*STUDIES ON THE ACTIVE CENTER OF ALKALINE PHOSPHATASE OF E. COLI**

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Alkaline phosphatase of E. coli is a zinc metalloenzyme,[1] and the activity of the enzyme can be reversibly abolished and restored by the removal and addition of zinc ions at the active site.[3] The metal has also been thought to stabilize the structure of the enzyme.[4] When assayed in 1 *M* Tris buffer, pH 8.0, the activity, measured by formation of p-nitrophenol from p-nitrophenylphosphate, is significantly higher than in veronal.[2] This increased activity is due to phosphotransferase activity of the enzyme, with Tris acting as the acceptor, as demonstrated by Wilson, Dayan, and Cyr.[5] A number of di-, poly-, and amino hydroxy compounds also accept phosphate: O-phosphorylethanolamine has been identified as the product with ethanolamine.[5]

We have recently performed inorganic and organic modifications, designed to characterize both the metal and substrate binding sites of the enzyme, and found that some of these affect the hydrolase and the phosphotransferase activities differentially. This paper summarizes the results and implications of such recent experiments.

Materials and Methods.—Alkaline phosphatase was extracted from *E. coli*,[6] purified by DEAE-cellulose chromotography, and crystallized from $(NH_4)_2SO_4$.[7]

The preparation of the apoenzyme, measurements of hydrolase and phosphotransferase activities, determination of protein concentration and zinc content, as well as control of metal contamination have all been described.[2, 3, 5, 7–9]

Chemical modifications were performed on both the native and apoenzymes; excess Zn^{2+} was added to reconstitute the latter after the modification reactions. Acetylation, photooxidation, and iodination were performed as previously described.[10–12] Oxidation with N-bromosuccinimide[13] (NBS) was performed in 0.01 *M* Tris, pH 7.5, 22°. The enzyme was coupled with 5-diazonium-1-H-tetrazole[14] (DHT) by adding different volumes of freshly prepared DHT, 0.195 *M*, to 1×10^{-6} *M* enzyme in 0.8 *M* $NaHCO_3$ (pH 8.8) at 22°. Solutions were diluted and assayed for activities after 1 hr.[15]

Results and Discussion.—Photooxidation of native alkaline phosphatase in the presence of methylene blue does not significantly affect activity,[16] but on similar treatment the apoenzyme loses capacity to rebind zinc; concomitantly, both activities are diminished, and the extent is determined by the dosage. Results of experiments with Rose Bengal[17] are quite analogous (Fig. 1). Histidine residues of both the native and apoenzymes are progressively destroyed as a function of the exposure but the rate is significantly faster for the apoenzyme (Fig. 1). Zinc protects free histidine from photooxidation,[18] and the data in Figure 1 suggest that it protects three histidyl residues of this enzyme, to which zinc may be bound. If histidyl residues are involved, this number protected (Fig. 1) is insufficient to account for the stability constants of the zinc metalloenzyme,[19] suggesting additional ligands which remain unknown so far. The rate of oxidation of tryptophan is not affected by zinc; loss of tyrosine could not be demonstrated, and the enzyme does not contain cysteine.[4]

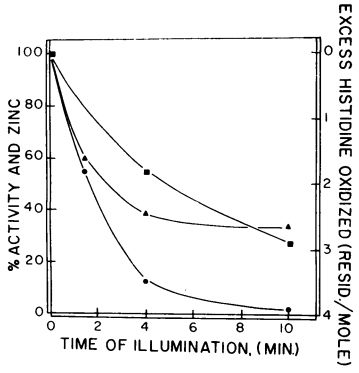

FIG. 1.—Illumination of apo- and native alkaline phosphatase in the presence of Rose Bengal. Protein (2.6 mg) and Rose Bengal (10 μg) in 1.05 ml of 0.01 *M* Tris pH 7.5 were irradiated at 20° using a 375-w "Photoflood" lamp 4 inches from the sample. Aliquots were diluted and excess Zn^{2+} was added to the apoenzyme samples which were assayed for total activity (●) and for protein-bound zinc (▲), the latter after dialysis to remove excess Zn^{2+}. These are plotted as percentage of the non-illuminated control. There was no change in phosphotransferase/hydrolase ratio. Activity and zinc content of the native enzyme did not change on illumination for up to 10 min.

By amino acid analysis six of the 17 histidine residues of the native enzyme were destroyed at 10 min, without change in activity. Histidines oxidized in the apoenzyme in excess of those oxidized in the native enzyme are plotted (■).

Replacement of Zn^{2+} by Co^{2+} at the active site: Like the native or reconstituted zinc enzymes, cobalt alkaline phosphatase is an active hydrolase; the specific activity is about 10 per cent of that of the zinc enzyme[2] (Table 1). In contrast to the zinc enzyme it has no phosphotransferase activity: the amount of p-nitrophenol formed is equal to the amount of inorganic phosphate (P_i) released, whether or not an acceptor is present (Table 1). The addition of Hg^{2+} did not alter the extremely low activities of the present apoenzyme preparations.

The virtual abolition of the phosphotransferase activity on substitution of cobalt for zinc is reminiscent of the curtailment of dual specificity of carboxypeptidase, on replacement of zinc at the active site of that enzyme by cadmium, mercury, or lead.[21] These metallocarboxypeptidases are active esterases, but they do not hydrolyze peptides. Cobalt carboxypeptidase, like the nickel and manganese derivatives, hydrolyzes both peptides and esters, although the ratio of these activities differs from that of zinc carboxypeptidase, similar also to observations on cobalt and zinc carbonic anhydrases B of rhesus monkey.[22]

Alkaline phosphatase consists of two identical subunits.[23] In terms of metal content the most active, though physicochemically uncharacterized, enzyme preparation previously analyzed contained 2.8-gm atoms of zinc per molecular weight of 80,000.[1] Electrophoretically and ultracentrifugally homogeneous enzyme prep-

TABLE 1

ACTIVITIES OF ZINC AND COBALT ALKALINE PHOSPHATASES

Phosphate acceptor	Product	Apoenzyme	Native enzyme	Apoenzyme plus Zn^{2+}	Apoenzyme plus Co^{2+}
			(μmoles/min/mg protein)		
None	A	0.05	21.4	18.6	1.64
	B	—*	20.9	17.9	1.70
Tris	A	0.05	32.5	27.1	1.06
	B	—	17.2	14.2	1.04
Diethanolamine	A	—	33.5	28.8	1.21
	B	—	16.6	14.7	1.15
Glycerol	A	—	21.3	19.5	1.58
	B	—	14.6	13.4	1.55

To apoenzyme, 1×10^{-5} *M* in 0.01 *M* Tris pH 7.5, containing 0.04 gm atoms zinc/mole, was added a 3-fold molar excess of Zn^{2+} or Co^{2+}. Aliquots were added to p-nitrophenylphosphate, 3×10^{-3} *M*, the respective acceptors, all at 1 *M*, and pH 8 buffers to a final volume of 1.3 ml and incubated at 22° for 5 min. The control and glycerol experiments were in 0.02 *M* veronal–1 *M* NaCl pH 8. The reaction was terminated by addition of 1 ml of 5 *N* H_2SO_4; p-nitrophenol[5] (A) and P_i[20] (B) were measured. The P_i released is a measure of the hydrolase activity: the difference between the amount of p-nitrophenol and P_i is equal to the phosphate transesterified and, hence, is a measure of the phosphotransferase activity.[5]

* Not measured.

arations of higher specific activity contain even more zinc,[24] which is essential for the catalytic activity and probably the maintenance of structure.[4] The failure to observe significant enzymatic activity with enzymes in which metals other than Co^{2+} are exchanged for Zn^{2+} (ref. 2) may be due to the lack of binding of these metals under the conditions employed for substitution, and to the condition of assay.

In addition, it could be postulated that the ligands for metals at the active site and for those pertinent to stabilization could be different. While the coordination characteristics of zinc and cobalt might be suitable for both of these sites, other metals may only be bound at one of them.

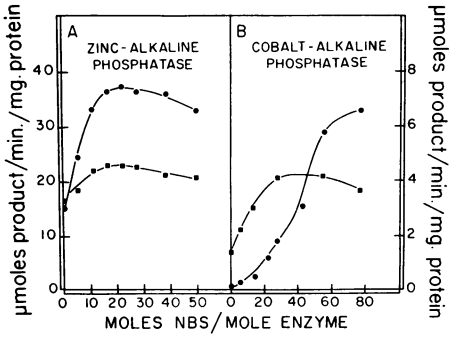

FIG. 2.—Effect of NBS on the phosphotransferase (●) and hydrolase (■) activities of zinc and cobalt alkaline phosphatases. Native zinc (*A*) or cobalt (*B*) alkaline phosphatase, the latter prepared by mixing apoenzyme with a 10-fold molar excess of $CoCl_2$ in 0.01 *M* Tris pH 7.5, were reacted with the indicated amounts of NBS. After 30 min, aliquots were diluted and assayed for hydrolase and phosphotransferase activities in 1 *M* Tris pH 8.0 as in Table 1. The left ordinate refers to the activities of the zinc enzyme, the right to those of the cobalt enzyme.

Exposure of zinc and cobalt enzymes to N-bromosuccinimide: At pH 4, N-bromosuccinimide (NBS) oxidizes tryptophanyl residues of proteins preferentially.[13] The reagent is less specific at pH 7.5, employed here to preclude loss of zinc. Depending on the conditions and the protein, NBS oxidizes tyrosine, methionine, histidine, cysteine, and disulfide bonds in addition to tryptophan.[25]

Reaction of zinc phosphatase with a 22-fold molar excess of NBS more than doubles the phosphotransferase activity and slightly increases hydrolase activity (Fig. 2*A*).[26] Transphosphorylation to diethanolamine and glycerol by NBS-treated enzyme is similarly increased. When phosphotransferase activity is maximally increased, between one and two tryptophanyl residues[27] and about eight out of 19 tyrosyl residues are destroyed, as determined by two different methods for tryptophan[28, 29] and by amino acid analysis,[30] but no histidine is lost. Possible effects on methionyl residues, disulfide bonds, and peptide bonds are under study.

Exposure of cobalt phosphatase to as much as a 50-fold molar excess of NBS at pH 7.5 increases the hydrolase activity to three times that of the control (Fig. 2*B*). Remarkably, this generates phosphotransferase activity in the cobalt enzyme. Zinc contamination was excluded analytically as the possible basis of this observation. At the present time we have not as yet differentiated the distinct possibility of oxidation of cobalt by NBS from its effect on amino acid residues in the vicinity of the metal.

Reaction with 5-diazonium-1-H-tetrazole (DHT): DHT couples with a number of amino acid residues of proteins, producing colored tyrosyl and histidyl derivatives.[14, 15] Up to a 2000-fold molar excess of DHT over the enzyme diminishes both hydrolase and phosphotransferase activities to about the same degree, i.e., 36 and 29 per cent of the control; color is not observed under these conditions which, however, we expected to result in coupling with lysine. But when the molar excess of DHT is increased to 20,000, spectral maxima characteristic of azotyrosine and

azohistidine[15] are produced. Concomitantly, while the hydrolase activity falls further to 11 per cent, the phosphotransferase activity now *rises* to 47 per cent of the control, a significant increase not only in the specific activity, but also in the phosphotransferase/hydrolase ratio.

In contrast to the changes on reaction with NBS, those of coupling with DHT are superimposed on initial decreases in both activities. On acetylation with acetic anhydride, a similar decrease in both activities occurs which may be related to the formation of ϵ-N-acetyllysyl residues. Curiously, a 50–100-fold molar excess of either acetic anhydride or acetylimidazole does not cause the formation of O-acetyltyrosyl groups in this enzyme. Perhaps modification of lysyl residues can alter the conformation of the enzyme sufficiently to render enzymatically critical residues accessible to subsequent chemical modification.

A phosphate complex, involving an "active" seryl residue,[31] has been suggested to play a part in the mechanism of action of alkaline phosphatase[5] either by reacting with water to yield inorganic phosphate, or with an acceptor to form the new phosphoester product. Kinetic data in such a scheme are consistent with the breakdown of the enzyme-phosphate complex as the rate-controlling step.[5] It is not known, however, whether water or other acceptors bind to the enzyme. Alterations of acceptor (substrate) binding sites or of the environment around the active serine might change the accessibility of the seryl phosphate to water or the organic acceptor and thus could readily shift the phosphotransferase/hydrolase ratio. The effect of additional inorganic and organic modifications and of their combinations on these two activities and on composition and structure should clarify the nature of the active site and center of this enzyme.

Work from this laboratory has previously demonstrated changes in the dual specificity of carboxypeptidase A from bovine pancreas due to both inorganic and organic chemical modifications.[21] Increases in esterase activity and decreases in peptidase activity result both from the substitution of various metals for the native zinc atom and from acetylation, succinylation, iodination, photooxidation,[21] coupling with diazonium compounds,[15] and nitration.[32] Such functional consequences of chemical modifications have been attributed to various combinations of altered catalytic efficiency, substrate binding, and inhibition.[33] The present findings on alkaline phosphatase of *E. coli* are seen to represent another example of the same general phenomenon which will require detailed analysis and delineation in these terms.

The work of Wilson, Dayan, and Cyr[5] has added alkaline phosphatase to the ever-increasing group of enzymes with dual specificity which present novel opportunities for the exploration of active centers, since certain chemical modifications actually preserve one of the catalytic processes even while abolishing the other. The delineation of the chemical basis of these functional changes is continuing.

Summary.—Like the native zinc enzyme, cobalt alkaline phosphatase is an active hydrolase, but it does not catalyze phosphate transfer. Treatment of zinc alkaline phosphatase with NBS doubles its phosphotransferase activity and now *generates* this activity in the cobalt enzyme. The functional effects of exposure to N-bromosuccinimide and coupling with 5 diazo-1-H-tetrazole are discussed. Evidence of the participation of histidine in Zn^{2+} binding is also presented.

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[3] The designation "active center" will refer to all those features of primary, secondary, tertiary, and quaternary structure of the enzyme—*including* the "active site"—which are required for substrate binding, specificity, or catalytic action. The "active site" will refer specifically to the metal-binding site and the active seryl residue.[31]

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[26] Similarly, exposure of the native enzyme to a 24-fold molar excess of iodine at 22° also doubles the phosphotransferase activity and slightly increases the hydrolase activity.

[27] An 8-fold molar excess of NBS at pH 4.5 (0.1 *M* acetate) oxidizes 1.9 tryptophan residues of the native enzyme compared with 0.3 residues at pH 7.5 (0.01 *M* Tris), but the increase in phosphotransferase activity after treatment at pH 4.5 is only 115% of the native compared to 190% for that treated with NBS at pH 7.5. Assays were performed at 1 *M* Tris pH 8.0. When NBS was used at pH 4.5, the samples were neutralized and excess Zn^{2+} was added before assaying.

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