaline phosphatase (Hull and Sykes, 1974, 1975a,b). It was found that the tyrosine residues do not exhibit significant internal mobility and the differences in their ^{19}F dipolar relaxation properties are thus described by differences in their interaction with other protein residues, i.e., "buriedness".

The theoretical expressions for dipolar relaxation make it possible to calculate the effective Σr^{-6} for each fluorotyrosine residue in the labeled protein, given measurements of relaxation times and knowledge of the motional properties of these residues (see Experimental Procedure). These Σr^{-6} values are plotted vs. chemical shift for each tyrosine in Figure 6. Considering that many factors influence chemical shifts, we feel that this rough correlation with estimated Σr^{-6} terms is significant. The van der Waals mechanism generally gives downfield shifts, and it appears that tyrosines with shifts downfield of the denatured protein (58.0 ppm) exhibit increased relaxation due to closer contact with neighboring protons. Tyrosine No. 10 has the least efficient relaxation and exhibits a T_1 and line width very close to that calculated for an isolated tyrosine tumbling at a rate appropriate for alkaline phosphatase (Hull and Sykes, 1975b), but this residue has a shift significantly different from that expected for an isolated tyrosine. This suggests the influence of a charged group, which cannot contribute

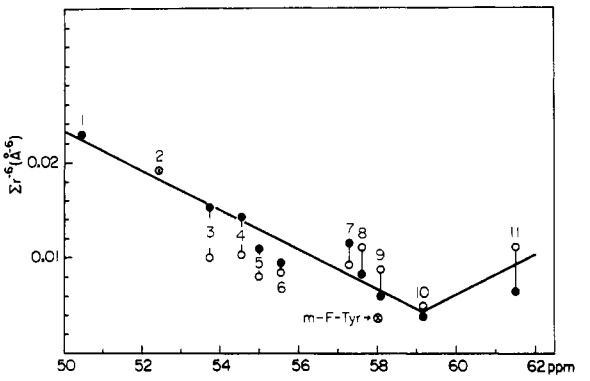

FIGURE 6: Correlation of the ^{19}F chemical shift in fluorotyrosine alkaline phosphatase with the internuclear interaction Σr^{-6} between fluorine and protons. The Σr^{-6} term is calculated from the dipolar linewidth (○) and T_1 (●) for ^{19}F at 94 MHz (see Experimental Procedure), and these values are plotted vs. the chemical shift in D_2O buffers (Table IV). The data for *m*-fluorotyrosine is given by (⊗). The solid line does not represent any particular theoretical best fit.

to Σr^{-6}, or ring current effects. A neighboring carboxyl group is one possibility. Tyrosine No. 11 is unique in its large upfield shift, insensitivity to pH, P_i binding, or even loss of zinc. This residue must be in a very stable and protected region of the protein.

The terms "buried" and "exposed" are used quite often when discussing the environment of aromatic residues. Perturbations to the uv absorption or titration behavior of the hydroxyl group are generally taken to indicate "buriedness". Another means of determining exposure to solvent has been suggested by P. C. Lauterbur (private communication). Since fluorine can participate in hydrogen bonding with exchangeable protons, exposed fluorines will have a H-bonding contribution to their chemical shifts which will be susceptible to a H–D isotope effect when the solvent is changed from H_2O to D_2O. The result is that fluorine shifts are to *lower* field in H_2O compared with D_2O. This difference in shift for the two solvents is defined as the solvent isotope shift (SIS), and for fluorocarbons SIS values are generally in the range 0.15–0.22 ppm (B. Kaufman, M.S. Thesis, State University of New York at Stony Brook). Since the dipolar Σr^{-6} term from the ^{19}F relaxation of fluorotyrosines in alkaline phosphatase reflects interaction with the protein and exclusion of solvent, it seems likely that some correlation should exist between this "buriedness" parameter and the "exposure" parameter of SIS.

Chemical-shift measurements of fluorotyrosine alkaline phosphatase and *m*-fluorotyrosine in H_2O and D_2O buffers are summarized in Table IV, along with observed SIS values and estimated Σr^{-6} terms for each tyrosine residue. The correlation between SIS values and Σr^{-6} is shown in Figure 7. The precision of the shift measurements is generally limited to ±0.05 ppm. Thus, tyrosines No. 1–4, 8, and 11 show a very small or zero SIS value, indicating that the fluorine atoms of these residues experience no significant contact with the solvent. On the other hand, tyrosines No. 5–7, 9, and 10 give SIS values significantly different from zero, indicating varying degrees of exposure to solvent. Tyrosine No. 10, for which the data are most precise, has a SIS value essentially the same as free *m*-fluorotyrosine as well as a similar Σr^{-6}. Thus, this residue appears to be completely "exposed" based on the criteria of SIS and Σr^{-6}, while at the same time exhibiting a perturbed chemical shift and anomalous pH titration behavior. Admittedly,

Table IV: ^{19}F Chemical-Shift Data and Solvent Isotope Shift (SIS) for Fluorotyrosine Alkaline Phosphatase.[a]

Tyrosine	$\delta^{TFA}(D_2O)$[b]	$\delta^{TFA}(H_2O)$[c]	SIS[d] δ_{av}	SIS[d] Two samples	*Dipolar* Σr^{-6} $(10^{-3}$ Å$^{-6})$[e]
1	50.46 ± 0.04	50.38 ± 0.14	0.08 ± 0.07	0.02	22.9
2	52.45 ± 0.03	52.39 ± 0.08	0.06 ± 0.04	0.04	19.1
3	53.75 ± 0.06	53.72 ± 0.11	0.03 ± 0.06	−0.01	12.7
4	54.56 ± 0.05	54.44 ± 0.13	0.12 ± 0.07	0.03	12.3
5	55.01 ± 0.05	54.90 ± 0.06	0.11 ± 0.04	0.11	9.5
6	55.57 ± 0.06	55.39 ± 0.08	0.18 ± 0.05	0.18	9.0
7	57.32 ± 0.05	57.23 ± 0.09	0.09 ± 0.05	0.14	10.4
8	57.64 ± 0.08	57.60 ± 0.12	0.04 ± 0.07	0.04	9.7
9	58.10 ± 0.03	57.97 ± 0.07	0.13 ± 0.04	0.25	7.4
10	59.16 ± 0.02	58.85 ± 0.02	0.31 ± 0.01	0.35	4.4
11	61.53 ± 0.05	61.50 ± 0.05	0.03 ± 0.04	0.07	8.8
m-F-Tyr[f]	58.019	57.735		0.284	3.56
Denatured AP[g]		57.94			

[a] The protein contains its full complement of zinc and is saturated with inorganic phosphate (2 P_i/dimer). All shifts are relative to a capillary of trifluoroacetic acid (TFA). [b] Average shifts and standard deviations for four different protein samples (0.1–0.3 mM dimer) in 10 mM Tris, 0.1 N NaCl, D_2O, pH 7.8. [c] Average shifts and standard deviations for four different protein samples (0.1–0.3 mM dimer) in 0.1 M Tris-Cl, H_2O, pH 7.8–8.0. [d] The solvent isotope shift is defined here as SIS = $\delta^{TFA}(D_2O) - \delta^{TFA}(H_2O)$. This gives the convention that a positive SIS value represents an *upfield* shift in D_2O relative to H_2O. The SIS values have been calculated using the average shifts that appear in this table and are also calculated using data from just two samples containing the same protein sample dissolved in the two buffers listed in footnotes *b* and *c*. [e] The Σr^{-6} term represents the total internuclear interaction between ^{19}F and protons on tyrosine or other neighboring residues and can be calculated from the dipolar relaxation of ^{19}F in D_2O solvent (Hull and Sykes, 1974, 1975b; see also Experimental Procedure). The tabulated values are the averages of the two values computed using the T_1 or line width of ^{19}F. [f] A 5-mM solution of *m*-fluorotyrosine was prepared in 10 mM Tris, 0.1 N NaCl, H_2O, pH 6.3, to give one chemical shift. This sample was lyophilized and redissolved in D_2O, and the pH meter reading was adjusted to 6.3 to give the other chemical shift. At this low pH range, the ^{19}F chemical shift is not sensitive to pH since the fully protonated form of the amino acid is present. The Σr^{-6} term was estimated from molecular models. [g] The shift is for denatured protein in the buffer of footnote *c*.

the correlations presented in Figures 6 and 7 are rather rough, but in view of the many factors involved in determining the parameters being studied, we consider it highly significant that qualitative correlations do exist.

Discussion

Metal Binding and Stoichiometry. The data presented in the previous section indicate that there are major structural differences between apo- and holo-alkaline phosphatase with at least 7 of 11 tyrosines per subunit experiencing changes in environment upon removal of zinc. Fluorine-labeled protein denatured in guanidine shows only a single ^{19}F resonance while the folded active enzyme gives 11 resolvable resonances. The fact that in the apoprotein all 11 tyrosines are no longer distinguishable and that the general movement of the resonances appears to be toward the denatured position implies that the conformational state of apoenzyme is less rigid or more open than the holoenzyme. A recent report on tritium exchange in alkaline phosphatase supports this interpretation (Brown et al., 1974). Tritiated approtein at pH 8 was found to lose all but 30–40 tritiums over a period of 6 h, while a Zn_4 protein retained 120 tritiums.

Examining the literature, one finds that efforts to determine the stoichiometry of metal binding and the nature of the metal sites has led to two classes of results. When native enzyme is treated with Chelex resin followed by *exhaustive* dialysis to remove any chelators, then under these conditions complete activity and structural integrity of the protein are restored with just 2 Zn/dimer (Csopak and Szajn, 1973). The same stoichiometry was observed for the Co(II) enzyme when prepared by Chelex treatment or chelator dialysis provided that exhaustive dialysis against chelator-free buffer followed (Applebury and Coleman, 1969b; Taylor et al., 1973). Several other experiments indicate 2 metals/dimer; the observation of the ^{31}P NMR line width of P_i during Zn, Co, and Mn titration of apoenzyme (Csopak and Drakenberg, 1973), the observation of ^{35}Cl NMR line widths during Zn, Co, Cu, Hg, and Cd titrations (Norne et al., 1974), the EPR spectrum of the Cu(II) enzyme (Taylor and Coleman, 1972; Csopak and Falk, 1974), and preparation in vivo of ^{65}Zn enzyme (Applebury and Coleman, 1969a).

Several other studies in which chelators were not scrupulously removed indicated four metal ions bind per dimer, probably as two distinct classes (Lazdunski et al., 1969a; Petitclerc et al., 1970; Chappelet et al., 1970; Lazdunski et al., 1970a; Lazdunski et al., 1970b; Simpson et al., 1968; Simpson and Vallee, 1968; Brown et al., 1974; Holmquist et al., 1975). Arguments have been made for two "structural" metals and two "activity" metals, but the results are not entirely consistent.

Our results with ^{19}F NMR and enzyme activity are similar to the behavior observed by other groups where the presence of chelators seems likely. The fact that complete enzyme activity, stable to the presence of NTA in the assay medium, was not obtained until at least 4 Zn/dimer were added suggests that even though the enzyme may be structurally intact with less than 4 metal ions per dimer, its activity is affected by NTA that is in some way interacting with the enzyme and/or the active site metals. The addition of excess zinc removes this effect and stabilizes activity.

Vallee has argued that the Zn_2 and Zn_4 dimers are distinct species (likewise for cobalt), but the data regarding activity of the Zn_2 and Co_2 species are not consistent (Simpson and Vallee, 1968; Brown et al., 1974; Anderson and Vallee, 1975). The study of partially reconstituted proteins is complicated by the possible differences between kinetically formed initial species and final equilibrium species. This problem is compounded by the probable pres-

ence of chelators in many enzyme preparations unless extreme precautions are taken. Differences observed in protein activity when titrated with zinc or cobalt may reflect different relative affinities of enzyme and chelator for these metals.

By observation of the visible absorption and circular dichroism of the cobalt enzyme, it was found that 2 Co/dimer (giving maximal activity) bound to chelexed enzyme (Taylor et al., 1973) in sites that are probably of distorted tetrahedral geometry. On the other hand, protein treated with chelators gave results indicating that the first two cobalts bound in octahedral sites (without activating the enzyme) and a second pair of cobalts bound to tetrahedral sites yielding active protein (Simpson and Vallee, 1968).

Our results also indicate multiple cobalt sites in the presence of chelator (Figure 2). With the addition of 2, 4, and 6 cobalts, distinct ^{19}F spectral changes were observed. As with the zinc enzyme stable activity was not restored until between 4 and 6 cobalts were added. The behavior of the ^{19}F chemical shifts is of interest because they reflect both the influence of the metal on the protein structure and the direct influence of the paramagnetism of Co(II). The Co(II) bound to the protein will produce paramagnetic contributions to both the line width and chemical shift of nearby nuclei. These effects depend on the internuclear vector and angle factors which relate the orientation of this vector with respect to the electronic g tensor of the cobalt (La Mar et al., 1973). Under favorable circumstances, distance information can be obtained by a study of paramagnetic effects, but in this case the presence of multiple sites makes quantitative analysis quite difficult.

A direct comparison of fully reconstituted zinc and cobalt enzymes suggests that several tyrosines are not significantly perturbed by the presence of cobalt. If one assumes that there are no significant structural differences between cobalt and zinc enzymes, then the differences between spectra in Figures 1C and 2D are attributable to direct paramagnetic effects. Furthermore, if one assumes the closest possible correspondence between resonances in the two enzymes, then tyrosines No. 1, 2, 4, 6, 9, 10, and 11 appear to experience little or no paramagnetic effects while No. 5, 7, and 8 have shifted significantly and No. 3 has "disappeared". We might speculate that tyrosine No. 3, which is most perturbed by P_i binding (Figure 3) may, in fact, be quite close to the active site.

The binding of a metal ion to a specific protein site will have both a direct effect on the environment of nearby residues and an indirect effect on distant residues, which is transmitted through conformational changes in the tertiary and quaternary structure. By comparing spectra in Figures 1A, 1C, 2D, 3A, and 3D, we propose the following description of the tyrosine environments as monitored by their fluorine substituent.

(1) Tyrosines No. 6, 9, and 11 are in very stable environments which are remote from metal binding sites and unaffected by the conformational changes incurred by metal removal.

(2) Tyrosines No. 1, 2, 4, and 10 are also remote from the metal site but their environments are perturbed by metal removal.

(3) Tyrosines No. 3, 5, 7, and 8 are in environments that are relatively near the metal site and this environment is altered by conformational changes when metal is removed.

(4) Tyrosines No. 3-7 are sensitive to the binding of P_i.

The theory of paramagnetic effects predicts that the line

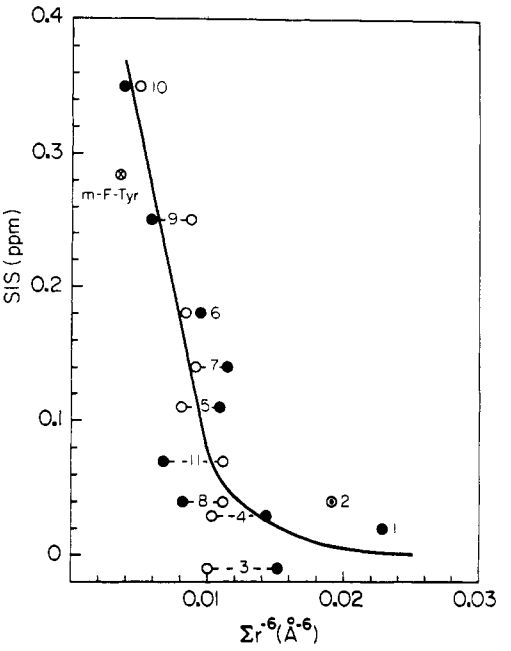

FIGURE 7: The correlation between solvent isotope shift (SIS) and dipolar Σr^{-6}. The solvent isotope effect on the ^{19}F chemical shifts of fluorotyrosines 1-11 of alkaline phosphatase is plotted vs. the dipolar Σr^{-6} for ^{19}F as calculated from the ^{19}F dipolar line width (○) and the dipolar T_1 (●). A positive SIS value indicates an upfield shift in D_2O compared with H_2O solvent. The Σr^{-6} terms were calculated from dipolar relaxation data obtained for protein in D_2O (Hull and Sykes, 1974, 1976b), and the SIS values are those calculated for the same protein sample in H_2O and D_2O buffers (see Table IV). The data for *m*-fluorotyrosine are given by (⊗). The solid curve does not represent any particular theoretical best fit.

width of fluorines in the cobalt enzyme will depend on r^{-6} and angular factors, while the chemical shift depends on r^{-3} and angular factors. Thus, perturbations to chemical shifts can occur at much longer range than line broadening. This can explain why several fluorotyrosine resonances are shifted but are not significantly broadened. The disappearance of tyrosine No. 3 in Figure 2D indicates that it is significantly shifted and/or broadened and may be rather close to the metal site. A rough calculation can be made after averaging over angular effects, to give some feeling for the distance factors involved (La Mar et al., 1973; Sloan et al., 1975). The paramagnetic contribution to line width will be less than 10 Hz when $r > 8.4$ Å, while the absolute value of the paramagnetic chemical shift contribution will be less than 2 ppm only when $r > 17$ Å. Thus, it would appear that, for all tyrosines except possibly No. 3, the fluorines are at least 8 Å from a metal site. Tyrosines in group (3) mentioned above are in the range of 10-20 Å from the metal, while tyrosines in groups (1) and (2) are probably >20 Å distant. The importance of angular factors is demonstrated by the opposite directions of paramagnetic shifts observed for tyrosine No. 5 compared with No. 7 and 8. For comparison, the dimensions of the protein dimer are about 80 × 60 × 60 Å, and the distance between the two important metal sites is 32 Å (Knox and Wyckoff, 1973).

Phosphate Binding at Alkaline pH. There is as much confusion in the literature concerning the stoichiometry of phosphate binding as with the question of metal binding. Coleman titrated the Co_2 dimer at alkaline pH and found 1 P_i/dimer completed the spectral change (Applebury and Coleman, 1969b), while Vallee found that the Co_4 dimer required 2 P_i/dimer to complete the spectral change (Simp-

son and Vallee, 1968). While most of the data to date have indicated the tight binding of only one P_i at alkaline pH, there is also some evidence for nonintegral stoichiometry of 1.5 P_i/dimer (Applebury et al., 1970) and nonintegral burst kinetics (Bloch and Schlesinger, 1973, 1974). All of the authors appear to agree that there is anticooperativity in P_i binding (Applebury et al., 1970; Simpson and Vallee, 1970; Lazdunski et al., 1969b) with values of K_D for the first site in the range 0.6–10 μM and for the second site $\gtrsim 10^{-5}$ M. The anticooperativity observed by equilibrium dialysis techniques was sensitive to ionic strength and became enhanced at high ionic strength. The high K_D for the second phosphate could explain the 1.0/dimer stoichiometries observed in titration experiments, especially at high ionic strength. Nonlinear Lineweaver–Burk plots for *p*-nitrophenyl phosphate have also been observed, implying anticooperativity in substrate binding (Simpson and Vallee, 1970). The presence of tightly bound endogenous phosphate (1.6–2.1/dimer) on the native enzyme has been observed (Bloch and Schlesinger, 1973), and these authors argue that the presence of unsuspected endogenous phosphate could have produced curved Scatchard plots in equilibrium dialysis experiments even when the two binding constants are equal. However, this explanation could not account for the curved plots observed with purged enzyme. Furthermore, Bloch and Schlesinger found that prolonged dialysis could remove one of the endogenous phosphates and this removal was enhanced by high ionic strength.

Our results of Figure 3 are consistent with the tight binding of P_i to two apparently indistinguishable sites. We have already mentioned that a reasonable upper limit for the dissociation constant for the second phosphate is 5 μM, based on the linear titration of the ^{19}F spectrum with phosphate, and this value is considerably lower than literature values, even when considering the low ionic strength used in our experiments. Furthermore, the appearance of two separate resonances 3a, 3b in spectra 3B and 3C implies that phosphate dissociation is slow on the NMR time scale. For the simple one-step scheme $E + P_i \leftrightarrow E'\cdot P_i$, the observed difference in shift between peaks 3a and 3b requires the off-rate for phosphate to be $k_{-1} < 400\ s^{-1}$ for either site. If the on-rate for phosphate is $\sim 10^8\ M^{-1}\ s^{-1}$, then we have $K_D < 4$ μM. The stability of spectrum 4D to dialysis is also consistent with these calculations. While the inhibitor 2-hydroxy-5-nitrobenzylphosphonate ($K_D = 3 \times 10^{-5}$ M) can be easily removed by dialysis, under similar conditions no phosphate is removed.

A possible criticism is that the purging process may leave the protein in a metastable state (spectrum 3A), which is converted "irreversibly" by a single turnover back to the state represented by 3D, even after phosphate has dissociated. This type of hysteresis is ruled out for the following reasons. First, we have observed one batch of native fluoroenzyme (as isolated) to be in a mixed state similar to that represented by spectrum 3C, even though the protein has not been subjected to purging. Secondly, if phosphate did not bind very tightly and purging did produce a metastable state, then less than stoichiometric amounts of P_i should *catalytically* convert *all* of the enzyme to state 3D. This is not observed.

Thus, our results indicate that at pH 8 there two distinct conformational states of alkaline phosphatase. The equilibrium between these states is controlled by inorganic phosphate but is not affected by phosphonate or arsenate. While the conformational change exhibited here is much less dramatic than the change caused by metal removal, nevertheless five tyrosines (No. 3–7) are perturbed by phosphate binding, implying something more than simple binding of P_i to a static active site. Tyrosine No. 3 shows the largest perturbation upon P_i binding and spectra of the cobalt enzyme also suggest it is near the metal (active) site. It should be mentioned that the peptide sequence surrounding the active serine residue is Asp-*Tyr*-Val-Thr-Asp-*Ser*-Ala-Ala-Ser-Ala so that at least one tyrosine should be in the general vicinity of the active site. Anticooperativity is not ruled out by our experiments, but the second phosphate must bind, in our experiments, much tighter than has previously been assumed.

Phosphate Binding at Acid pH. The conversion of the noncovalent $E\cdot P_i$ complex into an E–P covalent phosphoryl enzyme at pH <6 has been studied in considerable detail by Wilson and others (Wilson and Dayan, 1965; Cohen and Wilson, 1966; Barrett et al., 1969; Levine et al., 1969; Reid et al., 1969; Reid and Wilson, 1971; Applebury et al., 1970; Lazdunski et al., 1969b). The pH dependence of E–P formation is different for each of the transition metals that bind to alkaline phosphatase. Since the phosphoryl enzyme is 10^6 times more stable than expected for a simple phosphate ester, direct coordination of the metal with phosphate in the phosphorylation process is implicated. More detailed discussion of the equilibrium between $E\cdot P_i$ and E–P is reserved for the accompanying paper (Hull et al., 1976).

As was mentioned in the Results section there was little difference between phosphate-free and phosphate-saturated enzyme at pH 4.3, as judged by ^{19}F NMR of the fluoro-labeled protein. Furthermore, there were only small differences between these spectra at low pH and the purged enzyme at pH 8. It has also been observed that, below pH 6, the visible absorption, circular dichroism, and magnetic circular dichroism of Co(II) enzyme are the same whether or not phosphate is present, in contrast to the marked differences observed at alkaline pH (Taylor et al., 1973). Thus, it appears that formation of the phosphoryl enzyme E–P does not involve the same conformational change associated with $E\cdot P_i$.

Catalytic Mechanism. Bloch and Schlesinger have discussed in detail the evidence supporting the general scheme outlined below (Bloch and Schlesinger, 1973, 1974; Halford and Schlesinger, 1974).

$$\begin{array}{ccccc} E' + ROP & \overset{2}{\rightleftharpoons} & E'\cdot ROP & \overset{5}{\rightleftharpoons} & E'\text{—}P + ROH \\ \uparrow\downarrow 1 & & \uparrow\downarrow 3 & & \Vert\ 6 \\ E + ROP & \underset{4}{\rightleftharpoons} & E\cdot ROP & & \Vert \\ E + P_i & \underset{8}{\rightleftharpoons} & E\cdot P_i & \underset{7}{\rightleftharpoons} & E\text{—}P + HOH \end{array}$$

At acid pH, dephosphorylation (steps 6 + 7) is rate limiting. However, the pre-steady-state transient phase at acid pH and the rate-limiting step at alkaline pH, which have been shown to be mechanistically identical (Halford and Schlesinger, 1974), have in the past been assigned to the conformational change from E to E′ (steps 1 or 3) prior to phosphorylation by substrate. This suggestion was first made by Halford et al. (1969) before the discovery by Bloch and Schlesinger (1973) of the endogenous phosphate found in preparations of alkaline phosphatase. Subsequently, the influence of P_i on the kinetic properties of the en-

zyme was recognized due to its displacement of the E ↔ E′ equilibrium which lies far to the side of E′ in the absence of phosphate, though it was assumed that the dissociation of the noncovalent E·P_i complex (step 8) was fast (Bloch and Schlesinger, 1973).

The results with the fluorotyrosine alkaline phosphatase support the general scheme shown above. Under observation here are the conformational changes that accompany transitions away from the E′ state of the free enzyme in the absence of any ligands. There were no discernible changes in the conformation of the protein upon binding 2-hydroxy-5-nitrobenzylphosphonate to form a complex presumably analogous to E′·ROP nor in the formation of the covalent phosphoryl enzyme (E′–P) with inorganic phosphate at low pH. But direct evidence has been presented for a conformational change linked to the noncovalent binding of P_i at pH 8.0 (E′ + P_i → E·P_i). The lifetimes of many of the intermediates within the catalytic pathway have been examined by ^{31}P NMR (Hull et al., 1976) and a complete discussion of the mechanism of alkaline phosphatase is reserved for that paper. The conformational change within the catalytic turnover of alkaline phosphatase, for which this paper provides the first direct evidence, had previously been considered as one of the rate-determining steps of the mechanism. But these ^{19}F NMR results provide no support for such a role of the conformational change within the enzyme kinetics. Moreover, the ^{31}P NMR data in the accompanying paper indicate the dissociation of P_i from the noncovalent E·P_i intermediate to be rate limiting at pH 8.0. The known rate of the decomposition of the covalent phosphoryl enzyme (E′–P) at pH 8.0, measured by quenched-flow techniques and found to be approximately twice as fast as the turnover number of the enzyme (Aldridge et al., 1964; Reid and Wilson, 1971), is not in contradiction with the proposal that the rate of dissociation of noncovalently bound phosphate from E·P_i determines the turnover number of this enzyme at its optimal pH values.

Summary

The purpose of this report has been twofold: first, to provide specific information concerning the conformational and catalytic properties of alkaline phosphatase, and secondly to illustrate the general usefulness of studying proteins labeled in vivo with fluoro amino acids. Similar work is in progress on the gene 5 DNA-binding protein from bacteriophage fd labeled with fluorotyrosines (Anderson et al., 1975). We have demonstrated that ^{19}F NMR allows one to observe individual residues in large proteins and determine their motional properties and the degree of interaction with solvent and other protein residues. Furthermore, ^{19}F responds to small changes in its chemical and magnetic environment caused by both local and more general perturbations of protein structure, allowing for the determination of binding stoichiometries and the direct observation of conformational changes that may be of mechanistic importance. The signal to noise characteristics of ^{19}F NMR make it possible to study individual residues at protein concentrations of 10^{-4} M.

This report demonstrates the tight binding ($K_D < 10^{-5}$ M) of 2 mol of inorganic phosphate per mole of alkaline phosphatase dimer and provides direct evidence for a significant conformational change when purged enzyme binds phosphate or turns over substrate. In none of this work, or that to be discussed in the accompanying manuscript, have we been able to detect any form of subunit interaction. Recent work with a hybrid alkaline phosphatase dimer has also argued strongly for independent subunits (Bloch and Schlesinger, 1974), in contrast to considerable kinetic data requiring a single active site or negatively cooperative subunits (Chappelet-Tordo et al., 1974). Since our work involved protein concentrations of about 10^{-4} M, cooperativity may have been masked, but the results described here are not consistent with the strong negative cooperativity between sites observed by others. Since it has been reported that the aminotyrosine derivative of alkaline phosphatase exhibits little cooperativity under conditions where the wild-type enzyme displayed strong negative cooperativity (Simpson and Vallee, 1970), one might argue that the fluorotyrosine derivative also lost the cooperativity that is associated with the wild-type enzyme. However, the fluorotyrosine enzyme exhibits identical transient and steady-state kinetics when compared with the wild-type protein. Likewise, the amino derivative has unperturbed hydrolase activity. Hence, any differences that might exist between wild-type and fluoro enzymes with regard to phosphate binding cannot be of significance in the catalytic mechanism. In other words, the argument that the fluoro enzyme has lost cooperativity leads to the conclusion that complete negative cooperativity or a flip–flop mechanism is not necessary for catalytic efficiency. Alkaline phosphatase exhibits an extraordinary affinity for phosphate (the product of catalysis) at alkaline pH, and the dissociation of this noncovalent phosphate and the concomitant E → E′ conformational change limits the steady-state turnover of substrate. If subunit interaction or cooperativity were important, the most effective use of such interactions would be in assisting the E → E′ transition and dissociation of phosphate.

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