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EFFECT OF EDTA ON *ESCHERICHIA COLI* ALKALINE PHOSPHATASE

H. CSOPAK, K.-E. FALK AND H. SZAJN

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg (Sweden)

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SUMMARY

The effect of EDTA on alkaline phosphatase from *Escherichia coli* (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), and on the cobalt and copper derivatives of the protein, has been studied by measurements of enzyme activity and electron paramagnetic resonance (EPR) spectra.

From dialysis experiments on an EDTA-contaminated apoenzyme it was found that EDTA binds to the metal-free protein. In the complete absence of EDTA only two Zn^{2+} or Co^{2+} per enzyme molecule were required for full enzyme activity. Reports in the literature that more than two metal ions are necessary may be explained by varying levels of EDTA contamination in the enzyme and apoenzyme preparations.

EDTA also affects the EPR spectra of copper alkaline phosphatase, thus accounting for the two types of signals previously reported and their different behaviour to phosphate. Replacement of Zn^{2+} by Cu^{2+} in the growth medium results in a copper alkaline phosphatase which, however, seems to be contaminated with EDTA.

INTRODUCTION

The alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of *Escherichia coli* is a polymeric enzyme whose functional unit is believed to be a dimer. Only one structural gene for the protein has been demonstrated, indicating that the dimer probably consists of identical subunits[1]. Alkaline phosphatase is a zinc metalloenzyme which catalyzes both the hydrolysis of phosphate monoesters and the transfer of phosphate from such esters to acceptor alcohols[2]. The total number of strongly bound zinc atoms is uncertain, but at least two are essential for full enzymic activity[3–10]. It has also been suggested that Zn^{2+} plays a role in the maintenance of the structure of the protein[6,9]. The Zn^{2+} in *E. coli* alkaline phosphatase can be replaced by several divalent transition metal ions, of these only Co^{2+} gives rise to significant activity[3,4]. However, substitution of Zn^{2+} by Co^{2+} changes the specificity of the enzyme; Co^{2+}-alkaline phosphatase is an active phosphohydrolase lacking phosphotransferase activity[5].

Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

The object of the present paper is to investigate the relationship between protein and metal in the presence and absence of EDTA. This chelating agent is frequently used during isolation of the enzyme from the bacterial cells and in the preparation of apoenzyme. It is suggested that variations in these procedures are responsible for the apparent inconsistencies in metal stoichiometry.

MATERIALS AND METHODS

Reagents

All chemicals were reagent grade. Metal solutions were prepared from spectrographically pure metal sulfates. Dithizone was obtained from Fisher Laboratories, Springfield, N.J., U.S.A., Chelex 100 (200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif., U.S.A., DEAE A23 cellulose from Whatman, Kent, England, 1,10-phenanthroline and EDTA from Merck, Darmstadt, W. Germany, and 2-(*N*-morpholino)ethanesulfonic acid (MES) and *p*-nitrophenyl phosphate (Sigma 104) from Sigma Chemical Company, St. Louis, Mo., U.S.A. Metal-free solutions were prepared as described previously[11] and glassware was treated according to THIERS[12].

Preparation of Zn^{2+}-alkaline phosphatase

Bacterial growth and preparation of Zn^{2+}-alkaline phosphatase were carried out by a modification of the method described by MALAMY AND HORECKER[13]. An account of improved methods for the growth of bacteria and the preparation and purification of alkaline phosphatase will be reported[14].

Concentrations of protein were determined by absorbance at 278 nm as described by MALAMY AND HORECKER[13].

Preparation of the apoenzyme

The apoenzyme was prepared from Zn^{2+}-alkaline phosphatase by treatment with Chelex 100 (200–400 mesh)[11]. The metal-free enzyme was then extensively dialyzed (6–7 days) against large volumes of metal-free buffer or metal-free water.

Biosynthesis of Cu^{2+}-alkaline phosphatase

E. coli strain C_4F_1, derived from strain K-12, was a generous gift of Dr. A. M. Torriani, the Biological Laboratories, Harvard University, Cambridge, Mass., U.S.A. The bacteria were grown in acid cleaned flasks[12]. The culture medium was as for Zn^{2+}-alkaline phosphatase, except that Cu^{2+} replaced Zn^{2+}. The growth media were extracted with dithizone prior to addition of $CuSO_4$ in various concentrations (1–30 μM). The bacteria were grown for 20 h at 37° in 10-l batches. The cells were harvested, and the Cu^{2+}-protein was isolated with lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) in the presence of EDTA, according to MALAMY AND HORECKER[13]. After release of the protein and centrifugation, the Cu^{2+}-alkaline phosphatase solution was applied to a column of DEAE-cellulose, previously washed with 0.02 mM 1,10-phenanthroline, and then equilibrated with 5 mM MES buffer, pH 6.0, in 0.05 M NaCl. The protein was eluted from the column with 5 mM MES buffer, pH 6.0, in 0.125 M NaCl. The flow rate was 0.8 ml/min and 4-ml fractions were collected.

Enzyme activity

Activity was assayed spectrophotometrically on a Zeiss M4 QIII instrument, using 1.0 mM *p*-nitrophenyl phosphate[13] in 1.0 M Tris buffer, pH 8.0, at 25°. For activity measurements under metal-free conditions 1,10-phenanthroline at the final concentration of 0.1 mM was included in the assay mixture to remove traces of contaminating heavy metal ions. A unit of enzyme activity and the corresponding specific activity were defined as described previously[13]. A molecular weight of 80 000 (ref. 13) was assumed for alkaline phosphatase. Specific activities of the enzyme preparations used in the present work were 2500 ± 200.

Metal analyses

Metal analyses were performed as described earlier[11,15,16].

Spectral measurements

Absorption spectra were measured with a Cary Model 15 recording spectrophotometer. EPR spectra were recorded with a Varian E-3 spectrometer.

RESULTS

Titration of apoalkaline phosphatase with Zn^{2+} in the presence of EDTA

Fig. 1 shows the titration of apoalkaline phosphatase with zinc in the presence of various amounts of added EDTA. Under EDTA-free conditions a direct proportionality is found between enzyme activity and the amount of metal ion added, until the ratio of Zn^{2+} to enzyme is close to 2; further addition of Zn^{2+} causes no increase in activity. To obtain full activity when EDTA is present it is necessary to add more than two Zn^{2+} per protein molecule. The same activity is reached at all EDTA concentrations.

In another experiment a sample of apophosphatase containing a 30-fold molar excess of EDTA was dialyzed against metal-free water. Aliquots were withdrawn at intervals and titrated for activity with Zn^{2+}. The curves were of similar shape to those presented in Fig. 1. The linear parts of the titration curves were extrapolated to 100% activity and the obtained number of Zn^{2+} per enzyme molecule is plotted as a function of the dialysis time in Fig. 2. It is seen that this number is reduced during dialysis, but in order to reach the value of two Zn^{2+} per enzyme molecule very extensive dialysis has to be performed. A copper-EDTA solution was also dialyzed under the same conditions and it was found that the concentration of copper-EDTA in the dialysis bag decreased to about 10% in 15 h.

Titration of apoalkaline phosphatase with Co^{2+} in the presence of EDTA

Apoalkaline phosphatase titrated with Co^{2+} in the presence of EDTA behaves in the same way as when Zn^{2+} is used. The only difference is that the maximum activity is about 15% of that of the zinc enzyme. Here also, the effect of EDTA is to increase the amount of metal ion needed to reach maximum activity. The titration of apoenzyme with Co^{2+} in the absence of EDTA shows a proportionality between activity and added metal until the ratio of Co^{2+} to enzyme equals two.

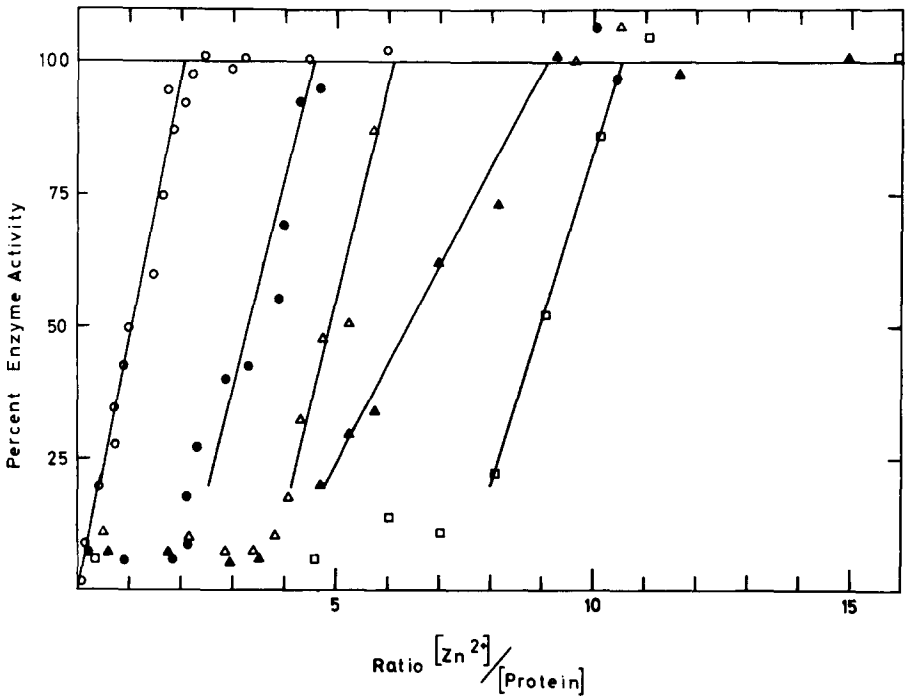

Fig. 1. Titration of apoalkaline phosphatase with Zn^{2+} in the presence of EDTA. 10 μM apoalkaline phosphatase in 0.01 M Tris–HCl buffer (pH 8.0) was incubated with EDTA for 10 min at 25°. The following EDTA/protein ratios were used: ○, 0.0; ●, 4.0; △, 6.0; ▲, 8.7; and □, 10.6. These solutions were titrated with $ZnCl_2$. After each addition of Zn^{2+} and incubation of 5 min at 25° small volumes were withdrawn and the enzyme activity assayed as described in MATERIALS AND METHODS.

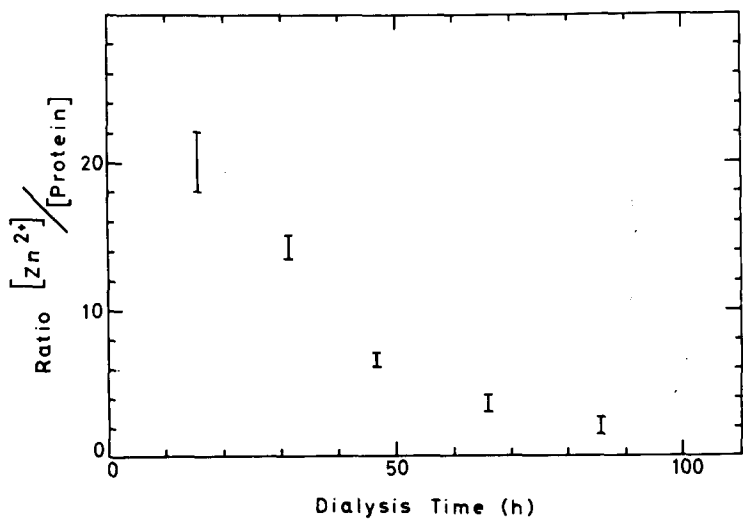

Fig. 2. Titration of EDTA-containing apoalkaline phosphatase with Zn^{2+} after dialysis. To 20 μM apoalkaline phosphatase in 0.01 M Tris–HCl buffer (pH 8.0) EDTA was added to a concentration of 0.6 mM. After 30 min incubation at 25° the sample (1.2 ml) was dialyzed against a 100-fold excess of metal-free water. The dialysis was carried out for 5 days with daily changes of dialysate. Samples of apoenzyme were removed each day, treated with different amounts of Zn^{2+}, and assayed. The linear parts of the titration curves were extrapolated to 100% activity and the obtained number of Zn^{2+} per enzyme molecule (with estimated errors) was plotted as a function of the dialysis time.

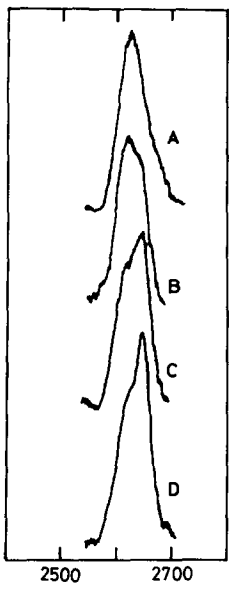

Fig. 3. Effect on the low field hyperfine line of the EPR spectrum from copper alkaline phosphatase. Low field hyperfine peaks from EPR spectra of copper alkaline phosphatase at pH 6.0 with different amounts of EDTA present. The protein concentration was 0.1 mM and the copper concentration 0.2 mM. The apoenzyme had been dialyzed for 7 days before the experiment. The mole ratios EDTA/protein were (A) 0.0, (B) 0.8, (C) 1.6, (D) 2.1. The microwave power was 5 mW, the modulation amplitude 10 G, and the temperature 77° K.

Effect of EDTA on the EPR spectrum of Cu^{2+}-alkaline phosphatase

The EPR spectrum of EDTA-free apoalkaline phosphatase treated with two moles of Cu^{2+} has the parameters $g_{\parallel} = 2.31$ and $A_{\parallel} = 155$ G. When EDTA is added at twice the molar concentration of protein the spectrum is altered and the parameters become $g_{\parallel} = 2.28$ and $A_{\parallel} = 150$ G. This change is best seen in the low field hyperfine line, as illustrated in Fig. 3. At intermediate concentrations of EDTA superpositions of the two spectra are obtained. Addition of Cu^{2+} to an apoenzyme which had not been dialyzed carefully enough also resulted in an EPR spectrum showing a mixture of the two forms in a ratio dependent on the dialysis time.

Biosynthesis of Cu^{2+}-alkaline phosphatase and the effect of EDTA on this protein

To investigate the incorporation of Cu^{2+} into alkaline phosphatase during growth of *E. coli* bacteria, Cu^{2+}-alkaline phosphatase was prepared as described in MATERIALS AND METHODS. The bacteria grew normally even in the presence of rather high concentrations of Cu^{2+}. Chromatography of these preparations gave rise to peaks at the same positions as for Zn^{2+}-alkaline phosphatase. When the cells were grown in the presence of 0.01 mM $CuSO_4$ the copper/protein ratio was found to be around 2. The EPR spectrum of this protein was identical to the spectrum of Cu^{2+}-alkaline phosphatase to which EDTA has been added in a 2:1 molar ratio (see Fig. 3d). Solutions of biosynthesized Cu^{2+}-alkaline phosphatase were dialyzed against metal-

free water or buffer. At intervals samples were withdrawn and their EPR spectra measured. The shape and amplitude of the signals were unchanged after prolonged dialysis.

DISCUSSION

In order to understand the mechanism of *E. coli* alkaline phosphatase, it is necessary to resolve the problem of the number of zinc atoms required for full enzyme activity. Most determinations of this number have been made by preparing the apoenzyme and then titrating with zinc until full activity is reached. During preparation of the apoenzyme and of the enzyme itself, EDTA or similar chelating agents are invariably employed. The experiments described herein clearly show that this may affect any determinations of metal stoichiometry.

From Fig. 1 it is seen that when EDTA is added to apoalkaline phosphatase more Zn^{2+} per enzyme molecule are needed to obtain full activity. Since the apparent stability constants for the zinc-EDTA and zinc-apoenzyme systems are $1 \cdot 10^{14}$ (ref. 16) and $1 \cdot 10^{11.4}$ (ref. 11), respectively, one would expect all the EDTA to be titrated before the apoenzyme. The early rise in the titration curves in Fig. 1 indicates that in fact this is not the case. Similar results are found when EDTA is dialyzed out of the apoenzyme solution (Fig. 2). It is therefore evident that some kind of interaction must exist between EDTA and protein. Very extensive dialysis is needed to remove all of the EDTA, suggesting that EDTA must be bound to alkaline phosphatase even in the absence of metal ions. If the EDTA is not carefully removed from the apoenzyme before titrating with Zn^{2+}, the resulting number of Zn^{2+} per enzyme molecule appears higher than 2. The presence of EDTA thus creates binding sites that are stronger than those responsible for the activity. However, the existence of weaker binding sites which are not related to the activity cannot be excluded.

Instead of dialyzing the apoenzyme before titration, some investigators[10,17] pass it through a Sephadex G-100 column. It seems probable that this procedure is not sufficient if EDTA is indeed bound to the apoenzyme. However, the discrepancies in the reported values of the zinc content do not appear to be a consequence solely of the use of EDTA during the preparation of apoenzyme. EDTA contamination may also derive from the original isolation of the enzyme, since alkaline phosphatase is released from the bacterial cells in the presence of rather high concentrations of EDTA. The EPR spectra of the biosynthesized Cu^{2+}-alkaline phosphatase support this idea.

Alkaline phosphatases in which Zn^{2+} has been replaced by paramagnetic ions, *e.g.* Co^{2+} (refs. 9, 10, 18–22) or Cu^{2+} (refs. 15, 22), have lately evoked considerable interest.

The results of the titration of the apoenzyme with Co^{2+} are essentially the same as for Zn^{2+}. In the presence of EDTA a higher ratio of Co^{2+} to enzyme is required to obtain full enzymic activity. A similar effect has also been reported with another chelating agent (8-hydroxy-5-quinolinesulfonic acid) in a spectrophotometric study of *E. coli* alkaline phosphatase[20]. Our results show that two cobalt ions per protein molecule are sufficient to produce a fully active enzyme and are in accordance with the results of APPLEBURY AND COLEMAN[20]. However, they disagree with those of SIMPSON AND VALLEE[9], who found that the first two Co^{2+} bound by the apoprotein

do not yield an active enzyme; addition of two more equivalents of Co^{2+} results in full activation. The reason for these divergent results might be related to the different methods by which the enzyme and apoenzyme have been prepared.

EPR spectra of Cu^{2+}-alkaline phosphatase have been reported to be sensitive to phosphate binding[15,22]. Another type of spectrum observed at pH 6, was unaffected by inorganic phosphate and substrates (Fig. 1C spectrum in ref. 15). The present work shows that a clean spectrum of the phosphate-sensitive species (Fig. 3a) only arises when Cu^{2+} is added after very long dialysis of the apoenzyme. Moreover, addition of EDTA to a sample giving the spectrum in Fig. 3a changes the EPR parameters to those of the spectrum reported insensitive to phosphate and substrates (Fig. 3d). Thus we think that only the spectrum with EPR parameters $g_{\parallel} = 2.31$ and $A_{\parallel} = 155$ G is typical of EDTA-free copper alkaline phosphatase at pH 6. The second spectrum with $g_{\parallel} = 2.28$ and $A_{\parallel} = 150$ G evidently represents a complex in which phosphate binding is altered by EDTA.

These experiments clearly demonstrate that great care must be taken when EDTA or similar chelating agents are used in the isolation of alkaline phosphatase or in the preparation of apoenzyme. We feel that the inconsistent reports of the zinc content are due to contamination by EDTA, and that the results presented in this paper favour two as the number of Zn^{2+} per enzyme molecule necessary for activity.

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