EFFECT OF PHOSPHATE ON MULTIPLE FORMS OF ESCHERECHIA COLI ALKALINE PHOSPHATASE

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Received August 1,1975

Summary

Alkaline phosphatase isolated from E. coli strain C-90 can be separated by DEAE-cellulose chromatography into three forms which contain 0.1, 1.2 and 1.9 moles of bound phosphate per mole of enzyme, respectively. However, the phosphate-free enzyme prepared from a mixture of all three forms chromatographs as a single peak, while readdition of phosphate again results in the separation of three forms. Hence, the chromatographic behavior of phosphatase is consistent with a variation in phosphate content of three forms.

Introduction

Large numbers of enzymes, when subjected to separation techniques capable of high resolution, have been shown to exist in multiple forms. While the molecular basis for such multiplicity has been established unambiguously in many instances (1), that of the separation of alkaline phosphatase into three forms by DEAE-cellulose chromatography (2-5) has remained obscure.

Following the initial observation of multiple phosphatase forms (6), it was established that a single cistron codes for the polypeptide chain (7) and, further, that the dimeric enzyme is composed of two identical subunits (8). Hence, Signer (9) suggested that the different forms cannot result from the random dimerization of genetically distinct subunits, but must arise from post-translational or epigenetic modifications of the enzyme. Indeed, it has been shown that the relative proportions of these phosphatase forms depend primarily upon the conditions under which the bacterium is grown (9-11).

Purified alkaline phosphatase has been shown to contain tightly bound phosphate. However, the stoichiometry reported has varied from 0.2 to 2 moles of phosphate per mole of enzyme (12-14). Since, theoretically, a variation in

the amount of bound phosphate could form the basis for the chromatographic separation observed, we have determined the phosphate content of the three phosphatase forms isolated from E. coli strain C-90. In addition, we have removed the bound phosphate from native phosphatase and examined the chromatographic properties of this phosphate-free enzyme, as well as of that to which phosphate was readded.

Materials and Methods

Alkaline phosphatase was isolated by osmotic shock of E. coli strain C-90 cells grown in 14 liter cultures as described previously (15). The water extract from the osmotic shock treatment was purified by batchwise absorption onto DEAE-cellulose (Whatman DE-52) and eluted with 0.1 M NaCl in 10 mM imidazole-Cl, pH 7.2, followed by chromatography on a 5 x 150 cm column of Sephadex G-150 (Pharmacia, 40-120 μ) in 10 mM Tris-Cl, pH 7.5. Multiple forms of phosphatase were separated on DEAE-cellulose as described in Figure 1. Apophosphatase was prepared using 8-hydroxyquinoline-5-sulfonic acid (16).

Enzymatic activity was measured with p-nitrophenyl phosphate (Sigma 104) in 1 M Tris-Cl, pH 8.0 at 25°. Units of activity are defined as μmoles of substrate hydrolyzed per minute using a p-nitrophenolate molar absorptivity of 1.68×10^4 at 400 nm. Protein concentration was determined spectrophotometrically using $A^{1\%}_{278}$ = 7.2 and all calculations involving molarity are based on a phosphatase molecular weight of 89,000 (15). Conductivity was measured at room temperature using a Radiometer type CDM 2d meter and expressed in mMho. Phosphate analyses were performed using an ammonium molybdate reagent as suggested by Bloch and Schlesinger (12). Certified phosphate standards (New England Reagent Laboratory) were utilized for reference. All phosphatase samples were hydrolyzed for 24 hr at 120° in 6 N HCl and dried under vacuum over NaOH pellets before analysis.

Results and Discussion

Alkaline phosphatase purified from E. coli strain C-90 with a specific activity of 39 units/mg contains 1.2 moles of phosphate per mole of enzyme before separation of the three forms on DEAE-cellulose (Table I). The activities of the three forms of phosphatase, when separated by chromatography (Figure 1, top), are similar, 40 to 44 units/mg (Table I). However, their phosphate contents, ranging from 0.1 for form 1 to 1.9 for form 3 (Table I), reflect approximately integral values and are consistent with their order of chromatographic elution: the form eluting last is most negative.

The possibility that this variation in phosphate content could account for the observed chromatographic separation was tested by chromatographing the phosphate-free enzyme. Removal of metal simultaneously removes bound phosphate (12). Hence, apoenzyme prepared from pooled fractions of all three forms

TABLE I

Activity and Phosphate Content of Phosphatase

Before and After Chromatography

Sample	Activity, Units/mg	Phosphate, Moles/Mole
Native[a]	39	1.2
Form 1	41	0.1
Form 2	44	1.2
Form 3	40	1.9

[a]Before chromatographic separation of phosphatase forms on DEAE-cellulose.

(Figure 1, top) contains 0.05 mole of phosphate per mole of enzyme. The metal-free protein is catalytically inactive, but activity is restored by addition of a 10-fold molar excess of both $Zn(SO_4)$ and $Mg(SO_4)$, followed by dialysis for 24 hr against the chromatographic buffer, 10 mM imidazole-Cl, pH 7.2. The chromatographic properties of this phosphate-free enzyme were compared to those of another sample treated identically, except that, in addition to the metals, a 10-fold molar excess of phosphate was added to the apoenzyme. Identical activities, 44 units/mg, were restored to both samples. Ninety percent of the phosphate-free enzyme emerges as a single peak from DEAE-cellulose (Figure 1, middle), and by comparison with Figure 1, top, can be identified as form 1. However, chromatography of apophosphatase, reconstituted in the presence of phosphate, again resolves three forms (Figure 1, bottom), eluting at conductivities typical of the three forms separated in the native enzyme (Figure 1, top). Therefore, it would appear that variations in bound phosphate account for the separation of alkaline phosphatase into its three forms by techniques dependent upon charge differences.

The presence of 1.6 to 2.1 moles of tightly bound phosphate per mole of

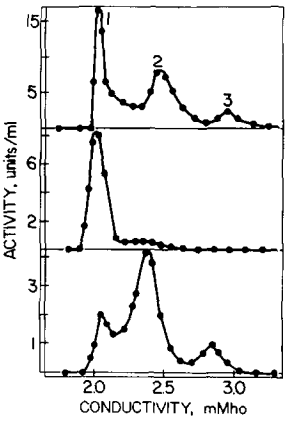

Figure 1. Separation of alkaline phosphatase forms. Alkaline phosphatase, 200 mg, was applied to a 2.5 x 40 cm column of DEAE-cellulose equilibrated with 10 mM imidazole-Cl, pH 7.2 and eluted with a 4 liter linear gradient of 10 to 70 mM NaCl in the same buffer (top panel). Apophosphatase, 15 mg, after reconstitution in the absence of phosphate was chromatographed on a 1.5 x 26 cm column of DEAE-cellulose using a 1.5 liter gradient of 10 to 70 mM NaCl in 10 mM imidazole-Cl, pH 7.2 (middle panel). An identical 15 mg sample of apophosphatase, but now reconstituted in the presence of phosphate, was chromatographed under the same conditions (bottom panel). For each chromatogram, phosphatase activity, in units/ml, is plotted versus the conductivity, in mMho, at elution.

purified enzyme was reported initially by Bloch and Schlesinger (12). Although subsequent studies have confirmed the presence of tightly bound phosphate, the molar stoichiometry reported has varied (13,14). This variation is now explicable, since the three forms contain different amounts of phosphate (Table I). Thus, the observed phosphate content of a mixture of these forms would be dependent upon the relative proportion of each present, which is influenced by the conditions employed for growth of E. coli cells utilized for enzyme isolation (6,9,10). In fact, Schlesinger et al., (11) has shown that addition of inorganic phosphate to E. coli cultures pulse labeled with ^{35}S-methionine causes the conversion of radioactive phosphatase form 1 to form 3. In parallel studies Schlesinger and coworkers (11,17) have found heterogeneity in the amino-terminal sequence of alkaline phosphatase isolated from another E. coli strain. These observations may bear upon the underlying structural basis of the differential binding of phosphate to the enzyme.

The fact that the binding of a small, charged molecule can give rise to chromatographically and electrophoretically distinct enzyme forms has been demonstrated for alcohol dehydrogenase of Drosophila (18). A similar situation is now observed for alkaline phosphatase of E. coli. The binding of approximately stoichiometric quantities of phosphate gives rise to three enzyme forms whose separation on DEAE-cellulose is consistent with differences in negative charge.

Acknowledgements

This work was supported by Grants-in-Aid GM-15003; W.F.B. is the recipient of Traineeship under GM-02123, both from the National Institutes of Health, of the Department of Health, Education and Welfare.

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