^{31}P Nuclear Magnetic Resonance Study of Alkaline Phosphatase: The Role of Inorganic Phosphate in Limiting the Enzyme Turnover Rate at Alkaline pH†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) was used to directly observe the binding of inorganic phosphate to alkaline phosphatase. Evidence for the tight binding of 1.5–2.0 mol of inorganic phosphate per dimer of alkaline phosphatase is presented. Two distinct forms of bound phosphate are observed, one predominating above pH 7 and representing the non-covalent E·P_i complex and the other predominating below pH 5 and representing the covalent E–P_i complex. The ^{31}P NMR line width of the E·P_i complex indicates that the dissociation of noncovalent phosphate is the rate-limiting step in the turnover of the enzyme at high pH.

Alkaline phosphatase from *Escherichia coli* (*E. coli*) (EC 3.1.3.1) is a dimeric zinc metallo-enzyme (mol wt = 86 000) which catalyzes the hydrolysis of a wide variety of phosphate esters and ^{18}O exchange from water into inorganic phosphate. From the early observations that this protein hydrolyzes $ROPO_3^{2-}$ esters at nearly identical rates, regardless of the pK_A of the leaving group RO^-, it was postulated that catalysis involved the rapid formation of a covalent phosphoryl enzyme intermediate. Subsequent rate-limiting hydrolysis or breakdown of this common intermediate would then result in a constant enzyme turnover rate, regardless of the nature of the R group. The proposed intermediate was found to be stable at acid pH and a phosphoserine group has been isolated (Schwartz and Lipmann, 1961; Engström, 1962; Schwartz et al., 1963). Stoichiometries of 1 P_i/dimer (Reid et al., 1969) at pH 5 and 2 P_i/dimer (Lazdunski et al., 1969b) at pH 4 have been reported for the phosphoryl enzyme.

At alkaline pH, a noncovalent Michaelis complex between enzyme and P_i was found to be more stable than the covalent complex. This complex is so stable that native enzyme, as isolated from *E. coli*, has been found to contain as much as 2 mol of noncovalently bound P_i per dimer (Bloch and Schlesinger, 1973). The equilibrium between covalent and noncovalent enzyme-phosphate complexes has been studied in detail and arguments presented for the importance of the phosphoryl enzyme intermediate in the catalytic pathway (Wilson and Dayan, 1965; Barrett et al., 1969; Levine et al., 1969).

In the accompanying report we have discussed experiments involving fluorotyrosine alkaline phosphatase (Hull and Sykes, 1976). The kinetic properties of this labeled protein were identical with those of the wild-type enzyme (Sykes et al., 1974). By observing the ^{19}F NMR[1] spectrum of the labeled protein, we found that titration of purged enzyme (phosphate-free) with P_i resulted in a conformational change that required 2 P_i/dimer for completion. The binding properties of each of the two phosphates were indistinguishable and an upper limit for K_D for either site was estimated to be 5×10^{-6} M. The extraordinarily tight binding of P_i at pH 8 and the conformational change observed led to the proposal that dissociation of *noncovalently* bound P_i was the rate-limiting step at high pH.

In order to provide further experimental data concerning the stoichiometry and stability of the noncovalent E·P_i complex, we report here the results of a ^{31}P NMR study carried out using wild-type alkaline phosphatase. Advantages to this approach include the *direct* observation of inorganic phosphate in equilibrium with enzyme and the possibility of observing enzyme–phosphate complexes under conditions where the protein is intact and catalytically active. The disadvantages are that ^{31}P NMR is a much less sensitive technique than ^{19}F or ^{1}H NMR. The ^{31}P resonances of phosphates in aqueous solutions tend to have much shorter T_2 relaxation times compared with T_1, and these relaxation processes are not so easily characterized for ^{31}P as for ^{1}H, ^{19}F, or ^{13}C. The long T_1's encountered even for enzyme-bound phosphate severely limit the efficiency of the repetitive pulsing technique used in Fourier transform NMR. Thus, the lower practical limit for phosphate concentration appears to be about 0.5 mM when using 12-mm NMR tubes at 40 MHz. For experiments with enzyme and phosphate at stoichiometric levels, this requires about 1 μmol of protein in a volume of 1–2 ml.

^{31}P NMR has been used earlier in studies of alkaline phosphatase. When a mixture of apoenzyme (10^{-5} M) and P_i (0.1 M) was titrated with Co(II) or Mn(II), the ^{31}P line width of P_i was not significantly affected until more than 2 mol of metal per dimer had been added. Subsequent addition of metal led to paramagnetic line broadening of the P_i resonance. Thus, the tight binding of 2 metals/dimer was confirmed (Csopak and Drakenberg, 1973). A direct ^{31}P NMR study of the binding of P_i at stoichiometric levels to Zn, Cd, and Co alkaline phosphatases has been recently

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[1] Abbreviations used: NMR, nuclear magnetic resonance; P_i, inorganic phosphate; NTA, nitrilotriacetic acid.

completed (Bock and Sheard, 1975). The concurrent independent work presented here confirms in detail and extends the fundamental observations of those workers.

In the sections to follow we will present evidence for the tight binding of 1.5–2 mol of P_i per mole of zinc alkaline phosphatase. There are two distinct forms of bound phosphate with one predominating above pH 7 and the other below pH 5, representing the noncovalent E·P_i and covalent E–P complexes, respectively. The ^{31}P line width of the E–P complex at low pH is consistent with its breakdown being the rate-limiting step at acid pH. Furthermore, the ^{31}P line width in the E·P_i complex indicates that dissociation of noncovalent phosphate takes place at a rate comparable to the turnover rate of the protein at pH 8, implying that the dissociation step is rate limiting at alkaline pH.

Experimental Procedure

Materials. Alkaline phosphatase from four different enzyme preparations was used to obtain the results presented in this report. Batches A, B, and C were prepared as previously described (Halford, 1971) from *E. coli* C90 provided by the Microbiological Research Establishment (Porton Down, Wiltshire, U.K.) except that the purified enzyme was not stored as a lyophilized powder. This material was dialyzed for 1–2 days against Tris buffer, pH 8, prior to use. Batch D was the generous gift of J. E. Coleman, and both zinc and cobalt enzymes were obtained as solutions in Tris buffer, pH 8. The enzyme as received will be referred to always as the "native" enzyme. For all calculations, an extinction coefficient of $E_{280}^{0.1\%} = 0.77$ (Rothman and Byrne, 1963) and a dimer molecular weight of 86 000 (Schlesinger and Barrett, 1965) were used. Protein OD measurements were made in 1 M Tris, pH 8, on Gilford or Zeiss spectrophotometers, and all concentrations refer to dimer.

Tris(hydroxymethyl)aminomethane (Tris) was the Ultra Pure grade from Mann Research Laboratories or Trizma from Sigma. Nitrilotriacetic acid (NTA) and *p*-nitrophenyl phosphate were from Sigma. The inhibitor 2-hydroxy-5-nitrobenzylphosphonate was prepared as previously described (Halford et al., 1969). All other chemicals were reagent grade and distilled water was supplied by Belmont Spring Water Co., Belmont, Mass. Buffers were made metal-free by extraction with 0.001% dithizone in CCl_4, followed by extraction with pure CCl_4 to remove dissolved dithizone, and finally aeration with N_2 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 recorded on a Cary 15 spectrophotometer, and specific activity was calculated from the initial absorbance slopes using $E_{400}^{mM} = 16.2$ for *p*-nitrophenol at pH 8 (Halford, 1971). The units of activity are micromoles of substrate cleaved per hour per milligram of protein and, for comparison, the pure crystalline enzyme has been found to have an activity of 3250 units (Malamy and Horecker, 1964). Since NMR samples involve very high protein concentrations, the sample was pre-diluted, 5 μl into 1 ml of 1 M Tris (metal-free), and then 5 μl of this dilution was assayed in 1 ml of metal-free assay medium so that absorbance changes of 0.1–0.3 OD unit/min were obtained.

Preparation of "Purged" Enzyme. "Native" alkaline phosphatase can contain as much as 2 mol of endogenous tightly bound phosphate per mole of protein dimer, and removal of this phosphate requires removal of the enzyme-bound metal ions (Bloch and Schlesinger, 1973). The enzyme sample (for this work 80–150 mg) in 2–4 ml of buffer was dialyzed against 500 ml of decreasing concentrations of NTA in 0.1 M Tris-Cl, pH 6.5–7 at 4 °C. Over a period of 3–4 days, the NTA concentration was decreased from 40 to 1 mM. The sample was then dialyzed against 1 mM $ZnSO_4$ in 0.1 M Tris-Cl, pH 8, for 2 days, and finally against 2–3 changes of metal-free buffer. This procedure produces a phosphate-free reconstituted holo-enzyme which is termed the "purged" enzyme. In a few cases the dialysis against metal was omitted, and the inactive apoprotein was obtained. Titration of this protein with zinc led to a linear increase in activity (pre-dilution and assay media contained 10^{-5} M NTA) with maximal activity obtained at 4 Zn/dimer. Purged cadmium enzyme was prepared in an identical manner by dialysis of apoprotein against $CdCl_2$.

pH Adjustment and Concentration of Samples. Protein samples from dialysis were passed through a millipore filter to remove precipitates and bacteria and were concentrated to about 1 ml in an Amicon Ultrafilter device using the PM-10 membrane. The sample was then transferred directly to the NMR tube and, after 1 or 2 washings of the membrane, the final volume was 1.5–1.6 ml. When it was desired to study a particular sample at different pH's, Tris or Tris-HCl buffers were used to dilute the sample to the desired pH followed by reconcentration with the ultrafilter.

NMR Techniques. Fourier transform ^{31}P NMR spectra at 26–28 °C were obtained at 40.5 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. A modified Varian Block Mode procedure was used to acquire spectra and is described in the accompanying report (Hull and Sykes, 1976). The following "standard" parameters were used for most spectra: spectral width 1500 Hz, acquisition time 0.25 s, pulse delay 0.75 s, pulse width 20 μs (56° pulse angle), sensitivity enhancement exponential filter time constant 0.06 s (contributing 5 Hz to the line width). Typically 25 000–50 000 transients were collected for each spectrum requiring 7–14 h. Samples were placed in 12-mm NMR tubes equipped with a bottom plug and Vortex plug (both Teflon) so that volumes of about 1.5 ml could be routinely handled. A 1-mm capillary containing a 0.2 M solution of sodium pyrophosphate in H_2O was used as both a chemical-shift reference and an area standard for area measurements. The chemical shifts reported here have been converted to a scale based on a capillary of 85% phosphoric acid, with upfield shifts defined as positive. The solvent H_2O resonance was used as a field-frequency lock.

For the work reported here, it was necessary to make measurements of the areas of ^{31}P resonances, and a polar planimeter was used for this purpose. The areas from different samples must be placed on a fixed scale which can relate areas to the concentration of various species. The absolute intensity or area of a particular resonance depends on several variables: concentration and sample volume, spectrometer gain, pulse width and timing, number of transients, spin–lattice relaxation time, and scaling of the data during computer manipulations. By using the *same* capillary reference and the same pulse width and timing for different samples, the area of the reference peak can be used as a standard to eliminate the variables of gain, number of transients, and data handling. Sample volumes did not vary significantly so that the areas of phosphate resonances when scaled or normalized to the reference peak should depend only on concentration and spin–lattice relaxation times T_1.

When the T_1 values for different species are not the same or if the T_1 values vary with concentration, then peak areas cannot be directly related to concentration unless pulse width and timing are adjusted so that complete relaxation of the z magnetization takes place between each pulse. This restriction would place a severe constraint upon the efficiency of the Fourier transform experiment. However, if T_1 values were approximately the same for different species and were insensitive to changes in concentration, then more efficient data acquisition at only partial saturation would not seriously affect the comparability of area measurements. Evidence to be presented in the next section will show that, under our pulse conditions and concentrations, the observed peak areas, after normalization to the reference peak, will directly indicate the relative concentrations of various species with sufficient accuracy.

A modification of the Varian program allows for the proper accounting for computer scaling in the Block Mode procedure. Spectra can be plotted directly on an absolute intensity scale, and the accuracy of this procedure has been confirmed by the excellent reproducibility of the reference peak intensity (±5%) in long-term experiments carried out over 2–3 days.

Results

The Stoichiometry of Phosphate Binding at Alkaline pH. Figure 1 illustrates an experiment performed with native zinc alkaline phosphatase (batch D) as obtained from J. E. Coleman. This enzyme had never been "exposed" to phosphate after release from the *E. coli* bacteria. Therefore, spectrum 1A represents the direct observation of endogenous phosphate bound to native enzyme at a concentration of 0.57 mM. The chemical shift of −3.4 ppm corresponds quite well with the position of bound phosphate on alkaline phosphatase reported earlier (Bock and Sheard, 1975). Spectra 1B and 1C were obtained after adding 0.94 and 1.87 mM inorganic phosphate to the native enzyme sample. It appears that the bound P_i resonance approximately doubles in area going from 1A to 1B, but is unchanged from 1B to 1C. A well-resolved free[2] phosphate resonance appears at −1.90 ppm in 1C and there is a small amount of free phosphate in 1B.

The nature of phosphate binding at alkaline pH has been generally considered to be a noncovalent Michaelis-type complexation, and we will use the symbol E·P_i to refer to this complex. From spectrum 1B it appears that the E·P_i resonance is not from just a single species but may involve a second species with a shift at ca. −4 ppm. Similar effects were reported earlier (Bock and Sheard, 1975), and a further clarification of this behavior will follow.

To determine the stoichiometry of phosphate binding, it is necessary to determine the amount of phosphate bound in 1A. For the analysis of the area data, we must assume that the signals for E·P_i and P_i in a repetitive pulsing experiment can be expressed as area = constant × concentration, where the constant is independent of concentration. If chemical exchange between two sites A and B is fast relative to the relaxation rate in either site ($1/T_1^A$ and $1/T_1^B$), then the observed relaxation rate for both species in any given sample will be the *same* exchange-averaged rate $1/T_1(\text{obsd}) = P_A/T_1^A + P_B/T_1^B$, where P_A and P_B are the fractional

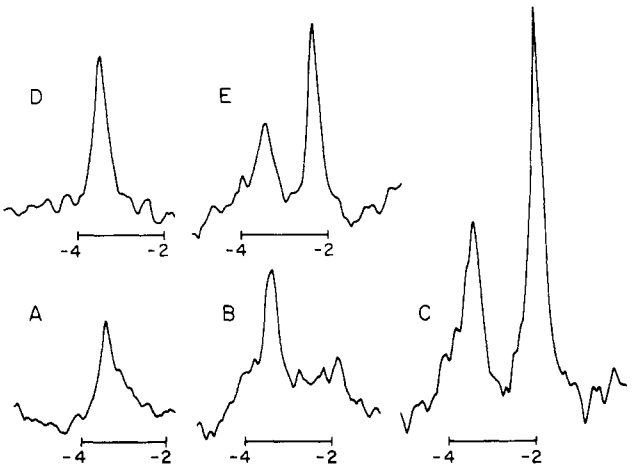

FIGURE 1: The binding of inorganic phosphate to native zinc alkaline phosphatase. The enzyme (batch D) as received was 0.57 mM dimer in 0.01 M Tris-Cl, pH 7–7.5 with specific activity 3250 units, and the ^{31}P NMR spectrum of the untreated native enzyme is shown in spectrum A. The addition of 0.94 mM and 1.87 mM P_i is shown in B and C, respectively. The chemical shifts of E·P_i and P_i are −3.37 and −1.90 ppm, respectively. The result of dialyzing the solution used for spectrum C against 2 changes of 0.01 M Tris-Cl pH 8 (2/1000) is shown in D with 0.46 mM dimer. The addition of 0.82 mM P_i is shown in E, with E·P_i at −3.42 ppm and P_i at −2.30 ppm. All spectra were taken using standard parameters. Spectra A, B, and C are plotted on matching vertical scales. Spectra D and E are plotted with a somewhat greater scale.

populations of sites A and B, respectively. In the enzyme–phosphate system, the lifetimes of free and bound phosphate are given as $1/\tau_{P_i} = k_{-1}(\text{E·P}_i)/(P_i)$ and $1/\tau_{\text{E·P}_i} = k_{-1}$ (see later discussion on kinetics), where k_{-1} is the dissociation rate constant. The value of k_{-1} is estimated to be 10–20 s^{-1} from line-width data, and variation of the pulse timing in our experiments indicates that T_1(obsd) is 1–2 s. Hence, the fast exchange condition $\tau/T_1 \ll 1$ applies, and the relative areas for P_i and E·P_i in any one sample directly reflect the relative concentrations of these species. If $T_1^A \neq T_1^B$, then as P_A and P_B vary through a series such as Figure 1, the T_1(obsd) will vary as well. It is found in several experiments that, as free P_i is increased from much less than E·P_i to 2–3 times E·P_i, that the area of E·P_i is constant within experimental error (Figures 1–3). Furthermore, the free P_i area appears to be linear in P_i concentration (Figure 4A). This behavior indicates $T_1^A \sim T_1^B$ and, under our experimental conditions, the use of areas to determine absolute concentrations through a series of samples appears justified. Given the validity of our assumption about the proportionality of area to concentration, it is a simple matter to calculate the concentration of E·P_i from the dependence of areas on the known concentration of free P_i added. The results are that E·P_i is 0.51 mM in 1A and 1.17 mM in 1C. Since the dimer concentration is 0.57 mM in 1A, it then appears that the native enzyme (batch D) contained 1 mol/mol of endogenous phosphate and is capable of binding tightly a second phosphate molecule. The question of just how tightly these phosphates are bound will be discussed in a later section. The free phosphate can be easily dialyzed away leaving only E·P_i as shown in 1D. Adding 1.8 P_i/dimer results in spectrum 1E. The area of E·P_i did not change from 1D to 1E, although there was a change in line width. This line-width change was not a reproducible effect (compare with Figure 3) and could not be characterized as a specific property of the enzyme–phosphate system. Com-

[2] The term "free" will be taken to mean phosphate *not* tightly bound to enzyme but which may associate very weakly (fast exchange limit) with nonspecific sites.

Table I: Summary of ^{31}P NMR Data for Alkaline Phosphatase Experiments.[a]

Species[b]	Chem Shift (ppm)	Line Width (Hz)	pH	Buffer[c]	Enzyme[d]	Metal
P_i	−2.78	3.7	8	A		
	−2.30	4.2	8	B	Native D	Zn
	−2.30	6–18	8	B	Native D	Co
	−2.52	15–20	8	C	Native B, C	Zn
	−1.87	6–20[e]	7	C	Purged C	Zn
	−0.30	~50	5.1	C	Purged B	Zn
E·P_i (I)	−3.40	8, 14	7–8	B	Native D	Zn
	−3.50	13	8	C	Native C	Zn
E·P_i (II)	−4.10 to −4.23	18–20	8	C	Purged A, B, C	Zn
E–P	−8.5 to −8.7	15–20	4.4–5.9	C	Purged B	Zn
	−8.53	17–22	7	C	Purged C	Cd
BP[f]	−20.04	4.4	8	A		
	−20.15	~50[g]	8	D	Purged A	Zn
E·BP	−22.02	~50[g]	8	D	Purged A	Zn

[a] ^{31}P chemical shifts are ±0.05 ppm relative to a capillary of phosphoric acid. Line widths are corrected for the instrumental line width and for free P_i generally refer to a range of concentrations where enzyme is 0.5–1.0 mM and total phosphate is 1–4 mM. [b] The species are: P_i, free phosphate; E·P_i, noncovalently bound phosphate; E–P, covalently bound phosphate; BP, 2-hydroxy-5-nitrobenzylphosphonate. [c] The buffers were: A, 0.1 M Tris, 1 N NaCl; B, 10 mM Tris-Cl; C, 0.1 M Tris-Cl; D, 0.1 M Tris-acetate. [d] Four enzyme preparations were studied. "Native" refers to enzyme as received; "purged" refers to enzyme treated with NTA and reconstituted as described in Experimental Procedure. [e] The concentration dependence of the line width is shown in Figure 8. [f] $^2J_{PH}$ = 19.0 Hz. [g] Line width includes the contribution due to $^2J_{PH}$.

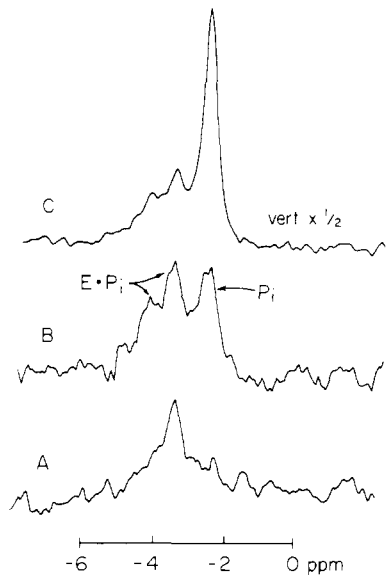

FIGURE 2: The titration of native zinc alkaline phosphatase with phosphate at pH 8.0. The native enzyme (batch B) was exposed to excess P_i and then dialyzed (2/500) against 5 changes of 0.1 M Tris-Cl, pH 8, to give spectrum A at a concentration of 0.95 mM dimer (specific activity = 2550 units). Spectra B and C were obtained after the addition of 1.03 and 3.05 mM P_i, respectively. The chemical shifts for E·P_i are −4.13 and −3.51 ppm while P_i appears at −2.51 ppm. Spectra A and B are on the same vertical scale, while C is at ½ that scale.

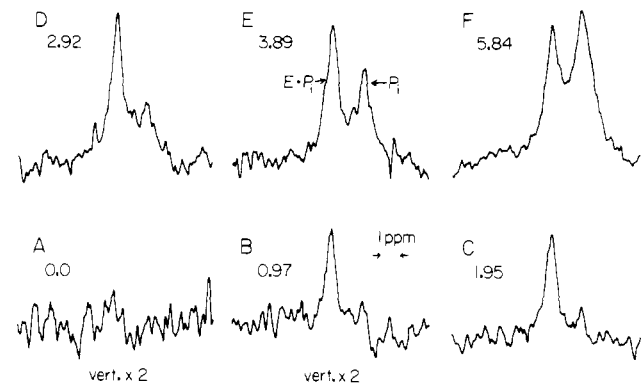

FIGURE 3: The titration of purged zinc alkaline phosphatase with inorganic phosphate. The ^{31}P NMR spectra have been recorded using the standard parameters described in Experimental Procedure, and each spectrum represents 28 000–50 000 transients requiring 8–14 h. The enzyme (batch B) was purged and reconstituted with final dialysis to pH 8 in 0.1 M Tris-Cl. The specific activity was 2160 units at the beginning and 2060 units at the end of the experiment. The purged enzyme (0.86 mM) was titrated with aliquots of 0.2 M NaP_i (in 0.1 M Tris, pH 8), and each spectrum is labeled with the ratio of total P_i added per dimer concentration. Spectra A and B are shown at twice the normal vertical scale. The chemical shifts of E·P_i and P_i are −4.20 and −2.66, respectively.

paring the areas in 1D and 1E, one finds the evidence is strong for 2 P_i/dimer binding tightly.

^{31}P NMR spectra obtained with another enzyme preparation (batch B) are shown in Figure 2. The native enzyme was exposed to phosphate and then dialyzed against 5 changes of 0.1 M Tris, pH 8, to give spectrum 2A. This demonstrates the stability of the E·P_i complex. Spectra 2B and 2C represent the addition of 1.1 and 3.2 P_i/dimer, respectively. The dialyzed enzyme (2A) thus contains at least one tightly bound P_i and in 2B one can observe two distinct resonances for the E·P_i complex at −3.51 and −4.20 ppm. These will be called E·P_i (I) and E·P_i (II), respectively. The ^{31}P NMR parameters for enzyme-bound phosphate under a variety of conditions are summarized in Table I. While it is tempting to assign resonances (I) and (II) to two nonidentical enzyme subunits, evidence obtained with four different enzyme preparations has indicated that the relative amounts of (I) and (II) are variable and susceptible to various enzyme treatments, especially the purging process. Table II summarizes data collected for all four enzyme preparations under a variety of conditions. What emerges from this comparison is that there appears to be a direct correlation between enzyme activity and the relative amounts of E·P_i (I) and (II). Native enzyme generally shows mostly (I) and high activity (3000–3200), while purged enzyme when exposed to phosphate often shows mostly (II) and low activity (2000). Enzyme displaying similar amounts of (I) and (II) has intermediate activity (2500). These data argue for the conclusion that the enzyme can exist in two distinct forms possessing different

Table II: The Correlation of E·P_i Chemical Shifts with Enzyme Activity.[a]

Enzyme[b]	Treatment[c]	Specific Activity	Fraction of E·P_i As I	Fraction of E·P_i Or II
A	Native + P_i, dialyzed	2500	1.0	0.0
	Purged + P_i	2000	0.0	1.0
B	Native + P_i	3150	0.5	0.5[d]
	Native + P_i, dialyzed	2550	0.6	0.4
	Purged + P_i	2170	0.0	1.0
	Purged + P_i, dialyzed 5 days	1600	0.0	1.0
	Purged + p-NPP	2000	0.0	1.0
C	Native + P_i	3150	1.0	0.0
	Purged + P_i	2250	0.3	0.7
	Purged + P_i, dialyzed 6 days	2500	0.4	0.6
D	Native + P_i	3250	0.7	0.3
	Native + P_i, dialyzed	3250	1.0	0.0

[a] The fraction of E·P_i appearing at −3.4 ppm (I) and −4.2 ppm (II) is tabulated along with the specific activity (μmol/h per mg) of alkaline phosphatase under a variety of conditions. [b] Four different batches of zinc alkaline phosphatase have been subjected to similar treatments. [c] Native, enzyme as received; + P_i, slight excess of p_i added; dialyzed, dialysis against metal-free 0.1 M Tris buffer, pH 8; purged, dialysis against NTA, then $ZnSO_4$, then buffer; + p-NPP, substrate *p*-nitrophenyl phosphate added. [d] This sample prior to extensive dialysis exhibited at least three bound P_i resonances and greater than 2 P_i/dimer stoichiometry.

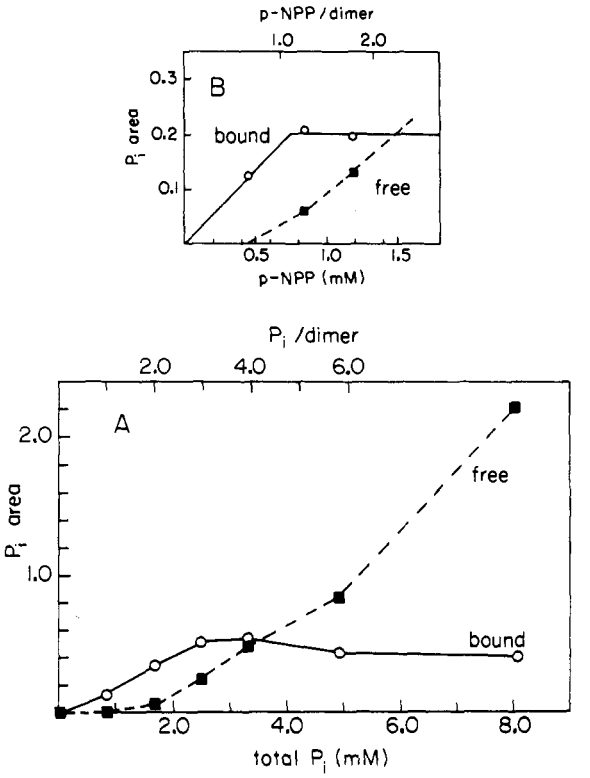

FIGURE 4: Titration of purged zinc alkaline phosphatase with inorganic phosphate and *p*-nitrophenyl phosphate at pH 8. The titration with phosphate is shown in A, where normalized areas (see Experimental Procedure) for free and bound P_i are taken from the spectra shown in Figure 3. An analogous experiment using *p*-nitrophenyl phosphate instead of phosphate is shown in B. The same protein sample used to determine A was used with a concentration of 0.68 mM, specific activity = 2000 units.

specific activities. Furthermore, these differences seem to be linked to the purging process. While purging appears to convert enzyme (I) into (II), prolonged dialysis resulted in a partial restoration of (I) in some cases.

A phosphate titration experiment performed with purged enzyme is illustrated in Figure 3. Spectrum 3A shows that the enzyme after purging contains no detectable phosphate. The addition of 1 P_i/dimer (3B) results in the appearance of a single well-resolved resonance at −4.2 ppm, corresponding to E·P_i (II). The addition of a second phosphate (3C) simply doubles the area of (II). Subsequent aliquots of P_i lead to the appearance of free P_i with no further change in E·P_i, and the line width of P_i appears to decrease with increasing concentration. These spectra demonstrate the tight binding of two indistinguishable phosphates to alkaline phosphatase. The protein used in this experiment exhibits a significantly lower activity but the same binding stoichiometry when compared with the maximally active protein used for Figure 1.

The peak area data from Figure 3 are presented graphically in Figure 4A. The detailed shape of these curves depends on the possible variation of the ^{31}P relaxation behavior of exchanging species as concentrations are varied (see above). There are also difficulties in measuring individual areas of overlapping peaks. Regardless of the shape of these curves, the point at which the two curves in Figure 4A cross represents a point of true equal concentrations. Furthermore, the equality of the relaxation rates for free and bound species was proved by demonstrating that a change in the pulse repetition rate did not significantly alter the relative areas of E·P_i and free P_i. Thus, the crossing point in Figure 4A confirms the binding of 2 P_i/dimer.

A similar experiment was performed using *p*-nitrophenyl phosphate rather than P_i, and the results are presented graphically in Figure 4B. This experiment indicates the binding of only 1 P_i/dimer after substrate turnover, in contrast to the results of Figure 4A. Further experiments of this type, with more data points, are needed to determine if there is indeed a fundamental difference in phosphate binding stoichiometry in the two situations represented by Figures 4A and 4B.

The pH Dependence of Phosphate Binding to Zinc Alkaline Phosphatase. The appearance of a new type of bound phosphate resonance at acid pH has been reported (Bock and Sheard, 1975), and our results presented in Figure 5 are virtually identical with the earlier work. Purged enzyme (batch B) was saturated with 2 P_i/dimer and washed free of excess P_i to give spectrum 5A. The pH was lowered in steps to give 5B, 5C, and 5E. Spectrum 5D is from another experiment in which free P_i was present. As the pH is lowered there is a conversion of bound P_i from E·P_i (II) at −4.2 ppm to a new form at −8.6 ppm which will be denoted E–P. It is reasonable to assume that this new low-field complex is, in fact, the covalent phosphoryl enzyme, since the stability of this intermediate at pH ≤5 has been well documented. The results of Figure 5 indicate that E·P_i and E–P are in equilibrium with each other at all pH's. The position of this equilibrium is shifted by changing pH, but the effect is completely reversible as indicated by spectrum 5F, which was obtained after raising the pH of sample 5E. The total amount of bound phosphate remains unchanged (2 P_i/dimer) throughout the pH range 4–8. The pH-dependent process being monitored does not involve a simple change in the ionization state of the phosphate since neither E·P_i nor E–P shows any systematic change in chemical shift, while

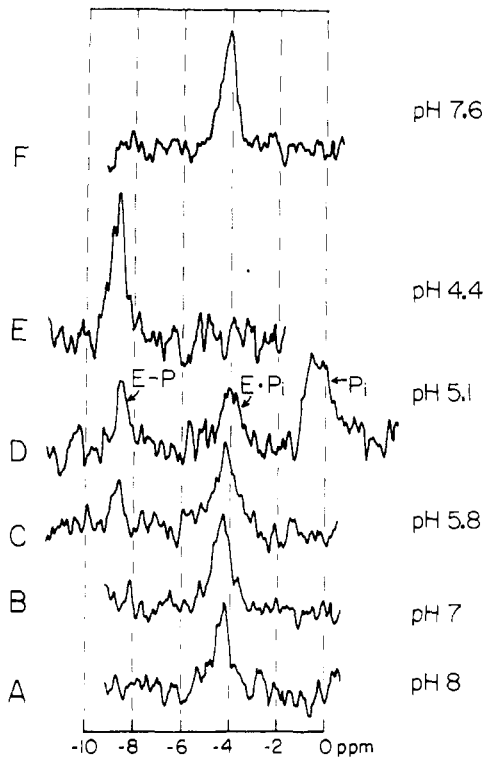

FIGURE 5: The pH dependence of phosphate binding. The ^{31}P NMR spectra (20 000–53 000 transients in 8–15 h) were taken with the standard parameters described in Experimental Procedure. The vertical scales of these spectra are not identical but vary over a 20% range. The purged protein (batch B) was saturated with P_i and then briefly dialyzed in 0.1 M Tris-Cl, pH 8, to remove free P_i using the Amicon ultrafilter. The pH was lowered by diluting the sample that gave spectrum A with 0.1 M Tris-Cl, 1.0 mM $ZnSO_4$, pH 2, and then reconcentrating using the Amicon device. The order of the spectra was A, B, C, E, F. Spectrum D is from another experiment with 0.70 mM protein (containing phosphate) and 1.7 mM free P_i added. Spectrum E was converted to F by dilution with 0.1 M Tris, pH 10. The chemical shifts of E–P and E·P_i are −8.6 and −4.2, respectively. At the end of the experiment, the enzyme specific activity was 2100 units.

free P_i moves upfield with decreasing pH. The process involved here is in the slow exchange limit with a rate constant less than 10^3 s^{-1}, based on the chemical-shift difference between E·P_i and E–P.

The chemical shifts of the two forms E·P_i and E–P are of considerable interest. The phosphate ester *O*-phosphoserine and proteins known to contain this residue exhibit ^{31}P shifts of −4.0 ppm at pH 8.6 and −0.1 ppm at pH 3.9 with $^3J_{PH}$ = 5.3 Hz (Ho et al., 1969). Since both E·P_i and E–P chemical shifts are pH independent, complete coordination of the ionized oxygens of $HOPO_3^{2-}$ is implicated, thus protecting the phosphate from protonation at low pH. Furthermore, the E–P complex exhibits a chemical shift that is 8.5 ppm downfield of that observed for a simple phosphoserine residue. This unique low-field resonance is well out of the range expected for phosphate monoesters (Bock and Sheard, 1975), but is within the range observed for five-membered ring cyclic esters. A recent empirical correlation between O–P–O bond angle and ^{31}P chemical shifts indicates that variation of this angle over only a 10° range can produce a 30-ppm variation in chemical shifts, and a narrowing of a O–P–O angle in phosphate by only about 2° could account for the 8.5-ppm downfield shift observed for E–P (Gorenstein, 1975). Association of phosphate with zinc also produces a downfield shift of about 2 ppm at pH 4 (Bock and Sheard, 1975), but this may reflect only the effect of displacing a proton.

When the areas corresponding to E·P_i and E–P are measured, the equilibrium between the two forms does not appear to follow a simple titration curve. However, the accuracy of these data is insufficient for detailed analysis. At pH 5 the concentrations of E–P and E·P_i are equal at 1/dimer. This corresponds very well with previous experiments where 1 site/dimer was found to be phosphorylated at pH 5 (Reid et al., 1969; Lazdunski et al., 1969b).

The Binding of Phosphate to Cobalt and Cadmium Alkaline Phosphatase. Cobalt alkaline phosphatase has been shown to bind at least one phosphate at pH 8 and can be phosphorylated at pH 4 (Lazdunski et al., 1969b; Applebury and Coleman, 1969b; Simpson and Vallee, 1968). Since Co(II) is paramagnetic, an enzyme-bound phosphate would suffer line broadening and chemical-shift perturbations that would depend on the distance vector from the cobalt and the orientation of that vector relative to the electronic *g* tensor of Co(II) (La Mar et al., 1973). Thus, the observation of the E·P_i complex for the cobalt enzyme would provide specific information with regard to the distance of the phosphate from the cobalt. In fact, after considerable effort, we have been unable to detect E·P_i for the cobalt enzyme by ^{31}P NMR, and other workers have met with the same difficulty (Bock and Sheard, 1975).

Cobalt alkaline phosphatase (specific activity 370) was obtained from J. E. Coleman in 10 mM Tris-Cl, pH 8. The addition of 2 P_i/dimer gave a ^{31}P NMR spectrum containing only a single resonance at −2.30 ppm. Subsequent addition of additional P_i only resulted in an increase in the resonance at −2.30 ppm. Brief dialysis of this sample removed the free P_i and spectra taken over a 100-ppm range for 25000 transients failed to detect the E·P_i species. The addition of P_i to the dialyzed sample at pH 7.7 again produced only a resonance at −2.21 ppm (free P_i). The facts that the free P_i chemical shift was constant with changes in concentration, its line width was similar to that observed with the zinc enzyme, and the absence of an E·P_i resonance are consistent with two conclusions: P_i is in fast exchange with an enzyme site removed from the metal site, or P_i is in slow exchange with a tight binding site very close to the metal. In view of the published data on cobalt alkaline phosphatase, we favor the second conclusion: P_i binds very closely to or directly to the cobalt and the large paramagnetic effects make E·P_i undetectable.

Using literature data concerning the electronic properties of Co(II), the perturbation to the ^{31}P NMR of E·P_i can be estimated (La Mar et al., 1973; Sloan et al., 1975). From *g* tensor data averaged over angular factors, one finds that a line broadening of about 100 Hz will occur at a distance of 4 Å. This amount of line broadening would make the E·P_i difficult to detect. Furthermore, the paramagnetic effect on the chemical shift at 4 Å is estimated to be in the range +85 to −170 ppm, depending on angular factors. This large effect occurs because the shift is proportional to r^{-3} while the line width is proportional to r^{-6}. Thus, direct binding of P_i to the cobalt can produce line broadening and shifts that would make E·P_i undetectable under our conditions. For r = 6 Å, we estimate a line width of 10 Hz and a shift of +28 to −56 ppm. In this case E·P_i should have been detected. Thus, it appears that P_i is bound with the phosphorus less than 6 Å from the cobalt.

The binding of phosphate to cadmium alkaline phosphatase has also been observed (Bock and Sheard, 1975), and

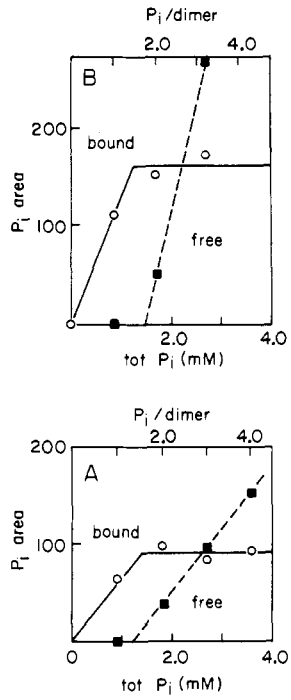

FIGURE 6: The titration of purged zinc and cadmium alkaline phosphatase with inorganic phosphate at pH 7. The protein (batch C) was purged and reconstituted by the standard method. Bound (O) and free (■) P_i areas are normalized to the reference (Experimental Procedure). Titration of the zinc enzyme at 0.89 mM in 0.1 M Tris-Cl, pH 7, specific activity = 2250 units, is shown in A. Titration of the cadmium enzyme at 0.81 mM in 0.05 M Tris-Cl, pH 7, is shown in B. The species E·P_i was a 2/1 mixture of two forms at −4.11 and −3.4 ppm, and P_i was at −1.85 ppm for the zinc enzyme. For the cadmium enzyme only a single bound peak for E–P was observed at −8.53 ppm.

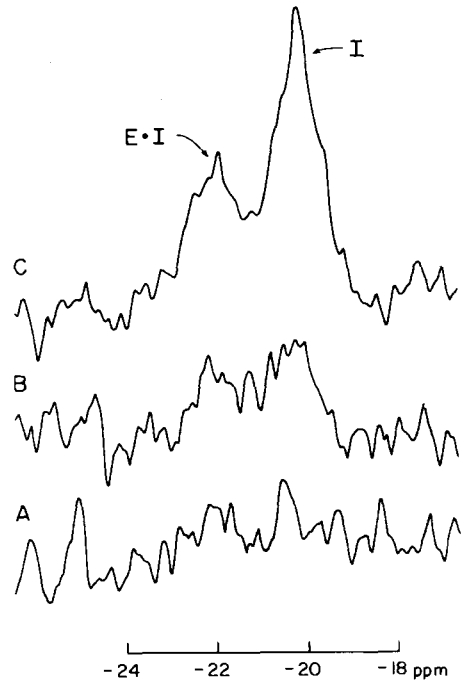

FIGURE 7: The titration of purged zinc alkaline phosphatase with 2-hydroxy-5-nitrobenzylphosphonate at pH 8. The enzyme (batch A) was purged by NTA dialysis and reconstituted by titration with zinc to a maximum activity of 2000 units at 4 Zn/dimer. The protein is 0.60 mM dimer in 0.1 M Tris-acetate, pH 8. Spectra A, B, and C are taken after the addition of 1, 2, and 4 mol of inhibitor per dimer. The chemical shifts of bound and free inhibitor are −22.0 and −20.15 ppm, respectively.

we have confirmed these results. At pH 7 a single bound phosphate resonance appears at −8.53 ppm corresponding to the E–P complex. This agrees with earlier observations that the cadmium enzyme is inactive and is readily phosphorylated even at neutral pH (Lazdunski et al., 1971; Applebury et al., 1970). Titration experiments of purged zinc and cadmium enzymes (batch C) at pH 7 are presented in Figure 6. The number and precision of the data points are insufficient for an accurate determination of the binding curve, especially in 6B. The lines drawn represent the behavior expected if phosphate binding is very tight. Figure 6A indicates that the stoichiometry of the zinc enzyme is only about 1.5 P_i/dimer. For the cadmium enzyme, the data are less complete, but 6B is consistent with a stoichiometry of between 1.0 and 2.0 P_i/dimer. It is not known why this batch of enzyme gave a 1.5 stoichiometry while the other batches exhibit 2.0 stoichiometry, but a 1.5 stoichiometry was also observed with the native enzyme C even before purging. Nonintegral stoichiometries of around 1.5 active sites/dimer have previously been reported for alkaline phosphatase preparations made in a different laboratory (Bloch and Schlesinger, 1973).

The Binding of 2-Hydroxy-5-nitrobenzylphosphonate to Zinc Alkaline Phosphatase. Using optical techniques this phosphatase inhibitor has been found to bind to the enzyme with a K_D of 3×10^{-5} M (Halford et al., 1969; Halford, 1972). While we did not observe any significant perturbation of the ^{19}F NMR spectrum of fluorotyrosine alkaline phosphatase in the presence of a large excess of inhibitor (Hull and Sykes, 1976), binding of the phosphonate to wild-type enzyme is confirmed by the results in Figure 7. In the absence of enzyme, the inhibitor displays a ^{31}P signal that is a triplet centered at −20.04 ppm with $^2J_{PH}$ = 19.0 Hz and a line width of 4.4 Hz (see Table I). Spectra A, B, and C in Figure 7 were obtained after adding 1, 2, and 4 mol of inhibitor per mole of purged zinc enzyme (batch A). The presence of a bound and free species can be seen at −22.0 and −20.15 ppm, respectively. The downfield shift upon binding is similar to that observed for P_i, but the unresolved character of the spectra indicates that exchange is faster than that for P_i. Using another enzyme preparation (batch C) inhibitor binding in the fast exchange limit was observed at pH 7. Only a single exchange-averaged resonance was detected with a chemical shift that varied from −20.91 ppm to −20.41 ppm for inhibitor concentrations of 1 to 3 mol/dimer, and the line width decreased in concert with the upfield shift toward the free position. The results indicate that the Michaelis complex of enzyme with phosphonate is weaker than with P_i but involves similar perturbations of the phosphorus chemical shift.

Chemical Exchange and the Kinetics of Phosphate Binding. For a chemical exchange discussion, the interaction of phosphate with enzyme may be written as in (1).

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{-}P + HOH \qquad (1)$$

At this point we are concerned only with the environment of the phosphate and, therefore, make no indications concerning the conformational state of the enzyme. Furthermore, no distinction can be made between the two binding sites per dimer so that E will represent either binding site. The three different states of phosphate shown in (1) can be simultaneously observed at pH 5, and the variation of pH does not produce significant changes in the shifts of E·P_i

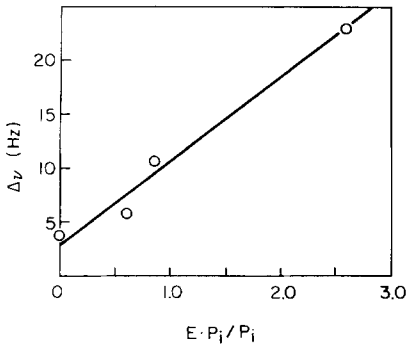

FIGURE 8: The ^{31}P line width of free P_i as a function of the enzyme: phosphate ratio. The observed line width of the free P_i resonance at −1.87 ppm is plotted vs. the $E{\cdot}P_i/P_i$ ratio as determined from the area measurements. The sample contains 0.90 mM zinc alkaline phosphatase (batch C) that was purged of P_i, specific activity = 2240 units. The sample was titrated with P_i up to a concentration of 3.6 mM. The data point at zero fraction bound was taken from a spectrum of P_i in enzyme-free buffer. The slope of the solid line is 7.7 s^{-1}.

and E-P. Therefore, we will assume that a slow exchange situation is obtained at all pH's, meaning that the lifetime of each species is sufficiently long compared with the chemical shift between species. Expressions for the line width of each species become

$$\pi\Delta\nu_{P_i} = \pi\Delta\nu_{P_i}^0 + k_1[E] = \pi\Delta\nu_{P_i}^0 + k_{-1}[E{\cdot}P_i]/[P_i] \quad (2)$$

$$\pi\Delta\nu_{E\cdot P_i} = \pi\Delta\nu_{E\cdot P_i}^0 + k_{-1} + k_2 \quad (3)$$

$$\pi\Delta\nu_{E\text{-}P} = \pi\Delta\nu_{E\text{-}P}^0 + k_{-2} \quad (4)$$

The rate constants k_{-1}, k_2, k_{-2} are all first order with the concentration of water taken into k_{-2}. The terms $\Delta\nu_i^0$ are the line widths that would be observed for each species i in the absence of exchange.

From eq 2 one finds that the line width of the free phosphate resonance should decrease as phosphate is added and a plot of $\Delta\nu_{P_i}$ vs. $[E{\cdot}P]/[P]$ should be linear with slope k_{-1}/π. This effect is illustrated in Figure 8, and the value of k_{-1} obtained at pH 7 is 24 s^{-1}. The line width of free phosphate may also contain contributions from fast exchange with nonspecific binding sites or trace paramagnetic impurities. In fact, the line widths of free P_i in different experiments were not very reproducible (compare Figures 1 and 3). The data for Figure 8 were from an experiment where the line widths were about as narrow as ever observed in these experiments.

From eq 3 an upper limit for k_{-1} may be obtained directly from the line width of the $E{\cdot}P_i$ resonances. From Table I the values of $\Delta\nu_{E\cdot P_i}$ are found to be 8–20 Hz which implies $k_{-1} + k_2 < 25$–60 s^{-1}, at pH 7–8. It is not possible to predict a value for $\Delta\nu_{E\cdot P_i}^0$ since relaxation of enzyme-bound phosphorus involves long-range dipolar relaxation and the possible contribution of chemical-shift anisotropy. Also from eq 4 and the line width of E-P, one finds that $k_{-2} < 60$ s^{-1} at pH 4–6. In the latter case $\Delta\nu_{E\text{-}P}^0$ is expected to be significant since the species is $-CH_2OPO_3$, which has a ^{31}P-^1H coupling constant and dipolar relaxation not occurring for $E{\cdot}P_i$ or P_i. Finally, from Figure 5 one finds that the line width of E-P is not sensitive to pH, while the line width of $E{\cdot}P_i$ increases from 19 Hz at pH 8 to about 30 Hz at pH 5.1.

Since the ^{31}P NMR results place certain constraints on the various rate constants for the interaction of P_i with alkaline phosphatase, it is worthwhile to compare these results with kinetic data that have appeared in the literature.

Table III: Transient Kinetic Rate Parameters for Alkaline Phosphatase at Low Ionic Strength, 20 °C.[a]

pH	k_f (s^{-1})	k_d (s^{-1})	Ref
4	50	0.06	*b*
5	21	0.3	*b*
5.5	21	0.6–1.0	*c*
5.7	25	0.8	*d*
6	10	2.0	*b*
7	5	9	*b*
7.3		16	*e*
8	8–10	40	*b*
8	15	100	*f*
8	19 (25 °C)[j]		*g*
8.4		115	*e*
5.4	0.046 (0 °C)[h]		*e*
5.5, 8.0	0.13 (25 °C)[i]		*g*

[a] The two rate parameters obtained from stopped-flow kinetics are k_f, the rate of forming E-P from phosphate ester substrates, and k_d, the rate of dephosphorylation or breakdown of E-P. These are generally referred to as k_2 and k_3, respectively, in the literature cited. All experiments were at ionic strength ≤0.1. [b] Fernley and Walker, 1966, 1969; μ = 0.02. [c] Bloch and Schlesinger, 1974; μ = 0.1. [d] Ko and Kézdy, 1967; μ = 0.05. [e] Aldridge et al., 1964. These experiments measured the break down of an E-^{32}P complex by pH jump. [f] Halford and Schlesinger, 1974; μ = 0.1. [g] Chlebowski and Coleman, 1974; μ = 0.1. [h] The rate of incorporation of ^{32}P inorganic phosphate into enzyme to form E-^{32}P was monitored. [i] The rate of ^{18}O exchange into P_i was monitored. The turnover rate in mol/(mols^{-1}) was calculated from the V_{max} value in the reference assuming two sites per dimer. [j] Calculated from V_{max} (μ = 0.1) assuming two sites per dimer.

The general approach in the analysis of stopped-flow transients observed for substrate hydrolysis has been to define two rate parameters which we will distinguish as k_f and k_d, the apparent first-order rate constants for the formation and decomposition of the E-P intermediate.[3] The steady-state turnover rate becomes $k_{cat} = k_f k_d/(k_f + k_d)$. The pertinent stopped flow results are presented in Table III. There is some difficulty in comparing these results because of ionic strength effects and the question of whether k_{cat} is based on moles/mole of enzyme or active sites and how active site concentration is determined. Two basic observations have been made: at low pH, k_d is rate determining and, at high pH, k_f is mostly rate determining.

The kinetic results from the literature may now be compared with the ^{31}P NMR data. According to eq 1, k_{-2} is equal to k_d, and the data in Table III predict an exchange line width for E-P of $\lesssim 1$ Hz over the pH range 4–6. This is in accord with our observation that $\Delta\nu_{E\text{-}P}$ is insensitive to pH and probably represents the true bound line width $\Delta\nu_{E\text{-}P}^0$ with only an insignificant contribution from k_{-2}. Furthermore, even at high pH, k_{-2} is still smaller than the frequency difference between the chemical shifts of $E{\cdot}P_i$ and E-P so that slow exchange conditions still apply. The rate constant k_2 represents the formation of E-P from $E{\cdot}P_i$, and this is estimated to be 0.1–0.2 s^{-1} from ^{18}O-exchange and ^{32}P-labeling experiments (see bottom of Table III). This rate has been found to be insensitive to pH (Schwartz et al., 1963; Chlebowski and Coleman, 1974), and is orders of magnitude slower than the rapid phosphorylation of enzyme by phosphate esters. It appears then that the equilibrium between $E{\cdot}P_i$ and E-P is governed by the pH dependence of k_{-2}, and one sees that, in the vicinity of pH 5, k_2

[3] In the literature of alkaline phosphatase kinetics, these rate constants are generally referred to as k_2 and k_3, respectively.

$\sim k_{-2}$, in agreement with our observation of equal amounts of E·P_i and E–P.

The value of k_f has previously been considered to represent a slow enzyme isomerization, rate limiting at alkaline pH, that occurs *subsequent* to rapid phosphate release (Bloch and Schlesinger, 1973). However, the ^{31}P NMR line-width data for E·P_i (and free P_i) require $k_{-1} \lesssim 25$ s^{-1} at pH 8. This leads directly to the conclusion that k_{-1} is the rate-limiting step k_f at alkaline pH. The exchange line width predicted for E·P_i from k_f in Table III is consistent with the NMR observations, and the increase in k_f at lower pH is in accord with the observed line broadening of E·P_i. Rate-limiting dissociation of P_i is also consistent with the observation that purged enzyme exhibits very rapid pre-steady-state kinetics (Bloch and Schlesinger, 1973, 1974; Chappelet-Tordo et al., 1974). Thus, the line width of E·P_i is a measure of the rate-limiting step at high pH, the breakdown of the stable E·P_i complex.

Discussion

Stoichiometry of Phosphate Binding. Using ^{31}P NMR techniques we have observed directly the noncovalent E·P_i complex which has generally been assumed to be the stable form of bound phosphate at alkaline pH. Titration experiments have shown that both the native and purged enzyme are capable of binding as many as 2 P_i/dimer, although in some cases stoichiometries of 1.0 and 1.5 were observed. Previous workers have also found that the native enzyme as isolated from *E. coli* may contain as much as 2.0 mol of endogenous phosphate per mole of dimer. In our studies with both the fluorotyrosine and wild-type phosphatases, the presence of endogenous phosphate has been confirmed, and the quantity was found to vary from one enzyme preparation to another. ^{19}F NMR studies of fluorotyrosine alkaline phosphatase have demonstrated that titration of the purged enzyme with P_i leads to a NMR spectral change (conformational change) that exhibits the same 2 P_i/dimer stoichiometry observed in the ^{31}P NMR studies reported here. The occurrence of a conformational change that is stabilized by phosphate is supported by the observation that tetrameric enzyme (formed at pH >8 when excess Zn is present) dissociates to dimers when phosphate or substrate is added (Halford et al., 1972).

At acid pH we have directly observed the quantitative conversion of E·P_i to E–P, the covalent phosphoryl intermediate. This process exhibits a pH dependence that appears to deviate from a normal sigmoid titration curve. At pH 7, no E–P is detected; at pH 5, E–P and E·P_i are equimolar; and at pH 4.4, no E·P_i is detected. The pH dependence observed by ^{31}P NMR is similar to that found for the incorporation of $^{32}P_i$ into enzyme (Reid et al., 1969; Lazdunski et al., 1969b). ^{31}P NMR studies revealed that, at pH 7, P_i bound to the cadmium enzyme only in the E–P form but with the same stoichiometry as that observed for E·P_i with the zinc enzyme. The chemical shift and line width of E–P were the same in both cadmium enzyme at pH 7 and zinc enzyme at pH 4–5. This is consistent with the expectation that there will be no significant chemical exchange line width for E–P in the zinc enzyme ($k_{-2} \approx 0.05$–2.0 s^{-1} over this pH range) and certainly not for the cadmium enzyme which is inactive and unable to catalyze ^{18}O exchange into P_i (Applebury et al., 1970).

Considerable evidence exists in the literature to indicate that the two phosphate binding sites are not equivalent; i.e., that one binds more strongly than the other (Applebury et al., 1970; Simpson and Vallee, 1970; Lazdunski et al., 1969b, 1971). The first phosphate was found to bind with a K_D of about 1 μM, while the second exhibited anticooperative binding with a K_D greater than 10^{-5} M. The negative cooperativity has been found to be very sensitive to ionic strength and was smallest at low ionic strength. Our experiments were performed at high enzyme concentrations and low ionic strength, which in part explains our inability to observe any anticooperative behavior. From the P_i titration behavior of the fluorotyrosine enzyme, an upper limit for K_D for either phosphate is 10^{-5} M. In the ^{31}P NMR experiments the protein concentration is higher so that the upper limit for K_D is correspondingly higher ($\sim 5 \times 10^{-5}$ M). More importantly, the ^{31}P results demonstrate that the *dissociation* rates of the two bound phosphates are indistinguishable (see Figures 3A and 3B) with $k_{-1} \lesssim 25$ s^{-1} at pH 7–8.

As was described in the Results section, the chemical shift of the E·P_i form exhibited two distinct values which appeared to correlate with enzyme activity. The purging procedure generally converted the enzyme from its high activity form (3200) to a lower activity form (2000) with a change in the E·P_i chemical shift. Enzyme samples with intermediate activity show ^{31}P resonances for both forms of E·P_i. The activity of enzyme once purged was quite stable thereafter, rarely falling below 2000 and often increasing after other treatments. A survey of the literature indicates that the majority of preparations used by other groups displayed activity also in the range 2000–3300. The fluorotyrosine enzyme also lost activity to a minimum of 2000, but this was over a much longer period of time. The fluorotyrosine enzyme showed no detectable differences in its ^{19}F NMR spectrum that could be correlated with activity and certainly no denatured protein. Both wild-type and fluorotyrosine enzyme exhibited the same phosphate stoichiometry, regardless of activity. Thus, results presented here suggest the enzyme is capable of existing in two forms with different activity and different E·P_i chemical shifts. It has not been determined as yet whether hydrolase or transferase activity is being affected.

^{19}F NMR work with the fluorotyrosine enzyme (Hull and Sykes, 1976) indicated that titration of purged enzyme with P_i or *p*-nitrophenyl phosphate produced the same spectral change requiring 2 mol/mol of dimer for completion. This behavior was observed both in the presence and absence of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate. ^{31}P NMR studies of P_i binding gave similar stoichiometry; however, one experiment involving titration with substrate indicated the tight binding of only 1 P_i/dimer (Figure 4B). The bulk of the data indicates a maximum binding stoichiometry of 2 P_i/dimer, and we consider the deviations from this value in particular experiments to be due to the limitations of reproducibility of the enzyme system and the accuracy of the spectroscopic methods.

The Catalytic Mechanism. The ^{31}P NMR results presented here and the ^{19}F NMR data discussed in the accompanying manuscript (Hull and Sykes, 1976) lead to two fundamental conclusions concerning the binding of phosphate to alkaline phosphatase. The lifetime observed for the E·P_i noncovalent complex at alkaline pH is sufficiently long that the dissociation of this complex must be (or at least contribute to) the rate-limiting step in the catalytic mechanism at alkaline pH. The other basic observation is that the purged enzyme undergoes a distinct conformational change when inorganic phosphate binds at alkaline pH or when

there is substrate turnover. The conformational change observed by ^{19}F NMR, however, is *not* induced by the binding of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate.

In addition, the covalent phosphoryl enzyme intermediate has been directly observed by ^{31}P NMR to be in equilibrium with the E·P_i complex. Our studies with the fluorotyrosine enzyme and previous work with Co(II) alkaline phosphatase (Taylor et al., 1973) indicate that binding of phosphate to purged enzyme at low pH (to form the phosphoryl intermediate) does not produce the conformational change observed at high pH. Furthermore, while substrates $ROPO_3^{2-}$ rapidly ($>10^3$ s^{-1}) phosphorylate the purged enzyme with release of ROH, inorganic phosphate is very inefficient in this reaction and is also slow to incorporate ^{18}O from labeled water.

Consideration of all of these properties of alkaline phosphatase and its interaction with phosphate leads directly to the following mechanism for catalytic action. The exact phosphate binding or active site stoichiometry of the enzyme is still uncertain. The majority of our experiments indicate a maximum of two, apparently indistinguishable, sites. Therefore, the following mechanistic discussion will refer to a single catalytic site.

$$E' + RCH_2P \overset{K_D^I}{\rightleftharpoons} E'\cdot RCH_2P \qquad (5)$$

$$E' + ROP \overset{K_D^S}{\rightleftharpoons} E'\cdot ROP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'\text{—}P + ROH \qquad (6)$$

$$E' + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'\text{—}P + HOH \qquad (7)$$

Equation 5 represents the nonproductive bimolecular interaction of enzyme with phosphonates. The phosphate-free enzyme exists in the conformation E′ and this state is not altered by the binding of phosphonates. Equations 6 and 7 represent the two halves of the catalytic cycle and are connected by the common intermediate E′-P. Phosphorylation of the enzyme by substrate and transphosphorylation to an alternative alcohol take place through (6) in the forward and reverse directions, respectively. The initial complexation of substrate is assumed to involve the same association step observed with phosphonates, and hence the intermediate E′·ROP is indicated (no conformational change). The complexation steps in (5) and (6) are expected to have similar dissociation constants. Exact values for K_D^I and K_D^S will depend to some extent on the individual properties of RCH_2P and ROP. The phosphorylation step k_3 is rapid at all pH's ($>10^3$ s^{-1}, 20 °C (Chappelet-Tordo et al., 1974)), and the rate of transphosphorylation is governed by k_3 together with the concentration of the alcohol [ROH]. The release of *inorganic* phosphate occurs through the reverse direction of (7). In eq 7 we have introduced the conformational change induced or stabilized by P_i binding. The E conformation is produced uniquely by phosphate binding. All other states of the enzyme are written as E′. This is not to indicate that there are no changes whatsoever in forming E′-P from E′ since phosphorylation must involve local changes in the active site region. The state E·P_i represents a more general change in protein structure as visualized by ^{19}F NMR studies of the fluorotyrosine enzyme. The ability of substrate (via turnover) to induce the same conformational change observed with P_i is a direct consequence of the linking of (6) and (7) through the common state E′-P.

In the Results section, eq 7 has already been analyzed in terms of the influence of the rate parameters on the ^{31}P line widths. The mechanistic step commonly referred to as "dephosphorylation" is k_{-2} (the concentration of HOH is included to give a first-order rate constant), and the pH dependence of this step is given by k_d in Table III. It is most important to bear in mind that dephosphorylation should not imply dissociation; these are separate steps in the mechanism. At pH $\lesssim$6, E′-P can be observed and $k_{-2} \lesssim 1$ s^{-1} is the rate-limiting step in catalysis. At high pH dephosphorylation is rapid ($k_{-2} \sim 100$ s^{-1}) and the rate-limiting step becomes the dissociation of phosphate coupled with the conformational change. The first-order rate constant for this process is directly observed by ^{31}P NMR to be $k_{-1} \lesssim 20$ s^{-1} in 0.1 M Tris, pH 7–8, 26 °C. From Table III it can be seen that the kinetic constant k_f is consistent with our estimates of k_{-1}.

In the mechanism outlined above, all species except E′·ROP have been observed directly under equilibrium conditions by NMR. The existence of E′·ROP is simply a logical consequence of the observed binding of and competitive inhibition by phosphonates. This mechanism differs fundamentally from those presented earlier (Halford, 1971, 1972; Halford et al., 1972; Bloch and Schlesinger, 1973, 1974) in that the earlier mechanisms *assumed* the dissociation of phosphate to be rapid and that the slow step was a substrate-independent isomerization of the free enzyme. The availability of ^{31}P NMR data allows us to rule out these earlier mechanisms and state that the dissociation of phosphate from a conformationally altered enzyme is, in fact, the rate-limiting step at alkaline pH. Consequently, it is the combined steps of dephosphorylation k_{-2} *plus* dissociation k_{-1} that provide rate-limiting control of substrate turnover at *all* pH's, conveniently explaining the substrate independence of V_{max} at all pH's.

The kinetic constants (at low ionic strength) may be summarized as follows. Judging from observed values of K_m and K_i for substrates and phosphonate inhibitors, K_D^I and K_D^S are probably in the range 10^{-5} to 10^{-4} M. The phosphorylation step k_3 is $>10^3$ s^{-1}. The dephosphorylation rate k_{-2} varies from 0.06 s^{-1} at pH 4 to 100 s^{-1} at pH 8. The phosphorylation rate k_2 is about 0.2 s^{-1} and insensitive to pH. The dissociation rate k_{-1} is 10–20 s^{-1} at pH 8 and increases slightly to about 50 s^{-1} at pH 4. The observed K_D for P_i binding is $\lesssim 10^{-6}$ M consistent with $k_1 \gtrsim 10^7$ M^{-1} s^{-1}.

The stopped-flow kinetic behavior predicted by the scheme described above is as follows (Fernley and Walker, 1969; Bloch and Schlesinger, 1973). When enzyme is purged of phosphate, it exists in the E′ state which is the conformation favored for catalysis. The addition of substrate results in a very rapid bimolecular association step followed by a rapid phosphorylation step k_3 to give E′-P and an instant burst of ROH. At acid pH, the steady-state phase follows with k_{-2} rate limiting. At alkaline pH, k_{-2} is fast and the formation of E′ from the E·P_i complex via k_{-1} becomes the steady-state rate-limiting step. When enzyme is saturated with phosphate, the addition of substrate cannot produce an instant burst. At alkaline pH, the steady state begins from time zero with k_{-1}. However, at acid pH the steady state is the breakdown of E′-P; hence, there is an initial transient burst of ROH which takes place at the first-order rate k_{-1}, i.e., the same mechanistic step involved in the steady state at alkaline pH.

Previous stopped-flow work (Halford, 1971) demonstrated that, when enzyme is in excess over substrate, the complete hydrolysis of substrate at pH 5.6 was a first-order

reaction. The rate constant *increased* from the constant value of the transient phase rate constant, observed when substrate was in excess, to much higher values as initial substrate was decreased below the enzyme level. This behavior required that an enzyme conformational change from an "inactive" to an "active" form *preceded* the binding of substrate. In the mechanism described above, this is the step involved with phosphate dissociation. Thus, the analysis described by Halford applies to our mechanism with E·P_i as the "inactive" enzyme form.

Stopped-flow studies have also shown that, as the free concentration of P_i is increased, the first-order rate constant for the transient phase of product release at low pH decreases. For example, at pH 5.5 1 mM P_i decreases the transient phase rate from 14 to 3.6 s^{-1} (Halford, 1971). This behavior is also predicted by the proposed mechanism. In the presence of phosphate, the transient phase at pH 5.5 for the production of alcohol product (observed by stopped-flow) can be written as follows:

$$E{\cdot}P_i \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E' + P_i \qquad E' + ROP \xrightarrow{k_p} E'{-}P + ROH \tag{8}$$

The first-order rate constant k_p contains a collection of constants and the concentration of substrate ROP. For the transient phase at acid pH, the breakdown of E′-P (k_{-2}) will be neglected. The reaction is initiated with enzyme mostly as E·P, and the transient phase ends with the enzyme mostly in the E′-P form. Thus, the transient phase release of ROH in the presence of phosphate can be shown to obey the first-order equation (9).

$$[ROH] = E_0[1 - e^{-bt}], \tag{9}$$

where

$$b = k_{-1}\left(\frac{k_p}{k_{-1} + k_p + k_1[P_i]}\right) \tag{10}$$

Since $k_p \gg k_{-1}$, this simplifies to

$$b = k_{-1}\left(\frac{1}{1 + k_1[P_i]/k_p}\right) \tag{11}$$

and as $k_1[P_i]$ becomes greater than k_p, there will be a decrease in b from its limiting value k_{-1}. The data of Halford indicate that, at 1 mM P_i, $k_1[P_i]/k_p \approx 3$ or $k_p/k_1 \approx 3 \times 10^{-4}$ M. A k_1 of 10^8 M^{-1} s^{-1} would give $k_p \sim 3 \times 10^4$ s^{-1}, which is reasonable since the steps involved in k_p have never been resolved by stopped-flow techniques.

The proposed mechanism also predicts the transient phosphorylation results of Wilson (Reid and Wilson, 1971). When ^{32}P-labeled substrates are used in a quenched-flow experiment, the production of the intermediate E′-P can be analyzed according to the following scheme:

$$E' + ROP \xrightarrow{k_p} E'{-}P \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E{\cdot}P_i \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E' + P_i \tag{12}$$

By applying a steady-state approximation to [E′], the concentration of E′-P is given by

$$aE_0 - b[E'{-}P] = Ce^{-bt} \tag{13}$$

where E_0 is the active site concentration, C is a parameter determined by the initial conditions, and

$$a = k_2 + (k_p k_{-1})/(k_p + k_1[P_i])$$
$$b = a + k_{-2} \tag{14}$$

When phosphate-free enzyme is used, then k_p produces a very rapid burst of E′-P, so we may assume that at $t = 0$, [E′-P] = E_0. Introducing this initial condition into (13) gives

$$[E'{-}P]/E_0 = (a/b) + [1 - (a/b)]e^{-bt} \tag{15}$$

Thus, the burst of E′-P decays with a rate constant b to the steady-state fraction given by a/b. When P_i is in low concentration so that $k_1[P_i] < k_p$, the constants for the reaction at pH 8 are

$$a = k_2 + k_{-1} \approx 10\text{–}20\ s^{-1}$$
$$b = a + k_{-2} \approx 110\text{–}120\ s^{-1} \tag{16}$$

This predicts that decay will be complete in 30–40 ms to a level of 10–20% E′-P, in excellent agreement with the results of Wilson et al. (1964; 10% E′-P after 40–50 ms). Since k_p is probably about 10^4 s^{-1} (see above), the limit $k_1[P_i] < k_p$ should be valid for $[P_i] < 10^{-4}$ M. Hence, P_i in the reaction mixture at 10^{-5} M should not influence the behavior, and, indeed, no effect was observed by Wilson. When enzyme is preincubated with P_i at pH 8 before mixing with ^{32}P substrate, so that at $t = 0$, [E′-P] = 0, then the solution to (13) becomes

$$[E'{-}P]/E_0 = (a/b)[1 - e^{-bt}] \tag{17}$$

in agreement with the observed effect of a rise in the E′-P level from zero to 10% at the rate b.

The transphosphorylation reaction has also been studied in detail (Wilson et al., 1964; Barrett et al., 1969). Acceptors such as Tris and ethanolamine were found to enhance the turnover of substrate without affecting the appearance of P_i. This is easily described by the proposed mechanism. Since an acceptor can react with E′-P through (6), it can enhance the production of E′, necessary for substrate turnover, without affecting the release of P_i through (7). This process is independent of substrate so that the ratio of transphosphorylation to P_i production remains constant for all substrates and is proportional to acceptor concentration, just as observed by Wilson.

Studies with tetrameric zinc alkaline phosphatase (Halford et al., 1972) have shown that the tetramer exhibits the transient kinetic behavior associated with the E′ conformation. However, after a single turnover or after incubation with P_i, the tetramer dissociated to dimer. On the other hand, tetramer was stable in the presence of the inhibitor 2-hydroxy-5-nitrobenzylphosphonate. If one assumes that the tetramer cannot be formed when the protein is in the E conformation, then the proposed mechanism conveniently explains all of these results. Tetramer is stable to phosphonate specifically because this inhibitor does *not* induce the conformational change observed to be the key step in the catalytic cycle and instead binds solely to the E′ conformation.

Finally, this mechanism is consistent with the very slow rate constants measured for phosphorylation of enzyme by P_i and ^{18}O exchange from water into P_i. Phosphorylation by P_i is expected to be slower than phosphorylation by substrates based on the high pK_A of the leaving group $^-$OH. Furthermore, the geometry of the E·P_i complex may be very unfavorable for this step. Thus, the rate constant k_2 is quite slow ($\approx$0.2 s^{-1}, see Table III), which is consistent with the ^{31}P NMR line widths observed for E·P_i as well as the observed pH dependence of the E·P_i ↔ E′-P equilibrium.

Regulatory Function of Phosphate Binding. One of the

striking properties of alkaline phosphatase is its lack of R-group specificity so that substrates with a wide variety of steric properties and whose leaving groups RO^- have very different pK_A's are all hydrolyzed at very similar rates. This property is a direct result of the basic mechanism (5)-(7), as outlined in the preceding section, where enzyme-bound phosphate proves to be the species which controls enzyme turnover at all pH's. However, alkaline phosphatase does have one particular property which is in essence an R-group specificity. When R = H, the substrate, inorganic phosphate, interacts with the protein in a unique manner by the formation of a very stable noncovalent complex with a protein conformational change. Thus the conformation of the protein differs between the noncovalent intermediates of (6) and (7), E′·ROP and E·P_i, respectively. Equations 6 and 7 contain all the intermediates that are directly observable and provide a complete description of the kinetic properties of alkaline phosphatase. However, for the purposes of gaining better physical insight into the nature of phosphate binding, we propose the following extension of (7):

$$\begin{array}{ccccc} E' + P_i & \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} & E'\cdot P_i & \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} & E'\text{—}P + HOH \\ & & k_i \downarrow \uparrow k_{-i} & & \\ & & E\cdot P_i & & \end{array} \qquad (18)$$

This mechanism is drawn by analogy from (6) under the assumption that alkaline phosphatase cannot distinguish $ROPO_3^{2-}$ from $HOPO_3^{2-}$ until after the initial bimolecular complex is formed. Furthermore, phosphate as $HOPO_3^{2-}$ should be able to form E′–P through the same intermediate complex that is proposed for $ROPO_3^{2-}$. Thus, we have introduced the intermediate E′·P_i, and (18) is now identical with (6), except that an additional isomerization step to the preferred conformation E·P_i can occur when R = H. This isomerization is unique for inorganic phosphate as substrate and, in essence, provides a regulating mechanism by trapping enzyme in an inactive state.

The ^{31}P NMR data have been analyzed in terms of the effective rate constants of eq 7. The question arises as to how these complex rate constants (without primes) may be expressed in terms of the individual steps (with primes) in (18). Since the lifetimes of phosphate in the three observable states P_i, E·P_i, and E′–P are readily expressed in terms of the kinetic parameters of (7) by eq 2-4, it is now of interest to consider what those lifetimes are in terms of the parameters of (18). Unfortunately, a complete solution requires knowledge of the properties of the new intermediate E′·P_i, particularly the magnitude of various rate processes relative to its chemical-shift difference from the other species. There are two simple limits to consider. First, assume that the ^{31}P chemical-shift difference between E′·P_i and E·P_i is small relative to the isomerization rates $k_i + k_{-i}$ so that the bound species is the exchange-average of the two forms, weighted toward E·P_i. Now, if the effective dissociation rate k_{-1} is slow compared with the difference in shift between P_i and E·P_i (= 400 s^{-1}), then slow exchange behavior between the two observable species P_i and E·P_i will be observed. The lifetime of the bound P_i is given by

$$1/\tau_{bound} = \frac{(k_{-1}' + k_2')[E'\cdot P_i]}{[E'\cdot P_i + E\cdot P_i]} = \frac{k_{-1}' + k_2'}{1 + K_i} \qquad (19)$$

where $K_i = k_i/k_{-i}$, the equilibrium constant between E′·P_i and E·P_i which will be >1, since E·P is the stable form. This leads to the relationships between prime and nonprime constants

$$k_{-1} = k_{-1}'/(1 + K_i) \qquad k_{-2} = k_{-2}'$$
$$k_1 = k_1' \qquad k_2 = k_2'/(1 + K_i) \qquad (20)$$

and the apparent K_D for noncovalent P_i binding becomes

$$K_D^{app} = K_D'/(1 + K_i),\ K_D' = k_{-1}'/k_1' \qquad (21)$$

Therefore, in this situation, the ^{31}P data would be consistent with the result:

$$k_{-1} = k_{-1}'/(1 + K_i) \simeq 20\ s^{-1} \qquad (22)$$

and enzyme turnover is limited by an effective dissociation rate k_{-1}, which is a product of the true dissociation rate k_{-1}' and the fraction of bound phosphate in the E′·P_i form.

Another possibility is that E·P_i and E′·P_i have different chemical shifts, not averaged by a slow isomerization process. The observed bound resonance corresponds to E·P_i whose lifetime is

$$1/\tau_{E\cdot P} = k_{-i} \simeq 20\ s^{-1} \qquad (23)$$

In this case the isomerization step k_{-i} is the slow step being monitored by ^{31}P NMR. Considering just the noncovalent complex, the apparent K_D and effective rate constants are defined as

$$K_D^{app} = [E'][P_i]/[E\cdot P_i] = K_D'/K_i$$
$$k_{-1} = k_{-i};\ k_1 = k_i/K_D' \qquad (24)$$

The complexity of multi-site exchange problems makes it difficult at this time to unambiguously choose between (19) and (23). However, there is some evidence which favors (19). First, the inhibitor 2-hydroxy-5-nitrobenzylphosphonate also has a ^{31}P-bound chemical shift that is downfield of the free species, similar to the situation with P_i. Hence, the chemical shift of E′·P_i is probably closer to that of E·P_i than to that of free P_i. Furthermore, the observation of stoichiometric burst kinetics with alkaline phosphatase requires that $k_{-1}' \ll k_i$ in scheme 18. This is because dephosphorylation of E′–P formed in a rapid burst must take place through E·P_i rather than directly through E′·P_i. The inequality $k_{-1}' \ll k_i$ leads to a condition upon k_1': namely that $k_1' \ll k_i/K_D^{app}$. If k_i was rate determining at 20 s^{-1} and with a value of K_D^{app} at 10^{-6} M, this consideration results in $k_1' \ll 2 \times 10^7\ M^{-1}\ s^{-1}$. Hence if (23) was valid, k_1' must be much smaller than a diffusion-controlled rate.

The chemical exchange situation described by (19)-(22) is the more likely one, and using the conditions k_{-1}'/K_i = 20, k_{-1}'/k_i = 0.1 (assumed), and $K_D^{app} = 10^{-6}$-10^{-7} M, a possible set of rate parameters is the following:

$$k_{-1}' = 10^3\ s^{-1} \quad k_{-i} = 200\ s^{-1} \quad k_{-2}' = 1\text{-}10^2\ s^{-1}$$
$$k_1' = 10^8\ M^{-1}\ s^{-1} \quad k_i = 10^4\ s^{-1} \quad k_2' = 5\text{-}10\ s^{-1}$$
$$K_D' = 10^{-5}\ M \quad K_i = 50$$
$$K_D^{app} = 2 \times 10^{-7}\ M$$

While the details of the individual steps of (18) remain undecided at this time, one feature remains clear: the breakdown of the noncovalent phosphate complex is the rate-limiting *process* at alkaline pH. It is not possible to specify what *step* in this breakdown is rate limiting, if any. As outlined above, one possibility is that enzyme turnover is controlled by dissociation from E′·P_i which is in reasonably rapid equilibrium with the favored E·P_i complex.

There are several attractive features of (18). First, it allows the enzyme to interact with P_i in the same manner as with all substrates, both in complex formation and phosphorylation–dephosphorylation. It allows the enzyme to recognize inorganic phosphate as a unique species (R = H) by means of a specific isomerization step. Finally, it gives the required rate-limiting behavior at high pH without putting severe constraints on the rate of any one individual step. Thus, phosphate is allowed to dissociate with a "normal" rate constant from a "normal" productive complex which is in equilibrium with a unique nonproductive E·P_i complex, whose stability controls the catalytic action of alkaline phosphatase.

The Nature of Phosphate Binding. Considerable literature data now exist which lead to some suggestions concerning the detailed nature of phosphate binding. It has been reported that reaction of alkaline phosphatase with 2,3-butanedione or phenylglyoxal results in the modification of about 15 arginine residues and nearly complete loss of activity (Daemen and Riordan, 1974). However, in the presence of excess P_i or phenylphosphonate, enzyme activity is unaffected by the reaction and two arginines per dimer are protected from modification. Furthermore, a completely modified protein shows reduced affinity for phosphate. These results implicate the role of 1 essential arginine per site in the binding of phosphate and phosphonates to alkaline phosphatase. A similar role for arginine has been found for several other enzymes, and crystallographic work has been reported for model guanidinium–phosphate complexes (Cotton et al., 1974).

Considerable data also exist to imply a direct interaction of phosphate with the metal in alkaline phosphate. Phosphate is known to inhibit the loss of zinc from alkaline phosphatase in the presence of chelators such as EDTA (Simpson and Vallee, 1968; Lazdunski et al., 1969a). Zinc is also lost from the enzyme at acid pH, and the dissociation vs. pH curve has a pK of about 5 (Applebury and Coleman, 1969a). The stability of the enzyme at acid pH is generally enhanced by the presence of substrates or inhibitors. ESR studies of the copper enzyme show that there are three nitrogen ligands at high pH but only two at low pH (Csopak and Falk, 1974; Taylor and Coleman, 1972), and the pK for the dissociation of this ligand (probably histidine) was found to be 7.6. Copper is bound more weakly than zinc to alkaline phosphatase and is lost by dialysis. The nature, stability, and pH dependence of phosphate binding are related to the particular metal ion incorporated in the enzyme (Applebury et al., 1970). Moreover, phosphate causes pH-dependent perturbations of the Co(II) enzyme visible spectrum and the Cu(II) enzyme ESR spectrum (Applebury and Coleman, 1969b; Csopak and Falk, 1974). Finally, ^{31}P NMR studies with the cobalt enzyme suggest that the phosphorus is less than 6 Å from the metal. The substitution of different metals into alkaline phosphatase probably does not change the basic nature of phosphate binding but does modify the individual rate constants of (7) or (18). For example, the E′–P complexes in the zinc and cadmium enzymes are indistinguishable by ^{31}P NMR but their stabilities and rates of formation differ by orders of magnitude. Recent work involving a comparison of the hydrolysis of phosphorothioate $ROPSO_2^{2-}$ and normal phosphate esters using both zinc and cobalt enzymes (Chlebowski and Coleman, 1974) argues for subtle influences of the metal ion on individual rate parameters without any basic change in the catalytic mechanism.

Considering the properties described above, a possible outline of the individual steps in (6), (7), and (18) is as follows. Inorganic phosphate, phosphate esters, or phosphonate inhibitors can bind in a reversible bimolecular step to an arginine residue in the active site. Further interaction with one coordination site on zinc, perhaps by displacement of a water molecule, is suggested by the increased resistance to chelators at neutral pH in the presence of phosphate. Phosphonate inhibitors cannot undergo any further steps and thus their binding is weak (eq 6). Inorganic phosphate (eq 18) possesses unique properties (the absence of a bulky R group) which allow it to induce or stabilize an enzyme conformational change (k_i) resulting in extraordinarily tight binding. Perhaps this change involves some rearrangement of the active site geometry which allows coordination of phosphate at its fourth oxygen by another residue. Substrate (eq 7), on the other hand, proceeds directly to form a phosphoryl enzyme intermediate by nucleophilic attack of a serine residue (k_3). This attack may be enhanced by additional coordination of the phosphate group by zinc with the displacement of one of the zinc's histidine ligands. The zinc's charge characteristics and the possibility of bond angle distortions could produce the large downfield ^{31}P chemical shift observed for the final phosphoryl intermediate. Active participation of the zinc could also facilitate the formation of a pentacoordinate phosphorus intermediate prior to the release of ROH. The loss of a histidine ligand from the metal ion at low pH in the absence of substrate is inferred from the ESR results with the Cu(II) enzyme and the dissociation of zinc from the native enzyme. The displacement of a zinc ligand in the phosphorylation process is also supported by the following argument.

The equilibrium between the phosphoryl and noncovalent complex may be expressed as

$$B_1H + L\cdot E\cdot O_3POH + SerOH + B_2^- \leftrightarrow B_1^- + HOH + E\cdot O_3POSer + B_2H + L \quad (25)$$

B_1H and B_2H are general acids with dissociation constants K_{A1} and K_{A2}, and L is a zinc ligand displaced by phosphate. The species L·E·O_3POH and E·O_3POSer represent E·P_i and E–P, respectively, where the exact nature of binding has not been specified. The equilibrium constant is

$$K_{eq} = \frac{K_{A1}[E'\text{–}P][L]}{K_{A2}[E\cdot P_i]} \quad (26)$$

If ligand L was not displaced, the ratio $[E'\text{–}P]/[E\cdot P_i]$ would be pH independent. But if the ligand was displaced and subject to a protonation equilibrium, then as [E–P] = [L + LH]

$$[L] = \frac{[E'\text{–}P]K_L}{K_L + [H^+]} \quad (27)$$

where K_L is the acid dissociation constant for the ligand LH. Substitution into (26) now yields

$$\frac{[E'\text{–}P]^2}{[E\cdot P_i]} = \frac{K_{eq}K_{A2}(K_L + H^+)}{K_LK_{A1}} \quad (28)$$

and the phosphoryl complex increases with increasing $[H^+]$ (lower pH). Formation of phosphoryl enzyme at low pH would serve to stabilize the zinc since the serine phosphate can take the place of a zinc ligand. The displaced histidine could even serve as the general base assisting the serine attack. The fact that the pK_A for histidine loss from Cu(II) is considerably higher than that inferred for zinc (7.6 vs. 5) may explain the poor stability of the Cu(II) enzyme.

It is interesting that the ^{31}P chemical shifts of the two forms E·P and E′-P show no significant pH dependence, implying that the two phosphate environments themselves are not altered by pH changes, but only their relative populations are affected. The pH-activity profile for alkaline phosphatase below pH 7 is similar to the pH dependence of the phosphorylation equilibrium (Lazdunski and Lazdunski, 1969; Lazdunski et al., 1969b; Fernley and Walker, 1966, 1969). This is in accord with the importance of k_{-2} (eq 7) in determining the position of the $E{\cdot}P_i \leftrightarrow E'{-}P$ equilibrium as well as being the rate-limiting step (k_d in Table III) in catalysis at low pH. At the same time, phosphorylation (k_2) is insensitive to pH.

The breakdown of E′-P requires the attack of water (k_{-2}) or another acceptor (k_{-3}), such as ethanolamine, probably assisted by a general acid-base residue. This is a pH-dependent process both because of the acid-base catalysis involved and the displacement of phosphate from zinc by the previously displaced histidine. At low pH this is the rate-limiting process. The presence of phosphate acceptors leads to an accelerated breakdown of E′-P (and hence catalysis) especially at low pH (Levine et al., 1969; Trentham and Gutfreund, 1968). However, attack on E′-P by water produces inorganic phosphate which induces a rearrangement to a tight complex. Dissociation from this complex then becomes the rate-limiting process at high pH.

Summary

In this report we have discussed a ^{31}P NMR study of phosphate binding to wild-type alkaline phosphatase and presented a basic catalytic mechanism 5-7 which includes all of those intermediates directly observable by NMR. ^{19}F NMR studies of fluorotyrosine alkaline phosphatase (Hull and Sykes, 1976) have demonstrated that a general conformational change takes place upon phosphate binding, with a stoichiometry of 2 P_i/dimer. From the ^{31}P NMR results, we have found that as many as two indistinguishable phosphates can bind to the enzyme, each with an effective dissociation rate of $k_{-1} \lesssim 20\ s^{-1}$. A phosphonate inhibitor 2-hydroxy-5-nitrobenzylphosphonate binds less tightly and does not induce a detectable conformation change. The fundamental conclusion from this work is that the lifetime of noncovalently bound phosphate is sufficiently long for its *dissociation* to be the rate-limiting process at alkaline pH. The conformational change produces a nonproductive stable complex E·P_i. Dissociation or ^{18}O exchange may take place through a common productive intermediate E′·P_i, similar to that involved in hydrolysis of substrates. This species would be present as only a transient intermediate in equilibrium with the favored species E·P_i. The magnitude of the equilibrium constant for isomerization K_i then governs the rate of phosphate release from the enzyme as well as the rate of ^{18}O exchange (see (20)). Thus, the release of phosphate from the enzyme controls the turnover rate at *all* pH's. At low pH the E′-P complex is very stable and its breakdown becomes rate limiting. At pH 8 the formation of E·P_i from E′-P is faster than turnover so that *dissociation* becomes the rate-limiting process. In general, it may not be possible to specify a particular individual mechanistic step as rate limiting, but at all pH's it appears that the overall release of phosphate via dephosphorylation and dissociation with a conformational change provides a simple, logical control mechanism for the hydrolysis of phosphate esters by alkaline phosphatase.

Our work has provided no unambiguous evidence for interaction between subunits or anticooperative behavior in the binding of phosphate, but such effects could have been masked by the high concentrations of enzyme used. If anticooperativity in phosphate binding does exist, it must involve the *association* rate constant since the dissociation rates as witnessed by ^{31}P NMR are indistinguishable. The binding of phosphate at high pH does not involve significant phosphorylation, while substrates must necessarily phosphorylate the enzyme. Since phosphate induces a conformational change that is important in regulating enzyme turnover, it is possible that this property of phosphate binding may produce site-site interactions (Lazdunski et al., 1971).

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An Analysis of the Autophosphorylation of Rabbit and Human Erythrocyte Membranes†

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ABSTRACT: The autophosphorylation of rabbit and human erythrocyte membranes has been studied under various experimental conditions. The phosphopeptides of the erythocyte membranes were identified using sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis followed by radioautography. The pattern of phosphorylation of membrane components differs with respect to the phosphoryl donor used (ATP or GTP) and to the pH at which the reaction is carried out. Both species appear to contain at least two distinct membrane-bound protein kinases. The human erythrocyte membrane contains a cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase and several substrates for this kinase. Only ATP can be used as a phosphoryl donor for this kinase. In contrast, the rabbit erythrocyte membrane does not contain a cyclic AMP dependent protein kinase but does contain a kinase which utilizes only ATP as the phosphoryl donor and is specific for certain endogenous substrates at low pH. Both the human and rabbit erythrocyte membranes contain a kinase which utilizes GTP, perhaps also ATP, as the phosphoryl donor. The substrates of these kinases are similar in both species.

Considerable interest has been focused on the problem of whether the enzymic phosphorylation and/or dephosphorylation of membrane proteins can result in altered functional properties of those proteins. Recent observations have implicated membrane phosphorylation in such diverse cellular processes as neuronal transmission (Greengard, 1975), ATPase activation (Knauf et al., 1974; Katz and Blostein, 1975), and insulin-stimulated glucose transport (Chang et al., 1974). In addition, it has been reported that certain muscular dystrophies may be associated with altered erythrocyte membrane phosphorylation (Roses and Appel, 1975; Roses et al., 1975).

In an attempt to further elucidate the factors controlling the processes of membrane protein phosphorylation, we have initiated studies dealing with rabbit erythrocyte membrane preparations. In contrast to results obtained with human erythrocyte membranes (Guthrow et al., 1972; Fairbanks and Avruch, 1974), we have not been able to detect any cyclic adenosine 3′,5′-monophosphate (cyclic AMP)[1]

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[1] The abbreviations used are cAMP, cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; NaDodSO$_4$, sodium dodecyl sulfate; mol wt, molecular weight.