A Proton Relaxation Rate Study of the Copper Analog of Escherichia coli Alkaline Phosphatase*

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RUTH S. ZUKIN† AND DONALD P. HOLLIS‡

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

Proton relaxation rates for the binary complex of the Cu²⁺ analog of Escherichia coli alkaline phosphatase and water have been measured. Titration of apoenzyme into copper salts shows approximately two tight copper-binding sites, \( K_i = 10^{-4} \text{M} \), and 13 weak sites per enzyme dimer molecule. Enhancement of \( 1/T_{1p} \) by a factor of 2 upon binding to enzyme is observed; this result for the first time suggests the participation of the metal in substrate binding. From the temperature dependence of \( T_{1p,*} \) and the fact that \( T_{1p} > T_{1p} \) it is concluded that \( T_{1p} \) is not exchange-limited. From the frequency dependence of \( T_{1p,*} \) and from the ratio of \( T_1 : T_2 \) we estimate a correlation time, \( \tau_m \), of \( 4.6 \times 10^{-9} \text{s}^{-1} \) which corresponds to the Cu²⁺-water proton distance \( r = 3.6 \text{ Å} \) when the number of ligand sites, \( q \), is 1 or \( r = 3.2 \) when \( q = \frac{7}{2} \). The addition of phosphate has little effect on \( T_{1p,*} \) for the copper enzyme. These results suggest that at least 1 water molecule or hydroxyl ion has access to the inner coordination sphere of the Cu²⁺ ion and that inorganic phosphate does not displace this ligand.

Escherichia coli alkaline phosphatase is a dimeric metalloenzyme which catalyzes the nonspecific hydrolysis of organic phosphomonoesters. The native enzyme, molecular weight = 80,000 to 86,000 (1, 2), contains 4 zinc atoms (3, 4) which can be replaced by numerous other divalent transition metal ions (4, 5). Only the zinc enzyme possesses complete catalytic activity; however, the cobalt and copper enzymes are partially active ( = 5% of the zinc-enzyme activity) analogue. Electron paramagnetic resonance data provide evidence that there are two specific tight copper-binding sites per enzyme dimer, as well as weak sites, and that the tightly bound copper atoms each bind at least three nitrogen ligands, probably histidyl side chains (11, 12). Moreover, EPR and Sephadex-binding studies suggest that the two tightly binding copper atoms compete for the catalytic zinc sites (11).

In the following section \( T_1 \) and \( T_2 \) are the observed spin-lattice and spin-spin relaxation times; \( T_{1p} \) and \( T_{2p} \) are paramagnetic contributions to \( T_1 \) and \( T_2 \); \( T_{1p,*} \) and \( T_{2p,*} \) are the values for the enzyme-bound metal; \( \tau_m \) is the correlation time; \( \tau_s \) and \( \tau_c \) are the rotational, electron spin, and chemical exchange correlation times, respectively; \( q \) is the number of ligands in the coordination sphere of the metal; and \( \epsilon_i \) is the observed enhancement of \( T_1 \). More detailed definitions and discussions of these terms can be found in the references (9, 13).

Alkaline phosphatase was isolated from E. coli C 90 (a generous gift from Dr. B. Bachmann of the Genetic Stock Center, Yale University) and purified by a modification of the original Malamy and Horecker procedure (11), using spheroplast formation and, in the final purification and concentration step, batch addition of triethylaminoethylcellulose (Bio-Rad). Only enzyme with specific activity \( \geq 50 \text{ pmoles mg}^{-1} \text{ min}^{-1} \) which corresponds to the maximal reported activity was used. The enzyme was found to be homogeneous by ultracentrifugation.

Apoenzyme was prepared by incubation of the purified native enzyme with Chelex (Bio-Rad) as described by Csopak (15). Copper-alkaline phosphatase was prepared by direct titration of spectroscopically pure CuSO₄ (Fisher "Spec-Pure"), CuCl₂ or Cu(NO₃)₂ ("Baker analyzed" reagent) into the apoenzyme. Zinc removal and copper incorporation were monitored by atomic absorption spectrometry.

Water \( T_S \) were measured using the pulsed NMR method of Carr and Purcell (10) with a nuclear magnetic resonance pulsed spectrometer operating at 8.1, 24.3, or 40.0 MHz. \( T_1 : T_2 \) ratios were measured on a Varian NMR spectrometer, equipped with Fourier Transform and operating at 220 MHz; \( T_1 \) and \( T_2 \) were determined by the null method and \( T_S \) were determined by line width measurements.

Fig. 1 shows the titration of 0.21 mM apoenzyme phosphate in 10 mM Tris·Cl buffer, pH 7.0, at 4°, with CuSO₄, CuCl₂, and Cu(NO₃)₂. \( 1/T_{1p,*} \) varies linearly with copper concentration and \( \epsilon_1 = \epsilon_2 = 2.2 \), until [Cu]/[enzyme] = 2; \( 1/T_{2p,*} \) versus [CuSO₄] then becomes nonlinear with a decreasing slope. \( 1/T_{1p,*} \) at 25° for a solution of 0.40 mM CuSO₄ at the same enzyme concentration is 0.26 s⁻¹; thus, the paramagnetic contribution to the spin-lattice relaxation rate shows a negative temperature coefficient.

The titration of CuSO₄ into the reconstituted Zn₂⁺-enzyme is also shown in Fig. 1; no enhancement within experimental error is observed. This result suggests that copper added to apoenzyme binds at the catalytic zinc sites. This possibility is substantiated by EPR data (Taylor and Coleman (11)) which show that addition of two copper to apoenzyme results in a spectrum with high g values and nitrogen splitting, but that addition of copper to Zn₂⁺-enzyme does not produce this effect. Moreover,
it is unlikely that these observations represent a conformational change in the apoenzyme when it is reconstituted with zinc, since neither ultraviolet optical rotary dispersion nor circular dichroism studies of alkaline phosphatase (17) show any change in the enzyme spectrum when zinc is added.

Fig. 2 shows a Scatchard plot (18) for the binding of CuSO₄ to apoalkaline phosphatase at 4°. As can be seen there are between two and three specific tight binding sites for copper, Kᵣ = 1.0 μM, and 13 weak sites, Kᵣ = 50 μM. The data were obtained from the titration of apoenzyme into a solution that is 0.5 mM CuSO₄ in 10 mM Tris-Cl, pH 7.0. Formation of the metalloenzyme was followed by the enhancement parameter, ε, obtained at 24.3 MHz; ε₀ = 2.2 was obtained from Fig. 1.

The data in Table I illustrate the frequency dependence of 1/T₁ₚ* for 0.125 mM copper-alkaline phosphatase in the same buffer at three copper concentrations and three frequencies. 1/T₁ₚ* is seen to be frequency-dependent for a given concentration. Fig. 3 shows a plot of T₁ₚ* versus ω². The correlation time, τₑ, as determined from (slope/intercept)², is 4.6 × 10⁻⁹ s.

Table II summarizes the T₁ and T₂ data at 220 MHz for 0.125 mM apoenzyme and 0.2 mM CuSO₄ in the above buffer. 1/T₁ₚ* is greater than 1/T₁ₚ* by a factor of 26. No chemical shift in the enzyme sample is detected upon broadening of the H₂O proton signal by copper.

Table III shows ρ calculated from the T₁/T₂ ratio and from the plot of 1/T₁ₚ* versus ω. As the value derived from the T₁/T₂ ratio represents a lower limit for ρ, 4.6 × 10⁻⁹ s is probably the better estimate. This corresponds to τᵣ = 3.6 A when q = 1.

Addition of a 2-fold excess of inorganic phosphate to 0.125 mM
apoenzyme, 0.250 mM CuSO4 at pH 7.0 results in no change of 1/T_{1p}^{*} with experimental error.

The data for the titration of apoenzyme with copper indicate the existence of two copper sites per dimer of alkaline phosphatase. These titrations have been carried out numerous times at a variety of enzyme concentrations and temperatures; a constant ε = 2 is always observed until [Cu^{II}]/enzyme = 2 after which ε decreases. The Scatchard binding plot, based on data for the titration of copper with apoenzyme, indicates between two and three tight copper sites per dimer; the inherent error in this analysis, however, is greater. Together, the data suggest that there are two tight copper sites, in agreement with atomic absorption data reported by Csapak (7), which also shows two copper sites, and in contrast to reports which show four sites for zinc, manganese, and cobalt (4).

T_{1p}^{*} has a negative temperature coefficient. In addition, T_{1p} > T_{2p}. These results clearly indicate that T_{1p} is not exchange-limited and that, therefore, \( f = T_{1p}^{*} = T_{2p} \). Thus, if \( \tau_B \) is known, \( f = T_{1p}^{*} \) can be used to calculate the interatomic distance, \( r \), between the water protons and the copper atom.

We have estimated \( \tau_B \) by two methods (Table III). First, we have determined \( \tau_B \) graphically by a plot of T_{1p}^{*} versus \( \alpha^2 \) (Fig. 2) (19). The square root of the slope/intercept affords a value of 4.5 \times 10^{-8} \text{s} for \( \tau_B \). (The fact that the plot is linear is good evidence that \( \tau_B \) is not dominated by \( \tau_B \) (9).) We have also calculated a lower limit for \( \tau_B \) from the T_{1}/T_{2} ratio (20); this method gives a value of 4.6 \times 10^{-8} \text{s} for \( \tau_B \). The two values for the correlation time and that, therefore, f = T_{1p}^{*} = T_{2p}.

Finally, it was noted that addition of excess phosphate had little effect on 1/T_{1p}^{*} for the copper enzyme. This result suggests that the phosphate does not replace a rapidly exchanging water in the first coordination sphere, although it may replace a slowly exchanging one.

Together these results suggest a tentative model for copper-alkaline phosphatase, with the following features: (a) at least 1 water molecule or hydroxyl ion can exchange on and off of the metal sites of the enzyme; (b) this water molecule or hydroxyl ion actually enters the first coordination sphere of the copper ion; and (c) inorganic phosphate binds at a site other than the copper site or it binds at the copper site without displacing the H_{2}O or OH^- ligand. It should be noted that these results do not exclude additional, slowly exchanging water molecules, which would not be detected by these methods.

It is significant that in the case of the partially active copper analogue of E. coli alkaline phosphatase, metal is implicated in H_{2}O binding, whereas this is apparently not true for the inactive manganese enzyme. These findings suggest the need for further investigation into possible correlations between H_{2}O-metal distances for the various phosphatase analogs and relative activities. These studies are now in progress in this laboratory.

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