Investigation of the Copper–Magnesium–Alkaline Phosphatase System

I. BERTINI, C. LUCHINAT, A. SCOZZAFAVA

Institutes of General and Inorganic Chemistry, University of Florence, Italy

A. MALDOTTI and O. TRAVERSO

Centro di Studio sulla Fotochimica e Reattività degli Stati Eccitati dei Composti di Coordinazione del C.N.R., Institute of Chemistry, University of Ferrara, Italy

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The effect of Mg^{2+} ions on Cu_2–alkaline phosphatase has been investigated with respect to the coordination geometry of the copper ion in presence and in absence of inorganic phosphate at pH 6.0.

It is somewhat puzzling that Cu_2AP binds only one phosphate ion giving rise to only one kind of ESR spectra consistent with a tetragonal structure. Cu_2Mg_4AP binds two phosphate ions, the resulting geometry being probably pseudotetrahedral.

Water ^1H T_1^{-1} measurements between 4 and 60 MHz have shown that water is present in the coordination sphere of the former derivative but not in the latter.

Introduction

Alkaline phosphatase from E. coli (AP) is a dimeric enzyme of molecular weight 95000 which catalyzes the hydrolysis of phosphate monoesters [1, 2]. The native enzyme contains two zinc ions (A and B sites) and one magnesium ion (C site) in each equivalent subunit [3–7].

The X-ray structure available at 6A of resolution has shown [8] that the two zinc ions in the same subunit are as close as 5A, while magnesium is 30A apart from the couple. The distances between the same metal sites belonging to different subunits are also very large (~30A).

The Zn_4Mg_2AP native enzyme or the Zn_4AP derivative are known to bind phosphate in a 2:1 metal:phosphate ratio [1, 9]. It seems that each substrate molecule interacts with a couple of Zn atoms in the A and B sites [10]. The enzyme partially reconstituted with two zinc ions (Zn_2AP) is able to bind only one phosphate ion [11, 12]. At acidic pH, phosphate binding occurs through the formation of a covalent intermediate resulting from phosphorylation of a serine residue [13, 14].

The enzyme reconstituted with other divalent transition metal ions shows different degrees of activity but the stoichiometry of the phosphate–enzyme interaction is the same, *i.e.* 2:1 and 1:1 for M_4Mg_2-AP and M_2AP derivatives, respectively.

In order to explain why the M_2AP derivatives are capable of binding phosphate only in one subunit, although the X-ray data indicate that the two subunits are equivalent, anti-cooperative effects were invoked: in the assumption that the M_2AP derivative contains the metals in the A sites, binding of one phosphate ion to one of the subunits would induce a modification in the other so that the latter is not capable of binding a second equivalent of phosphate.

Using ^{13}C, ^{31}P and ^{113}Cd NMR measurements on several cadmium AP derivatives, Otvos *et al.* recently proposed [10] that the anti-cooperative behavior can be rationalized in terms of phosphate-induced migration of metals, resulting in phosphate binding at a subunit containing metals in both the A and B sites, the other subunit being empty.

The three sites are known to display different stereochemistries: the A site is most probably five-coordinated, whereas the B and C sites are octahedral [15–17]. According to Otvos *et al.*, the A site con-

TABLE I. ESR Parameters of Some Copper Alkaline Phosphatase Derivatives, at pH = 6.0.

	$g_\parallel$	$g_\perp$	$A_\parallel$ (cm^{-1})
Cu_2AP	2.32	2.06	0.0162
Cu_2Mg_4AP	2.33	2.06	0.0162
$Cu_2AP \cdot 1P_i$	2.33	2.07	0.0141
$Cu_2Mg_4AP \cdot 2P_i$	2.36	2.07	0.0129

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tains four histidine nitrogen donors, the B site one, and no histidines are present in the C site [10].

Among bivalent ions, copper(II) appears to be the most suitable to monitor the type of sites occupied, since the ESR parameters are sensitive to the coordination geometry and the nature of the ligands [18]. Therefore, the clean ESR spectra which originates by adding increasing amounts of copper to the apoprotein up to a 2:1 ratio [19–22] (and which show spectral parameters typical of a tetragonal coordination), can be taken as indicative of a selective binding of copper to only one kind of the available sites, most probably to the A site consistently with the well known preference of copper(II) for nitrogen donors. Addition of only one equivalent of phosphate is enough to completely modify the ESR spectrum [19], which, however, does not show any evidence of heterogeneity and therefore would indicate that the two copper centers still possess the same chromophore.

The role of magnesium(II) ions in the function of alkaline phosphatase and in the spectroscopic behavior of its derivatives with several divalent metal ions has been recently emphasized [10, 17, 22]. It is now well established that magnesium has high affinity for its natural site C, moderate affinity for the B site, and negligible affinity for the A site [10]. Therefore, it seems to be the natural complement for studying the coordination of copper in the A site and possibly checking the hypothesis of metal migration in the Cu_2AP derivative upon addition of phosphate in a 1:1 ratio.

We have therefore investigated the Cu_2Mg_4AP system and its interaction with phosphate at pH 6.0 through EPR and NMR measurements. We have also repeated the early EPR measurements on the behavior of the Cu_2AP system upon addition of phosphate.

Experimental

Alkaline phosphatase from E. coli was obtained from Sigma and used without further purification. The enzyme was first dialyzed against TRIS-HCl 0.05 *M* pH 8; the apoenzyme was prepared according to the procedure described by Otvos and Armitage [5] and further dialyzed against 0.01 *M* sodium acetate, 0.1 *M* sodium chloride, pH 5.5.

Enzyme concentrations were determined spectroscopically at 280 nm using $E^{0.1\%}_{1\,cm}$ 0.77 [23]; a molecular weight of 95000 was assumed [5].

Apoenzyme solutions were concentrated by ultradialysis up to a final concentration of 0.5–1 m*M*. ^{63}Cu (Oak Ridge Laboratories) was added as copper sulfate to the above solutions in the required stoichiometric ratio. $MgSO_4$ solutions and KH_2PO_4/Na_2HPO_4 solutions were prepared from analytical grade reagents. Freshly bidistilled water was used throughout.

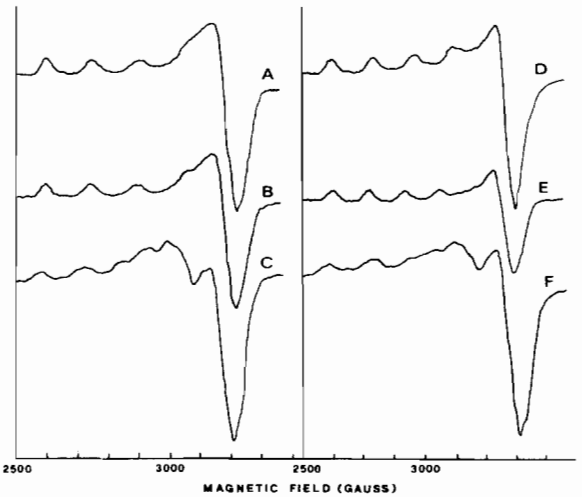

Fig. 1. ESR spectra of frozen solutions of copper alkaline phosphatase and its derivatives at 100 K, pH = 6.0. A) Cu_2AP, B) Cu_2Mg_4AP, C) Cu_4AP, D) $Cu_2AP{\cdot}1P_i$, E) $Cu_2Mg_4AP{\cdot}2P_i$, F) $Cu_4AP{\cdot}2P_i$.

Electronic spectra were recorded on a Cary 17D spectrophotometer. X-band EPR spectra were run on a Bruker 200TT or Bruker ER 200D at 100 K, and calibrated using diphenylpicrylhydrazyl (g = 2.0037).

Water proton longitudinal relaxation measurements were performed at room temperature in the magnetic field range 0.092–1.41 T (corresponding to proton Larmor frequencies of 4–60 MHz) using a Bruker CXP 100 FT instrument equipped with a Varian DA60 electromagnet.

Results and Discussion

The ESR Spectra

The ESR parameters for the various copper AP derivatives investigated are collected in Table I. As already reported, copper(II) ions added at pH 6.0 to alkaline phosphatase in a 2:1 ratio give rise to an EPR signal indicative of a single chromophore [19] (Fig. 1A) and assigned to copper(II) coordinated at the A sites. Such specificity of copper, which is also observed at alkaline pH [20], is at variance with the behavior of the more active cobalt(II) derivative [15] and possibly of the zinc(II) reconstituted enzyme itself [5, 10]. In the case of the M_2-derivatives with the latter two metal ions, addition of magnesium enhances the catalytic activity [10, 22], and apparently forces the catalytic metals to fully occupy the A sites [10]. In the present Cu_2-derivative, addition of four equivalents of magnesium at pH 6 causes only minor changes in the EPR spectrum, confirming that the two copper(II) ions are coordinated at the A

site even in the absence of magnesium. The minor changes (Fig. 1B) are attributed to conformational effects. The spectrum is slightly better resolved, and shows evidence of superhyperfine coupling with the histidine nitrogens of the A site. Four magnesium ions rather than two are required to develop these minor spectral changes. Since magnesium occupies first the C sites and subsequently the B sites, the presence of a metal in the nearby B site induces some changes although it does not substantially alter the coordination sphere of copper in the A site.

Binding of phosphate to metalloalkaline phosphatase at low pH values results in a more or less complete phosphorylation of a serine residue (Ser 99) [1] in the active site [12, 14]. As the pH is raised, phosphate is increasingly bound in a non-covalent fashion [24], probably through direct coordination to the metal. Both the overall affinity of phosphate and the equilibrium mixture of covalent *versus* non-covalent adducts depends, as well as on pH, on the kind of metal ions involved [1, 11, 25]. In any case, the presence of the metal is required for the phosphorylation to occur, and for the dephosphorylation to take place at alkaline pH. It is not well established whether the phosphate group of the phosphoryl enzyme is also coordinated to the metal.

No precise data are available on the fractional amount of phosphorylated adduct at low pH for the copper derivative [11]. Nonetheless the binding of phosphate at low pH, as monitored through ESR, follows the usual stoichiometry of 1:1 in the case of Cu_2AP (Fig. 1D) and 2:1 in the case of Cu_2Mg_4AP (Fig. 1E). However, the effects on the EPR spectra are strikingly different; in particular $A_\parallel$ is largely reduced by the presence of magnesium. Such small values of $A_\parallel$ have been observed only in pseudotetrahedral and distorted five coordinate complexes [18, 26, 27]. However, in the latter complexes the decrease in $A_\parallel$ is accompanied by an increase in rhombicity which is not observed in the present spectra. Therefore it is suggested that the copper in $Cu_2Mg_4AP{\cdot}2P_i$ is pseudotetrahedral. The derivative without Mg can be assigned a tetragonal geometry, probably five coordinate. The spectrum of $Cu_2Mg_4AP{\cdot}2P_i$ does not show evidence of heterogeneity, indicating that either the phosphoryl derivative is almost completely formed or the covalent and non-covalent adducts are spectroscopically indistinguishable.

The behavior of the Cu_2AP derivative remains however puzzling with respect to the interaction with phosphate, because if binding of phosphate is accompanied by migration of copper from one A site to the B site in the other subunit [10], the ESR spectrum should show evidence of two different copper chromophores. As a matter of fact a heterogeneous ESR spectrum completely different from that of the $Cu_2AP{\cdot}1P_i$ derivative is obtained when 4 equivalents of Cu and two equivalents of P_i are added to the apoenzyme (Fig. 1F). A further point which is not easily reconciled with the idea of a metal migration is that the ESR spectrum of the $Cu_2AP{\cdot}1P_i$ derivative develops instantaneously while, for instance, migration of cadmium from B to A sites requires a time of the order of several days [10].

Note that a heterogeneous spectrum would also be expected if the classical explanation for anti-cooperation is adopted, *i.e.*, binding of phosphate at the A site (the B site being empty) alters the A site in the other subunit to an A′ situation, incapable of binding another phosphate. Clearly, the anti-cooperative effects in alkaline phosphatase are far from being understood in detail.

Water Proton NMR Spectra

Assuming that the $Cu_2Mg_4AP{\cdot}2P_i$ system is close to a situation relevant from the point of view of the enzymatic function, *i.e.*, it may simulate an intermediate in the catalytic process, we have made a study using ^1H NMR spectroscopy.

The ^1H T_1^{-1} values of water solutions containing the systems Cu_2AP and $Cu_2Mg_4AP{\cdot}2P_i$ have been measured in the range of proton Larmor frequencies 4–60 MHz. The ^1H T_1^{-1} values are reported in Fig. 2 as compared to the apo AP solution values. The T_1^{-1} enhancements are indicative of exchangeable protons interacting with the paramagnetic center. The shape of the dispersion curve clearly indicates that the proton exchange time τ_M is not contributing significantly to the measured T_1 values, indicating that fast exchange conditions are met [28]. Furthermore, measurements at 4 and 35 °C have shown that the T_1^{-1} values are essentially temperature independent, while they are expected to exponentially increase with temperature in the case of exchange controlled processes. The T_1^{-1} values are sensibly larger for Cu_2AP solutions than for $Cu_2Mg_4AP{\cdot}2P_i$. At 60 MHz the ^1H T_1^{-1} value has also been measured for the Cu_2Mg_4AP, which is intermediate between the above two systems.

The frequency dependence of water proton relaxation has been analyzed in terms of the G and τ_c parameters according to the Solomon equation [29] as described elsewhere [30]. A reasonable value for the electronic relaxation times of copper ($\tau_c = 3 \times 10^{-9}$ s) in the Cu_2AP derivative is obtained, and a G value (3.0×10^{45} cm^{-6}) consistent with a regularly coordinated water molecule. Similar measurements at several frequencies were already performed on the Cu_2 derivative at pH 8, the conclusions being that a coordinated water molecule was present [31] although the G value calculated from the presented data is less than one half of our value at pH 6.0. If the chromophore is taken as substantially five-coordinated square pyramidal, consistent with the A values and with the presence of four nitrogen ligands [18], the water molecule can be placed in the axial position and

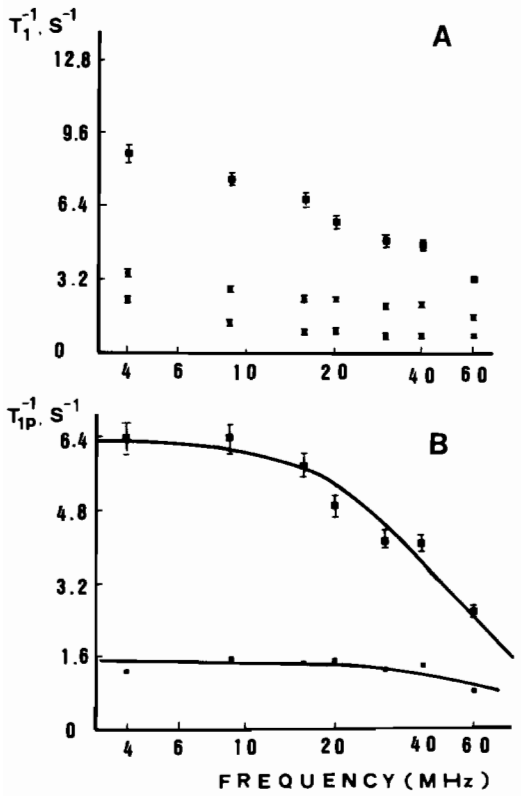

Fig. 2. A: ^1H T_1^{-1} values of water solutions of 8.7×10^{-4} *M* dimeric alkaline phosphatase at pH 6.0 at various magnetic fields. The data refer to apoAP, $Cu_2Mg_4AP \cdot 2P_i$, and Cu_2AP, in order of increasing T_1^{-1}. B: T_{1p}^{-1} values of Cu_2AP (upper) and $Cu_2Mg_4AP \cdot 2P_i$ (lower). The solid lines are the best-fit curves to the Solomon equation.

allowed to be coordinated at a somewhat longer distance (as often observed for axial ligands of copper) to account for the relatively small effect on water proton relaxation at high pH [31]. The paramagnetic effect in the $Cu_2Mg_4AP \cdot 2P_i$ derivative is strongly reduced, the G value being probably too small (1.0×10^{45} cm^{-6}) to be ascribed to coordinated water [30]. The EPR spectrum of the above derivative is indicative of tetracoordination, consistent with the removal of water; the resulting chromophore could result either from binding of phosphate and concomitant detachment of a histidine residue or, more likely, from an N_4 chromophore in which the presence of phosphoryl ester prevents the access of water to the coordination sphere. In this case the phosphoric ester would be only weakly interacting with the metal ion or not interacting at all.

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