THE SPECIFIC BINDING OF COPPER(II) TO ALKALINE PHOSPHATASE OF *E. COLI*

H.CSOPAK and K.E.FALK

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Göteborg, Sweden

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1. Introduction

The *E. coli* alkaline phosphatase, a dimeric zinc metalloenzyme [1, 2], forms a phosphoryl enzyme [3] which is an important intermediate in the catalytic mechanism [3–7]. The number of zinc atoms per protein molecule varies in different preparations [8–11]. However, only two appear to be required for catalytic activity [12, 13]. Two zinc ions bind strongly to the enzyme and the binding of these has been studied by various methods [14, 15]. Several other transition metal ions can be substituted for zinc in alkaline phosphatase [2]; among these metalloproteins only the Co^{2+}-alkaline phosphatase has significant enzyme activity. The kinetic properties of the Co^{2+}- and the Zn^{2+}-enzymes have been extensively investigated [16, 17]. Co^{2+}-alkaline phosphatase possesses characteristic visible absorption bands [12, 18] and exhibits changes in both the circular dichroism and absorption spectra accompanying phosphate binding [12, 18]. Cu^{2+}-alkaline phosphatase has too low catalytic activity to be considered significant [2] but it induces phosphate binding [19] and it can probably form a phosphoryl enzyme. However, despite the fact that the Mn^{2+}- and Co^{2+}-phosphatases display little or no activity they are capable of forming stable phosphoryl intermediates [19]. Therefore, in the elucidation of the role of metal ions in the catalytic action, investigations carried out with the Cu^{2+}-alkaline phosphatase may be valuable.

The aim of the present paper is to provide preliminary data concerning the binding of Cu^{2+} to *E. coli* alkaline phosphatase. Displacement experiments suggest that Cu^{2+} ions are bound to the two specific binding sites occupied by Zn^{2+} ions in the native enzyme. The elctron paramagnetic resonance (EPR) spectra of the copper alkaline phosphatase give new information on the metal and phosphate binding.

2. Materials and methods

Alkaline phosphatase was purified as previously described [15]. The apoenzyme was prepared by treating the metalloprotein with Chelex 100 [15] Enzyme activity, protein concentrations and zinc analyses were performed as described earlier [15]. Cu^{2+}-alkaline phosphatase was prepared from the apoenzyme by the following method. $CuSO_4$ solution was added to the apoalkaline phosphatase (1.0×10^{-4} M in 0.125 M 2-*N*-morpholino ethane sulfonic acid buffer, pH 6.0), to give a final Cu^{2+}-concentration of 2.4×10^{-4} M, and the reaction mixture was equilibrated 24 hr at 7°. In all cases the Cu^{2+} analyses were performed [20] after extensive dialysis of the Cu^{2+}-enzyme against 0.125 M 2-*N*-morpholino ethane sulfonic acid buffer, pH 6.0, at 7°.

The equilibrium dialysis technique and the preparations of radioactive zinc solutions have been described previously [15]. Copper solutions were prepared from spectrographically pure $CuSO_4$. Other chemicals were analytical grade. Metal free solutions were obtained by extraction with dithizone [15] and

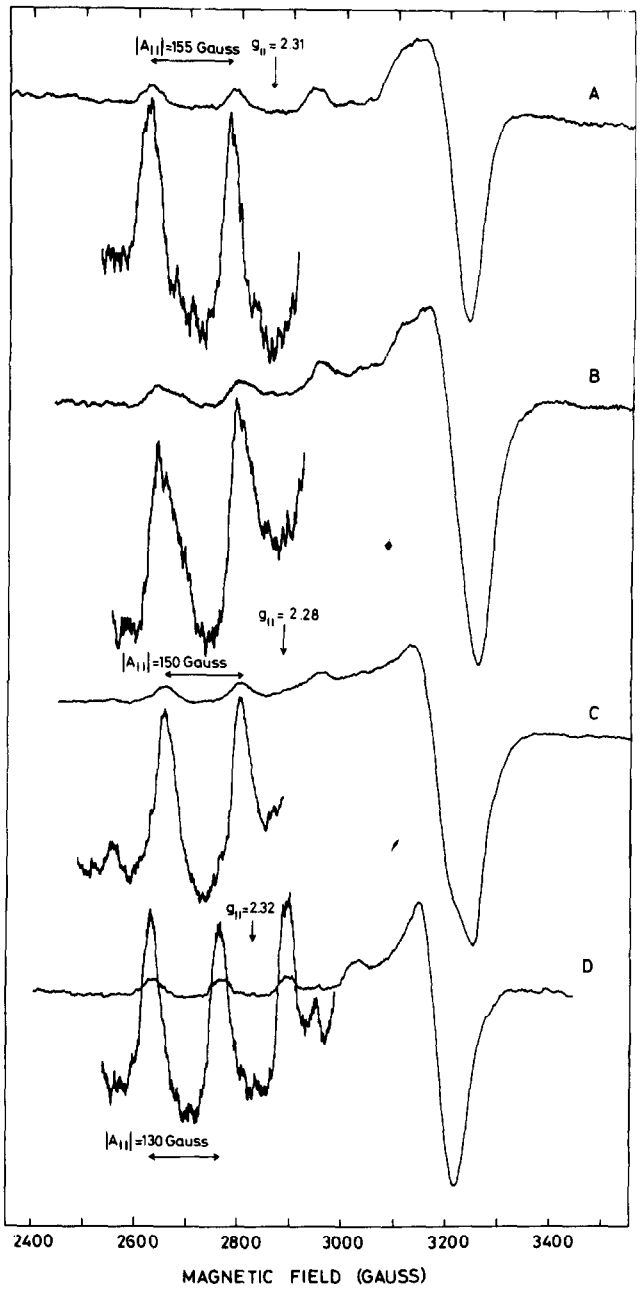

Fig. 1. EPR spectra of Cu^{2+}-alkaline phosphatase. Cu^{2+}-enzyme was prepared as described in Materials and methods. (A) 0.54 g atoms Cu^{2+} per mole of protein; (B) 1.16 g atoms Cu^{2+} per mole of protein; (C) 1.92 g atoms Cu^{2+} per mole of protein; (D) 0.91 g atoms Cu^{2+} per mole of protein and a half equivalent of K_2HPO_4, added 2 min before the spectrum was recorded. The spectra were recorded at 9.2 GHz at 77°K. The magnified parts of the spectra were recorded with ten times higher gain.

Table 1
The displacement of Cu^{2+} by radioactive Zn^{2+} from copper alkaline phosphatase at pH 7.35.

Enzyme (E)	Atoms Cu^{2+}/ mole of protein	Atoms Zn^{2+}/ mole of protein	Enzyme activity %
Apo-E	0.0	0.05	3
Zn*-E	0.0	2.30	100
Cu^{2+}-E	2.21	0.06	5
Zn*-E dialysed against Cu^{2+}	0.43	2.10	92
Cu^{2+}-E dialysed against Zn*	1.38	0.89	38

Protein concentrations, 1.0×10^{-5} M; metal concentrations, 3.0×10^{-5} M; buffer 0.05 M tris-HCl buffer; temperature, 25°; dialysis time, 4 days. Enzymatic activity is expressed as percent of the activity measured for Zn^{2+}-alkaline phosphatase which was treated under the same conditions as in the displacement reactions, except that Cu^{2+} was omitted. Zn* designates radioactive zinc.

glassware was washed in acid [21].

EPR measurements were carried out at 77°K in a Varian E-3 spectrometer at 9.15 GHz.

3. Results

3.1. *Binding of Cu^{2+}*

Cu^{2+}-enzyme was prepared as described in Materials and methods. The average number of metal ions bound after dialysis was 2.2 ± 0.2 g atoms per mole of enzyme. The initial addition of a large excess of $CuSO_4$ (4–6 g atoms Cu^{2+} per mole of protein) did not result in any increase of the final value.

Displacement experiments performed by the equilibrium dialysis techniques were utilized to determine whether the binding of the two Cu^{2+} and Zn^{2+} ions occurs at the same sites. The data in table 1 provide evidence that this is the case.

A solution of Cu^{2+}-alkaline phosphatase was incubated with radioactive zinc. Copper was gradually displaced with a concomitant increase of enzyme activity. When the Zn^{2+}-enzyme was incubated with Cu^{2+} a small loss of activity occurred and some displacement was observed.

3.2. *EPR spectra of Cu^{2+}-alkaline phosphatase*

The EPR spectra of alkaline phosphatase in the presence of varying amounts of Cu^{2+} are shown in fig. 1. Spectra recorded at low copper concentration have lines at 2625 and 2775 gauss. Increasing Cu^{2+} concentrations resulted in the appearance of additional lines at 2655 and 2800 gauss, then these new lines increase in intensity while the first lines decrease and finally disappear. At Cu^{2+}-saturation the spectra have only the lines at 2655 and 2800 gauss.

In addition, fig. 1 shows the spectra resulting when the protein is treated with K_2HPO_4 as described in the legend. Upon addition of approximately half an equivalent of phosphate at pH 6.0, the spectra show definite changes.

4. Discussion

4.1. *Cu^{2+} binding properties*

Approximately two metal ions are tightly bound to alkaline phosphatase, even when 6-fold excess of Cu^{2+} ions are initially added to the protein. The binding of Cu^{2+} is time dependent, indicating that a rearrangement of the chelating site is necessary.

The fact that two Cu^{2+} ions are tightly bound to each alkaline phosphatase molecule provides strong evidence that the copper alkaline phosphatase complex studied in this investigation involves the coordination of the Cu^{2+} to two specific sites of the enzyme. That this metal binds to the same sites as Zn^{2+} is indicated by the displacement experiments.

4.2. *EPR properties*

The analysis of EPR spectra (fig. 1) at different Cu^{2+}-saturation shows the existence of two spectral species, one dominating at low Cu^{2+} ion concentrations the other at high concentrations. Since pairwise binding of Cu^{2+} would give only one type of EPR spectra, this possibility is excluded. The spectra obtained at low Cu^{2+} concentrations probably represent enzyme molecules with one Cu^{2+} atom bound, while spectra recorded with Cu^{2+}-saturated enzyme (2.0 g atoms Cu^{2+} per mole of protein) represent enzyme molecules with very close to 2 metal ions tightly bound to each molecule of alkaline phosphatase. At the intermediate Cu^{2+} concentrations one

can estimate the relative proportions of each species for different levels of Cu^{2+}-saturation.

In the case of a random binding of Cu^{2+} to two equivalent sites, the theoretical distributions of each species can be calculated from the equations:

$$\frac{E_0}{E} = 1 - r + \frac{r^2}{4}, \quad (1)$$

$$\frac{E_1}{E} = r - \frac{r^2}{2}, \quad (2)$$

$$\frac{E_2}{E} = \frac{r^2}{4}. \quad (3)$$

E is the total amount of enzyme and is equal to $E_0 + E_1 + E_2$ and r is the average number of metal ions bound per protein molecule. The experimental values obtained from such EPR measurements agree within the experimental error with the calculated values and are in accordance with the model for random binding.

It is not known why alkaline phosphatase with one or two sites respectively, occupied by Cu^{2+} have different EPR parameters. However, it is possible that the binding of the second Cu^{2+} ion caused a conformational change affecting both sites.

The changes in both the circular dichroism and absorption spectra of Co^{2+}-alkaline phosphatase accompanying the phosphate binding [12, 18], and the fact that apoalkaline phosphatase does not bind phosphate [19] indicates that the metal ions of the enzyme play a very important role in the catalytic action, either in the formation of the active site by induced conformational changes or by coordination of the substrate molecule.

The EPR spectra obtained when Cu^{2+}-alkaline phosphatase is treated with half an equivalent of phosphate (fig. 1) show a shift of the lines of the spectra. The magnitude of the EPR spectral changes indicates direct interaction of the phosphate with the Cu^{2+}. The unique nature of the Cu^{2+} binding by alkaline phosphatase and the effect of phosphate and other substrates on the Cu^{2+}-alkaline phosphatase and other paramagnetic metallo-phosphates are being investigated by EPR techniques and the results will be reported in detail later.

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