^{31}P NMR OF ALKALINE PHOSPHATASE

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SUMMARY: Phosphate bound to E. coli alkaline phosphatase gives ^{31}P-NMR peaks at -3.5 and -8.5 ppm from phosphoric acid. A shift of -8.5 ppm cannot be correlated with any phosphorylated amino acid analogue. It is ascribed to a strained phosphoserine at the active site, with implications for catalysis. The two peaks trade intensity as the pH is varied, and at pH>6.5 only the upfield one is observed. In the cadmium enzyme the downfield peak is observed even at pH 7, which parallels the Cd-enzyme's failure to dephosphorylate at this pH. Enzyme-bound phosphate cannot be detected in the cobalt enzyme, implying that the phosphate binds close to the paramagnetic ions; a maximum P-Co distance of 3.3 Å is estimated.

Alkaline phosphatase from E. coli (EC 3.1.3.1.), a zinc metalloenzyme, binds inorganic phosphate tightly over a wide pH range, forming complexes which are thought to be reaction intermediates in the hydrolysis of phosphate monoesters (1,2). Accordingly the binding of phosphate to alkaline phosphatase has been intensively studied, but the high-resolution crystal structure is not available and hence the environment of phosphorus at the active site is unknown. Using ^{31}P NMR we have been able to detect phosphate bound to alkaline phosphatase and gain insight into its chemical nature.

MATERIALS AND METHODS

Alkaline phosphatase was isolated from Escherichia coli (strain CW3747, kindly supplied by Dr. Milton Schlesinger) as described by Schlesinger and Olsen (3). Assay by monitoring p-nitrophenyl phosphate hydrolysis in 1M Tris, pH 8, gave an average specific activity of 50μmol/min/mg protein at 23°C. Enzyme was quantitated by measuring absorbance at 280nm (2).

Apoenzyme was prepared by dialysis against 0.1M EDTA at pH 6 and then against deionized water, using ^{14}C-EDTA to monitor complete removal of chelator. To make the cobalt enzyme, 1.95 moles of $CoCl_2$ were added per mole of apoprotein at pH 8.0. Cadmium enzyme was prepared by dialysis against 1mM

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$CdCl_2$ in 10mM Tris at pH 7.1. Glassware was acid-washed, and buffers were treated with dithizone or Chelex resin.

NMR samples were prepared by concentrating enzyme in an Amicon ultrafiltration cell, followed by dialysis against the desired buffer. Spectra were recorded on a JEOL JNM-FT-100 operating at 40 MHz in Fourier-transform mode. 8 mm sample tubes were used, with D_2O in a concentric capillary to provide a field/frequency lock signal. Chemical shifts are reported relative to 85% phosphoric acid.

RESULTS AND DISCUSSION

^{31}P NMR spectra of alkaline phosphatase at low pH are shown in figure 1. Spectra obtained at pH values up to 9 are similar to the one at pH 6.45. Free inorganic phosphate produces the peak at highest field, and the nature of the other peaks, labelled I and II, may be inferred from their pH dependence. If enzyme at low pH is rapidly denatured, phosphorylation of a unique serine residue can be demonstrated (4), but at high pH no covalent phosphate is

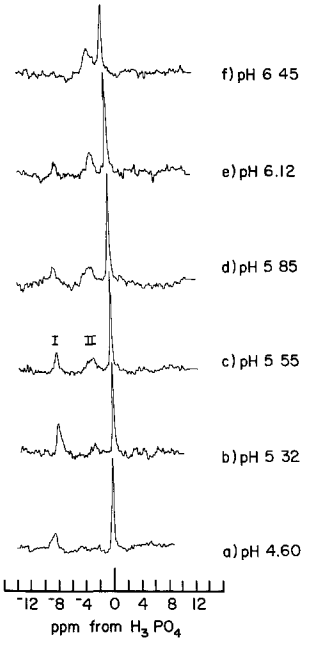

Figure 1: ^{31}P NMR spectra at 10°C of 1.8mM *E. coli* alkaline phosphatase dialyzed against 2.0mM sodium phosphate, 1.0M sodium chloride, 10mM sodium citrate, and 50μM sodium merthiolate, a growth inhibitor. About 23,000 transients were collected for each spectrum, with a repetition interval of 3.1 sec. The peaks at highest field are at the P_i position for the given pH.

detected (1,2). Kinetic studies indicate that the phosphorylated species obtained at low pH is a catalytic intermediate at both high and low pH (1,5). Since the area of peak I roughly follows the pH-dependent occurrence of the phosphoenzyme, we assign it to this species. Support for this is obtained by replacing zinc in the enzyme with cadmium, in which case only peak I is observed even at pH 7.1 (figure 2a), mirroring the known failure of the Cd-enzyme to dephosphorylate at high pH (2). Although no multiplet structure in peak I is resolved, proton decoupling narrows it by ∿7Hz, consistent with the phosphorus being bound as a CH_2OP moiety.

This identification of peak I is complicated, however, by its anomalous chemical shift. We have found no phosphate monoester or phosphoserine - containing protein with a shift near -8 ppm at any pH (table 1, ref. 6). Ring currents could not shift a phosphoserine resonance downfield so drastically (7), and the data of table 1 tend to rule out proximity to charged

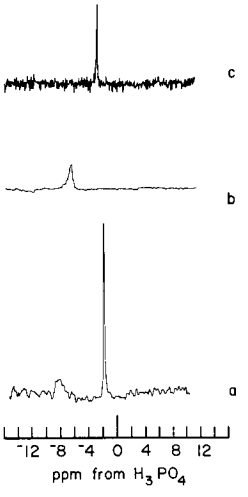

Figure 2: ^{31}P NMR spectra of E. coli alkaline phosphatase derivatives at 23°C. (a) Cadmium enzyme (1.5mM) in 10mM Tris, pH 7.1, with 4mM phosphate added. (b) Phosphorylated apoenzyme (0.8mM) in 0.1M Tris, pH 7.0 (proton decoupled). (c) Cobalt enzyme (1.5mM) in 50mM Tris, pH 8.0, with 4mM phosphate added. The upfield peak in (a) and the peak in (c) are at the P_i position for the given pH.

TABLE 1: ^{31}P chemical shifts of model compounds and phosphoproteins. Commercial products of highest available purity were used, except that phosphoimidazoles and phospholysine were synthesized according to (18) and (19) respectively.

Compound	pH	Chemical shift (ppm)	pH	Chemical shift (ppm)
O-phosphoserine*	4.3	-0.4	8.6	-4.0
O-phosphothreonine*	3.9	+0.9	8.6	-3.2
2-phosphoglyceric acid	5.5	-0.4	8.8	-3.6
phosphoglycollic acid	5.0	-0.3	8.4	-3.2
O-phosphoethanolamine	5.0	-0.7	8.0	-3.7
monoethylphosphate	2.8	-0.4	9.4	-3.8
phenyl phosphate	4.8	+3.2	8.1	0.0
α-naphthyl phosphate	5.0	+3.3	8.0	-0.2
p-nitrophenyl phosphate	4.7	+4.5	9.3	+1.7
pyruvate enol phosphate	2.4	+5.7	9.7	+2.0
phosphatidic acid, Na salt, in $CHCl_3$		-0.5		
phosvitin*	3.6	-0.3	9.3	-4.2
α-casein B in 8M urea*	4.0	-0.2	9.5	-4.3
ovalbumin †	5.2	-0.9,-0.5	7.1	-3.9,-3.6
N^{ε}-phospholysine	4.9	+0.8	8.6	-3.2
diphosphoimidazole	6.5	+4.9	9.5	+4.6
monophosphoimidazole			9.5	+1.0
acetyl phosphate	6.7	+2.3		
carbamyl phosphate	3.5	+6.6	9.5	+1.8
creatine phosphate	3.5	+5.3	8.7	+3.2

*from Ho, C., Magnuson, J. A., Wilson, J. B., Magnuson, N. S., and Kurland, R. J. (1969) Biochem. 8, 2074-2081.

† With proton decoupling, two sharp peaks of slightly different intensity are observed.

groups or hydrophobic environment as major factors. Since phosphate migration during the isolation of phosphopeptides is a possibility (8), it is important to consider that phosphate can not only form ester links to proteins but may also in principle bind as phospholysine, phosphohistidine, phosphoarginine, or acyl phosphate. Analogues of all of these have been examined, and none has a shift in the region of peak I. In addition, coordination of phosphate to the metal in the enzyme cannot be the sole explanation for the shift anomaly. When phosphorylated enzyme was dialyzed at pH 2, a procedure which removes zinc but preserves covalent phosphate, only a peak at the normal phosphoserine position was observed, and this was still true when the pH was raised to 6.6 in the presence of 6M guanidine. With guanidine omitted or subsequently removed by dialysis, however, the phosphorylated apoprotein gave a peak at -6.5 ppm (figure 2b) which is still very far downfield. Atomic absorption verified that this species was practically free of zinc; restoration of zinc at pH 7 partially restored both enzyme activity (~45%) and peak II of the native spectrum.

Having ruled out all obvious simple explanations for the shift of peak I we are left with only one known mechanism which can account for large downfield shifts -- steric strain. Studies of cyclic phosphate di- and triesters indicate that ^{31}P shifts are very sensitive to distortion of the phosphate's tetrahedral geometry (9). Such strain could clearly account in part for the kinetic lability of the phosphoenzyme, in view of the Westheimer mechanism (10) for nucleophilic displacement at phosphorus. It is most unlikely that I is a diester; such a hypothesis would be without experimental support and would introduce severe mechanistic complications. However, a hydrogen bond, with its directional character, could probably act in concert with a covalent link to serine to mimic a strained cyclic ester. This seems a most likely explanation for the anomalous peak I.

Peak II, which has a less remarkable but still substantial downfield displacement, is assigned to non-covalent phosphate at the active site on

the basis of its pH behavior. Further evidence for its catalytic importance is that it is never observed in apoenzyme, denatured enzyme, or cadmium enzyme. Distinct shoulders at -3.5 and -4.0 ppm are sometimes observed, but their significance is uncertain. Peaks I and II do not change width or position perceptibly with pH, indicating that each species has a constant state of protonation in the pH region where it is observed.

Kinetic information can be obtained from these spectra, since the widths of peaks I and II set minimum limits on the lifetimes of the corresponding species. These limits are roughly 30 and 10 msec respectively at 25°C. With a specific activity of ∿50μmol/min/mg for alkaline phosphatase at pH 8, a turnover time of ∿15 msec is calculated. At pH 5.5 the specific activity is more than one hundred-fold less (11), and the turnover time is correspondingly longer. At low pH there is clearly ample time for both species I and II to lie on the reaction pathway, but in the region of maximal activity the assumption that II is on the pathway means that dissociation of the enzyme-phosphate complex must be close to rate-limiting.

The cobalt alkaline phosphatase, which has partial catalytic activity (12) and binds phosphate both covalently and non-covalently (2,13), was also examined. At pH 8 no protein-bound phosphate peaks were observed, even with rapid pulsing, implying that phosphate binds close to the paramagnetic Co^{2+} ions. With more than about one phosphate per dimer a single sharp peak appeared at the P_i position (figure 2c), so as Csopak and Drakenberg (14) concluded from a ^{31}P NMR study at much higher phosphate/protein ratio, exchange must be slow. A maximum cobalt-phosphorus distance can be estimated, assuming that any peak must have a width >200 Hz to be undetectable. Taking g=2 and $T_{1e}<10^{-11}$ sec as a reasonable upper limit for electron relaxation times reported in Co^{2+} metalloenzymes (15), and ignoring the hyperfine term, the Solomon-Bloembergen equation (16) gives r<3.3 Å. A finite hyperfine interaction term would make this upper limit higher but would in itself presuppose that the cobalt and phosphorus are very close. The result is most consistent with binding of

phosphate in the first coordination shell at this pH. At lower pH, where the phosphoenzyme predominates (2,13), bound phosphate was still undetectable, but the P_i peak was broad, making interpretation ambiguous.

Similar observations to those reported here have recently been obtained in the laboratory of Dr. Joseph Coleman (17).

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