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THE ROLE OF Zn(II) IN CALF INTESTINAL ALKALINE PHOSPHATASE STUDIED BY THE INFLUENCE OF CHELATING AGENTS AND CHEMICAL MODIFICATION OF HISTIDINE RESIDUES

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Summary

Alkaline phosphatase from calf intestine (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) is reversibly inhibited at pH 8.0 by incubation with chelating agents. Complete reactivation may be achieved by stoichiometric addition of Zn^{2+}. Atomic absorption spectrometry was used to demonstrate the linear correlation between Zn^{2+} content and degree of reactivation. The reversibly inhibited enzyme contained 1 Zn^{2+} per subunit whereas 2 Zn^{2+} were found in both the reactivated and the native enzyme.

At more alkaline pH-values, inactivation by chelating agents becomes irreversible; under such conditions the inactivated alkaline phosphatase still contains 1 Zn^{2+} per subunit. The conformational changes resulting from the loss of Zn^{2+} and leading to irreversible inactivation were investigated by optical rotatory dispersion, immunological techniques, and ultraviolet and fluorescence spectroscopy.

Azocoupling of the alkaline phosphatase with diazonium-1-H-tetrazole and Zn^{2+} content measurement of azocoupled enzyme probes indicated that 2 histidine residues per subunit are involved in binding of the catalytically important Zn^{2+}.

Introduction

Alkaline phophatases (orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) from calf intestine, bovine kidney, human placenta

This work forms part of the doctoral thesis of H.A. Ensinger. It was presented in part at the Spring Meeting of the German Society of Biological Chemistry at Frankfurt/Main, March 12—15th, 1978. Abbreviations: TNBS, trinitrobenzene sulfonate; 1,8-ANS, 8-anilino-1-napthalene-sulfonate.

and intestine contain 4 gatom Zn^{2+} per mol enzyme [1—3]. Hiwada and Wachsmuth [4] have found 2 gatom Zn^{2+} per enzyme tetramer in the alkaline phosphatase from pig kidney. The literature contains contradictory results with respect to molecular weight and to the number of subunits in the active enzyme. The alkaline phosphatase from calf intestine has been characterized as a dimeric enzyme with a molecular weight of 140 000, which includes 12% carbohydrate [1]. The influence of 2 metal ions, Mg^{2+} and Zn^{2+}, has been discussed. Mg^{2+} enhances the catalytic activity of mammalian alkaline phosphatases to various degrees, the isoenzyme from kidney displays a larger activation than the enzyme from intestine or from placenta (Mössner, E., personal communication and refs. 2, 5 and 6). There is evidence that 2 Zn^{2+} per dimer of alkaline phosphatase from *Escherichia coli* have a structural function and that the other two are involved in the catalytic process [7,8]; similar properties have been reported from mammalian alkaline phosphatase [9].

Alkaline phosphatase from different mammalian sources is inactivated by various chemical agents specific for several amino acid residues [10,11]. However, there is a lack of experimental evidence, for instance by analysing the reaction products of the chemical modification, that amino acid residues, discussed in these papers, are really involved in the catalytic mechanism.

Materials and Methods

Calf intestinal alkaline phosphatase (grade I) and alcohol dehydrogenase from yeast (EC 1.1.1.1) were purchased from Boehringer Mannheim, Germany as a particularly purified batch (number 1057541). All other chemicals used were of the purest grade available.

Methylacetimidate was prepared according to the method of Pinner from acetonitrile in methanol saturated with HCl. Acetamidination of the enzyme and the determination of the incorporation of acetamidinogroups into the protein was carried out as described by Tuengler and Pfleiderer [12] in 0.5 M borate buffer (pH 8.8)/1 mM $MgCl_2$. Diazonium 1-H-tetrazole was prepared as described by Horinishi et al. [13,14]. Azocoupling of the enzyme was effected with a 50-fold molar excess of diazonium 1-H-tetrazole relative to the concentration of enzyme monomers (at pH 7.2 in 0.5 M borate/acetate buffer) without addition of Zn^{2+} and Mg^{2+}.

Protein concentrations were determined from the absorbance at 278 nm, $A_{1\,cm}^{0.1\%} = 0.76$ [1], by the biuret method [15] and by the method of Lowry et al. [16]. The activity of the enzyme was measured according to the recommendations of the German Society of Clinical Chemistry [17]. When measuring the constants of inactivation by chelating agent, 0.5 mM EDTA, was present in the assay medium to prevent reactivation during the assay.

Amino acid analyses were performed on a Biotronik LC 6000 according to the method of Spackman [18].

Immunological investigations, optical rotatory dispersion and ultraviolet and fluorescence measurements were performed as previously described [19].

Atomic absorption spectrometry

In an effort to minimize the amounts of enzyme for the zinc determination

we sought to employ a highly sensitive and accurate method. Flameless atomic absorption spectrometry with a carbon-tube atomizer seemed to be most suitable. Its lower detection limit is approx. $1 \cdot 10^{-13}$ g zinc. Because of the high danger of contamination and a background zinc ion concentration we carried out measurement in the range of $1.1—7.5 \cdot 10^{-11}$ g zinc by choosing suitable operating parameters of the instrument (Perkin Elmer 303, equipped with the graphite furnace HGA-72, the autosampling system AS-1 and a deuterium background corrector). Nevertheless it was necessary to treat all the vessels which were in contact with the alkaline phosphatase with a 2% solution of 8-hydroxyquinoline in ethanol/water (50/50, v/v). The applied chemicals were analyzed for their zinc content. Enzyme-free blanks handled in this manner were found to be free from zinc in the range of the detection limit. To examine the influence of the protein matrix on the zinc peak, the enzyme, alcohol dehydrogenase, from yeast with a known zinc content was compared at different concentrations ($1—4 \cdot 10^{-8}$ M) with pure aqueous Zn^{2+} solutions (1—15 parts per billion Zn^{2+}). We found that the dissociation temperature of 400°C avoided interferences from the protein. The chemical similarity of the alcohol dehydrogenase and the alkaline phosphatase permitted the use of aqueous standards to determine the Zn^{2+} content of the alkaline phosphatase. With respect to the enzyme monomer, the concentrations were varied between $1 \cdot 10^{-8}$ and $4 \cdot 10^{-8}$ M. Each sample was measured three times. The resonance wavelength was 213.9 nm, the slit 0.7 nm, nitrogen was used as a purge gas in continous flow mode, the sample dosage was 20 μl. The standard deviation of the method was $s_r = \pm 3.2\%$ (5 parts per billion).

Results

The influence of chelating agents

Incubation of alkaline phosphatase with 0.01—1 mM EDTA resulted in total inactivation of the enzyme. The inactivation follows first-order kinetics with a change in the inactivation constants at 50% residual activity, with the exception that this biphasic behaviour is not observed at 1 mM EDTA (Fig. 1). This change does not depend on the pH-value within the range pH 8.0—11.0. It also occurs when using either 1 or 0.5 mM 1,10-phenanthroline or 8-hydroxyquinoline. Lazdunski et al. [1] also found a non-biphasic first order inactivation at EDTA concentrations between 2 and 10 mM.

Incubation with 0.1 mM 1,10-phenanthroline at pH 9.8, which does not inhibit alkaline phosphatase from calf intestine, was used to remove non-specifically bound metal ions from the protein.

The pH-dependence of the inactivation by 0.5 mM EDTA, 1 mM, 1,10-phenanthroline and 0.5 mM 8-hydroxyquinoline at pH 8.0—11.0 is shown in Fig. 2. These results reflect the different binding constants of these agents for Zn^{2+}, as well as their different ability to interact with the enzyme as the charge of the protein surface changes. 1,10-phenanthroline ($pK = 4.84$) remains uncharged in the pH range investigated. The different inactivation rates observed between pH 8.0 and 11.0 are therefore due to the pH-induced changes in the environment of the Zn^{2+}-binding site. The rate of inactivation by EDTA decreases above pH 9.8 as negative charges of both, the enzyme and the com-

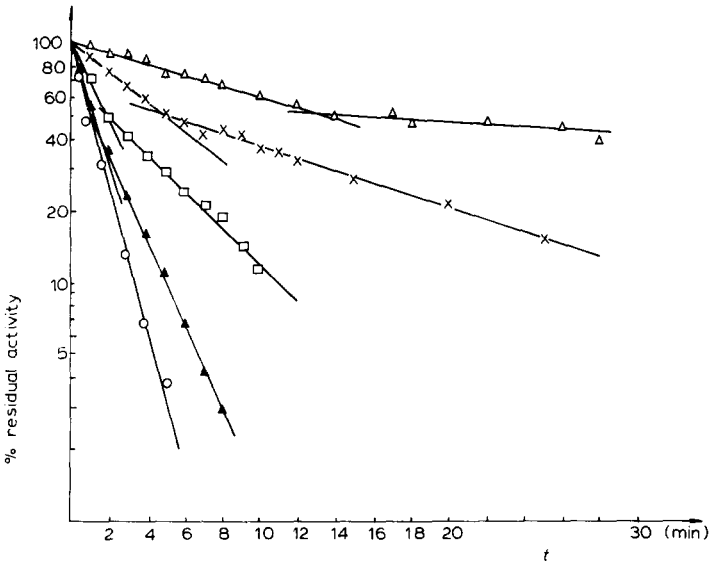


Fig. 1. Inactivation of alkaline phosphatase at pH 9.8 with EDTA. ○———○, 1 mM EDTA; ▲———▲, 0.5 mM EDTA; □———□, 0.1 mM EDTA; X———X, 0.05 mM EDTA; △———△, 0.01 mM EDTA.

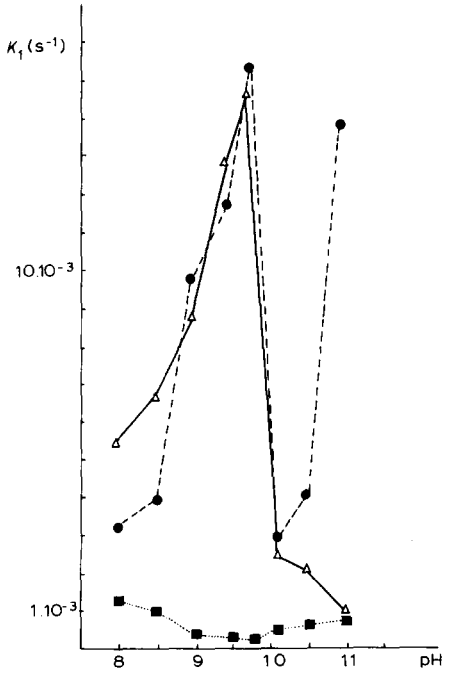


Fig. 2. pH-dependence of the initial phase inactivation constants, K_1. △———△, 0.5 mM EDTA; ●— — —●, 0.5 mM 8-hydroxyquinoline; ■. . . . . ■, 1 mM 1,10-phenanthroline.

plexing agent ($pK_1 = 10.26$) increase due to higher pH. Similar results have been found for alkaline phosphatase from pig kidney by Hiwada and Wachsmuth [4], who used chelating agents of different molecular size and charge at a constant pH.

Correlation of enzymatic activity and Zn^{2+} content

At pH 8.0, the alkaline phosphatase can be reversibly inactivated by incubation with EDTA, independent of the EDTA concentration. The removal of 1 Zn^{2+} per subunit causes total inhibition of enzymatic activity. The activity of the enzyme can be restored by gradually titrating the apoenzyme with $ZnCl_2$ solution.

The Zn^{2+} content was determined by atomic absorption spectrometry. The native and the fully reactivated enzyme contain 2 Zn^{2+} per monomer. The reversibly inactivated enzyme can be stored at pH 8.0 at 4°C for several days without losing its ability to reactivate after addition of Zn^{2+}. Only Zn^{2+} is required for reactivation of the enzyme, Mg^{2+} is not needed.

The inactivation with 1 mM EDTA at pH 9.8 in 1 M diethanolamine buffer resulted in a completely inactivated enzyme, which was also reported for the pig kidney enzyme by Ackermann and Ahlers [9]. The irreversibly inactivated enzyme still contains 1 Zn^{2+} per subunit, even after incubation for a long time and dialysis against 1 mM EDTA.

Conformational studies

The reversibly and irreversibly inactivated apoenzymes were compared with the native alkaline phosphatase by means of ultraviolet and fluorescence spectroscopy and optical rotatory dispersion. The reversibly inactivated enzyme at pH 8.0 showed no change. However, irreversible inactivation of the enzyme at pH 9.8 by incubation with 1 mM EDTA can be followed by ultraviolett difference spectroscopy. The increase of the absorption at 294 nm (Fig. 3b) is a measure for the irreversible inactivation of alkaline phosphatase. The spectra recorded at different time intervals are shown in Fig. 3a. The absorption maximum at 294 nm indicates some perturbation in the environment of tryptophan residues. The conformational change is also indicated by an overall decreased intrinsic fluorescence polarization of the irreversibly inactivated enzyme.

The titration of the native and inactivated enzyme with 8-anilino-1-naphthalene sulfonate at pH 9.8 (which indicates the number of hydrophobic binding sites) yields 1 site per monomer of the native protein and 2 binding sites for the irreversibly inactivated enzyme subunit (Table I). Enzymatic activity was not affected by 1,8-anilino-naphthalene sulfonate; the reversibly inactivated enzyme could be reactivated after 1,8-anilino-naphthalene sulfonate titration.

The ORD of alkaline phosphatase in the region of the Cotton Effect was recorded and the helix content was calculated according to Simmons et al. [20]. The irreversibly inactivated enzyme showed a slightly decreased helix content (26.9% as compared with 28.8% of the native one). Since the three-dimensional structure of the determinants on the surface of a protein is important for the antibody combining reaction, immunological investigations are a very sensitive method to examine changes in the conformation of proteins. No precipitation of the irreversibly inactivated enzyme was obtained: neither by

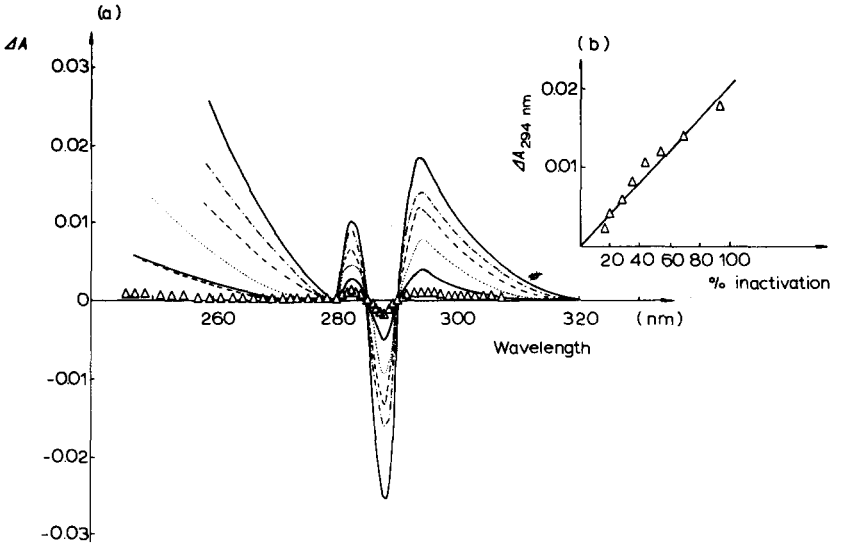


Fig. 3. (a) Time dependence of ultraviolett difference spectra of alkaline phosphatase at pH 9.8 incubated with 1 mM EDTA in 50 mM diethanolamine buffer ΔΔΔΔ, 50 min; ———, 75 min; · · · · · ·, 2 h; - - - - - -, 4 h; — · — · —, 6 h; ———, 21 h. (b) increase of the absorption at 294 nm vs. irreversible inactivation. tion.

the simple radiodiffusion technique according to Mancini [21] nor by the double diffusion test according to Ouchterlony [22]. Thus the small conformational changes indicated by the spectroscopic techniques cause total loss of antibody-binding capacity.

pH-dependent reversible inactivation at slightly acidic solution

A significant loss of enzymatic activity is observed by incubating the alkaline phosphatase in increasing acidic buffer medium from pH 7.0 to 5.0. The inactivation follows first order kinetics and again shows a change in inactivation constant at 50% residual activity. Based upon the pH-dependence of the rate constant of inactivation a pK value of 6.4 can be derived for the functional group or groups involved in the loss of enzymatic activity. This pK value corresponds to the pK value of the imidazole of histidine residues in proteins.

TABLE I

FLUORESCENCE TITRATIONS WITH 8-ANILINO-1-NAPHTHALENE SULFONATE

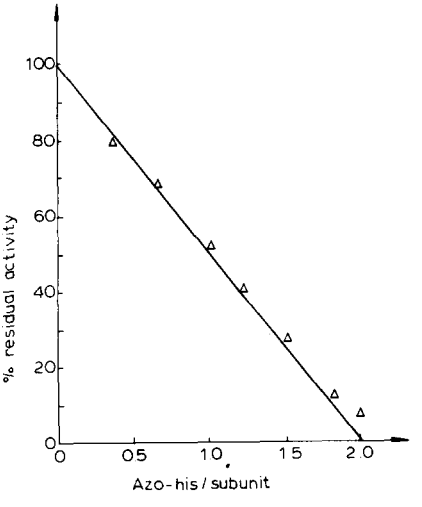
Alkaline phosphatase	Binding sites per subunit	Enzymatic activity (U/mg)	Relative fluorescence intensity (arbitrary units)
Native pH 8.0	0.46	1380	0.2
Reversibly inactivated pH 8.0	0.48	15	0.244
Native pH 9.8	0.96	1380	0.51
Irreversibly inactivated pH 9.8	1.95	10	1.00

Acetamidination and azocoupling of the enzyme

Chemical reaction of proteins with diazonium reagents leads to the modification of amino, imidazole- and phenolic groups. Diazonium 1H-tetrazole is considered to be a useful substance for elucidating the function of histidine and tyrosine residues in proteins. The extent of modification of both amino acid residues can be determined spectrophotometrically [23]. The triazenes formed by amino groups and diazonium 1-H-tetrazole during azocoupling have an absorption maximum near 340 nm and they obscure the absorption maximum of monoazotetrazole histidine at 360 nm. To avoid this disturbance the available NH_2 groups at the surface of the protein were blocked by methylacetimidate, which is specific only for amino groups [24]. The incorporation of acetamidino groups was determined by amino acid analysis and by the TNBS-test as previously described [12,25]. Of the 27.5 lysine residues per subunit, 21 could be blocked by acetimidate, resulting in an enzyme which was still fully active and showed no change in its catalytic properties.

The azocoupling of the acetamidinated protein resulted in a complete loss of enzymatic activity after modification of 2 histidine residues per monomer (Fig. 4). Azocoupling of the enzyme in the presence of β-glycerophosphate as substrate gave no inhibition of the enzyme and no azocoupling of histidine residues. So there is strong evidence that these histidine residues are located in the active site of the alkaline phosphatase. Under the conditions applied either in the presence or in the absence of substrate only a small portion of tyrosine were azocoupled, but without any influence on the catalytic activity of the enzyme.

The pK value of 6.4 found by inactivation of alkaline phosphatase in acidic



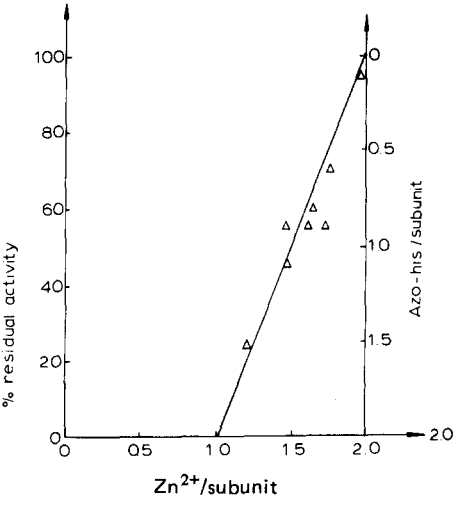


Fig. 4. Azocoupling of acetamidinated alkaline phosphatase. Relation between activity of the enzyme and azocoupling of histidine residues.

Fig. 5. Residual activity and rate of modification of histidine residues versus the Zn^{2+} content of alkaline phosphatase.

solutions and the results of the azocoupling demonstrate that histidine residues play an essential role in the catalytic mechanism. As shown in the first part of the paper one Zn^{2+} per monomer is also needed for enzymatic activity. It was therefore of interest to determine whether these histidine residues are ligands of the catalytically important Zn^{2+} or whether they play another role in the catalysis. Measurements of the Zn^{2+} content of the azocoupled protein by atomic absorption spectrometery furnished a correlation between the extend of modification of the histidine residues, the residual activity and the Zn^{2+} content of the azocoupled enzyme probes, Fig. 5. Obviously the 2 histidine residues which are azocoupled by diazonium 1-H-tetrazole, act as ligands of the catalytically important Zn^{2+}.

Discussion

In several Zn^{2+}-containing enzymes, like alcohol dehydrogenase, carboxypeptidase, human carbonic anhydrase and alkaline phosphatase from *E. coli* the metal ion is complexed by two or three histidine residues which appear closely in the amino acid sequence [26,27]. Other ligands are glutamic acid and cysteine residues. Sarkar and Iyer [28] have found that a Zn^{2+} complex with two histidines and one glutamic acid residue of a cyclic octapeptide is most stable in the pH range from 7.0 to 10.0 with a maximum at pH 8.2. We observed the same behavior, indicated by the reversible loss of activity below pH 7.0 and by the increase of the rate of inactivation by 8-hydroxyquinoline and 1,10-phenanthroline above pH 10.0 in the alkaline phosphatase from calf intestine. The pK value 6.4 indicates that the protonation of histidine residues is responsible for the acid inactivation of alkaline phosphatase. The azocoupling of histidine residues and the determination of the Zn^{2+} content of the azocoupled enzyme are a positive proof that 2 histidine residues per subunit of the enzyme are involved in binding the catalytically important Zn^{2+}. The lack of inactivation and the complete protection against azocoupling of these two histidine residues in the presence of the substrate β-glycerophosphate is also good evidence that the Zn^{2+} and the histidine residues are located in the active site of the enzyme.

ESR studies on the Cu(II) alkaline phosphatase from *E. coli* [29] indicated three nitrogen ligands at the metal-binding site, probably nitrogens from histidine residues.

Photo-oxidation of the apoenzyme and the native enzyme also from *E. coli* [30] showed in the case of the native enzyme that 2—3 of the histidine residues were protected by the metal ion.

The Zn^{2+} may play a similar role in the catalysis mechanism to that of the Zn^{2+} in human carbonic anhydrase. In the latter case the Zn^{2+} has three histidine ligands and a water molecule as a fourth ligand which can act as a nucleophile, also in its hydroxyl ion form, in the catalysis [31]. The presence of such a ligand on the Zn^{2+} of mammalian alkaline phosphatase, was derived from kinetic measurements of Ahlers [32], who proposed a water molecule as a Zn^{2+} ligand with a pK of 8.8. Similar results were found by Lazdunski et al. [33] with the enzyme from *E. coli*. They determined a pK of 9.3 in inactivation experiments and suggested also a water molecule as ligand of the catalytically active Zn^{2+}.

The role of other amino acid residues in the catalysis is not clear. The evidence of essential lysine residues for substrate binding [32,34,35] is very doubtful, as amidination of the enzyme does not change its catalytic properties.

The biphasic kinetics of inactivation observed when affecting the Zn^{2+}-binding site with pH, chelating agents or by chemical modification, indicates a nonequivalence of the two active sites. This is in good agreement with Lazdunski et al. [36] who found also the nonequivalence of the two active sites and postulated a half-of-the-sites-reactivity-mechanism like in the alkaline phosphatase from *E. coli* [27]. It is very interesting in this connection to note, that the native alkaline phosphatase possesses at pH 8.0 only 0.5 hydrophobic binding sites per monomer, e.g. 1 molecule of 1,8-ANS is bound to the dimer, probably to 1 subunit. At pH 9.8 the two subunits become equivalent as far as binding of 1,8-ANS is concerned.

The catalytically important Zn^{2+} not only has a function in the catalysis mechanism but also serves to stabilize the native conformation of alkaline phosphatase, especially at alkaline pH. Under these conditions the enzyme is strongly negatively charged (the isoelectric point lies within the range of 4.3—4.8 [37]). The removal of this first Zn^{2+} results in an increase of the number of hydrophobic binding sites and of the conformational flexibility of the enzyme as indicated by an overall decrease of the intrinsic fluorescence polarization. The dissociation constant for the zinc-enzyme complex is about 10^{-12} M [38]. Since the second Zn^{2+} could not be removed with EDTA under the applied conditions even from the irreversibly inactivated state of the enzyme, its K_D must be much greater or it must be inaccessible for chelating agents.

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