PHOSPHORUS-31 RELAXATION RATE STUDIES OF Mn^{2+} - ALKALINE PHOSPHATASE

by

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SUMMARY

Phosphorus-31 NMR relaxation rates for the ternary complex of manganese-alkaline phosphatase-phosphate have been measured and their temperature dependence studied. The exchange of phosphate into the complex is exchange limited with respect to the transverse relaxation rate but is fast with respect to longitudinal relaxation. The data show that the observed phosphate relaxation is an outer-sphere effect. The activation energy for phosphate exchange is E_a= 8 Kcal/mole as determined from the temperature dependence of the line width of the phosphorus resonance.

E.coli alkaline phosphatase is a metalloenzyme reported to contain 4 zinc atoms[1,2] per dimer of molecular weight 80, -86,000[3,4]. The zinc atoms can be replaced by a number of other divalent metal ions including Mn^{2+}, Co^{2+}, Cd^{2+}, and Cu^{2+} [2,5]. Whereas each of the metalloenzymes can bind inorganic phosphate, and the zinc and cobalt enzymes possess whole or partial catalytic activity[6,7], the apoenzyme is inactive and cannot bind the phosphate ion[7]. Thus, the metal ion is implicated in both the catalytic and binding actions of alkaline phosphatase.

Manganese alkaline phosphatase provides a paramagnetic probe for studying substrate binding by magnetic resonance techniques. We have therefore chosen to study the binding of phosphate to the manganese enzyme by ^{31}P relaxation measurements on the phosphorous atom of inorganic phosphate. In the following

Table 1. ^{31}P relaxation rates of inorganic phosphate (P_i) in the presence of free and enzyme-bound Mn^{2+}; all solutions are 10 mM P_i buffer, pH 6.0, 25° C.

Sample	[alkaline-phosphatase] x10^3M	$[Mn]_{total}$* x10^3M	^{31}P line width (Hz)	$1/T_2$ (sec^{-1})	$1/T_1$† (sec^{-1})
1	0.00	0.00	5 ± 2	18 ± 6	0.08 ± 0.01
2	0.00	0.10	>1000	>3141	
3	0.10	0.10	46	144	1.80 ± 0.2
4	0.00	0.40	>1000	>3141	
5	0.40	0.40	180	567	5.00 ± 0.5

*as determined by atomic absorption spectrometry.
†as determined by the plotting method; all spectra were obtained using a Varian XL-100 NMR spectrometer operating at 40.3 MHz and equipped with an F.T. accessory.

sections T_1 and T_2 are observed spin-lattice and spin-spin relaxation times; T_{1p} and T_{2p} are paramagnetic contributions to T_1 and T_2; T_{1M} is the spin-lattice relaxation of a small molecule in the first coordination sphere of a paramagnetic ion; T_{1p}^* and T_{2p}^* are T_{1p} and T_{2p}, respectively, for the enzyme-bound metal; τ_m is the chemical exchange correlation time; and ϵ_1 is the observed enhancement of T_1. More detailed definitions and discussions of these terms can be found in the references[8,9].

Results:

The data in Table I illustrate the ^{31}P line broadening of a solution of 10 mM phosphate buffer, pH 6.0, at 2 concentrations of manganous ion, both in the free and enzyme-bound forms. Comparison of the $1/T_2$ values, as determined from the line width, shows a marked deenhancement, $\epsilon_2 < 0.05$, of the phosphorous relaxation rate. The $1/T_1$ values for the phosphate-enzyme solutions are also shown in Table I. $1/T_2$ is greater than $1/T_1$ by a factor of approximately 100. Figure 1 shows the titration of 10 mM orthophosphate buffer, pH 6.0, with Mn^{2+}-alkaline phosphatase; $1/T_{2p}^*$ varies linearly with metalloenzyme concentration. No chemical shift is detected upon broadening of the ^{31}P signal of phosphate by enzyme.

Table II. Temperature dependence of ^{31}P relaxation rates of an inorganic-phosphate-enzyme complex; all solutions are 10 mM P_i buffer, pH 6.0, and 25 μM Mn^{2+}-alkaline phosphatase.

T(° C)	line width(Hz)	$1/T_2$(sec^{-1})	$1/T_{2p}$(sec^{-1})	$1/T_1$(sec^{-1})	$1/T_{1p}$(sec^{-1})
1	16 ± 2	50 ± 6	32 ± 6		
5	18	57	39	2.04 ± 0.2	1.96 ± 0.2
10	23	72	54		
20	26	81	63		
25	46	144	126	1.82	1.74
40	58	181	163	1.67	1.59

Table II shows the effect of temperature on ^{31}P line broadening of a solution of 10 mM P_i, pH 6.0, and 25 μM Mn^{2+}-alkaline phosphatase. $1/T_{2p}$ increases with increasing temperature. $1/T_{1p}$ values at 3 temperatures are also shown in Table II; the paramagnetic contribution to the spin-lattice relaxation rate shows a negative temperature coefficient.

Figure 2 shows an Arrhenius plot of $1/f\ T_{2p}^*$ vs. $1/T$. The activation energy, E_a, as determined from the slope, is 8.0 Kcal for this process.

Discussion:

The large positive temperature coefficient of $1/T_2$ shows that $1/f\ T_{2p}$ is dominated by $1/\tau_m$; i.e., ^{31}P spin-spin relaxation for the phosphate-enzyme complex is an exchange limited process. This fact enables graphic determination of the activation energy for the phosphate-manganese on-off exchange from the slope of an Arrhenius plot of $1/\tau_m$ vs. $1/T$; E_a, obtained in this manner, is 8 Kcal/mole. This value probably represents an ionization energy for the removal of negatively charged phosphate ion from the ligand sphere of a positively charged manganous ion.

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, $1/f\ T_{1p}^* = 1/T_{1M}$. Thus, if τ_c is known, $1/f\ T_{1p}^*$ can be used to

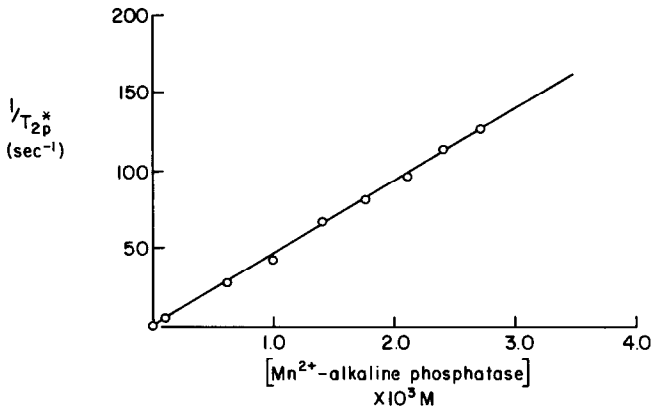

Figure 1: Titration of 10 mM phosphate buffer, pH 6.0, 25° C, with Mn^{2+}-alkaline phosphatase ($^1/T_{2p}^*$ is shown for the ^{31}P signal of inorganic phosphate). Alkaline phosphatase was purified by a modification of the original Mallamy and Horecker procedure[10]; apoenzyme was prepared by Chelex treatment as described by Csopak[11], then titrated directly with manganese, followed by exhaustive dialysis. Zinc removal and manganese incorporation were monitored by atomic absorption spectrometry.

calculate the interatomic distance, r, between the phosphorous and manganese atoms.

We have estimated τ_c from the T_1/T_2 ratio. This procedure assumes a small contact term in the Solomon-Bloembergen equation for $^1/T_{2M}$. (This assumption may be of limited validity as suggested by the relaxation rate studies of Nowak, et al[12], on enolase in which they calculate different τ_c values depending on whether they use proton or phosphorous relaxation rates.) The value which we calculate for τ_c is 1.86×10^{-8} seconds. This corresponds to r = 18 Å, a value much too large to represent inner sphere relaxation. Note, for example, that Cottam and Thompson[13] found a distance of ~9 Å for H_2O-Mn^{2+} for this same enzyme. The somewhat longer distance in the case of phosphate may reflect the long P-O bond (≈ 3 Å) or possibly a water molecule interposed between the manganous and phosphate ions.

The marked deenhancement observed for $^1/T_{1p}$ on binding corroborates the idea that outersphere relaxation is the main contribution to the observed T_1, but does not exclude the possibility of a very slowly exchanging phosphate occupying one or more inner sphere positions. The marked inequality of T_1 and T_2 may be due to an extension of the contact interaction into the second

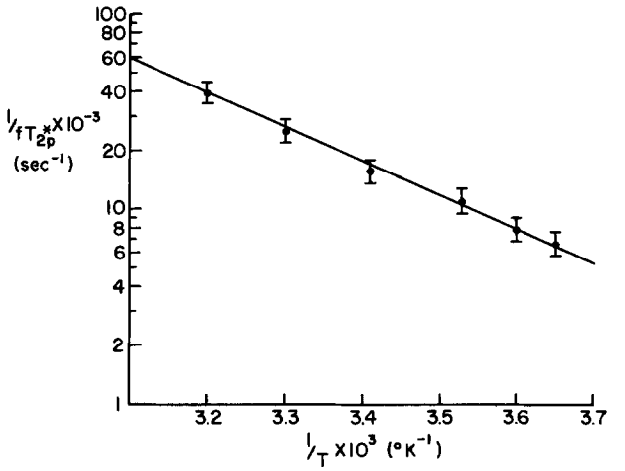

Figure 2: Arrhenius plot of $1/f\ T_{2p}^*$ (= $1/\tau_m$) vs. $1/T$ for a solution of 10 mM P_i, pH 6.0 and 25 μM Mn^{2+}-alkaline phosphatase. The activation energy is calculated from the slope according to the relation $\log 1/k = E_a/2.303\ k \cdot 1/T + \ln A$. E_a = 8.0 Kcal.

sphere as described by Alei, 1964[14], Brown and Drago, 1970[15], and as found by Nowak, et al, 1973[12], for enolase.

It is interesting to compare the ^{31}P relaxation rate studies on phosphate binding to alkaline phosphatase with the water proton relaxation rate data as reported by Cottam and Thompson[11]. They report a deenhancement, $\varepsilon_1 = 0.4$, for $1/T_{1p}$ of the H_2O protons, and attribute this deenhancement to outer-sphere relaxation. We have confirmed their results and, in addition, have found similar behavior for Co^{2+}-alkaline phosphatase (results to be published elsewhere) in two respects: 1) deenhancement is observed and 2) there are 4 indistinguishable metal binding sites. Both the PRR and the ^{31}P relaxation rate measurements for alkaline phosphatase suggest outer-sphere relaxation but do not exclude a very slowly exchanging inner sphere ligand.

It should also be noted that the value obtained for the rate constant $1/\tau_m$ for phosphate dissociation is about 10^5 sec^{-1} as compared to a value of about 70 sec^{-1} for the turnover rate of the enzyme under optimum conditions. Thus, the enzyme-phosphate interaction time which we observe is consistent with the interaction being part of the enzyme mechanism.

Conclusions:

From this work we draw the following conclusions:

1) From the ^{31}P NMR, an outer-sphere complex between Mn^{+2}-alkaline phosphatase and inorganic phosphate has been detected. The phosphorous-Mn^{+} distance is estimated as r = 18 Å.

2) The lifetime of the detected Mn^{+2} alkaline-phosphatase-inorganic phosphate complex is much shorter than the enzyme turnover rate, a fact consistent with the participation of the complex in the enzymatic mechanism.

3) From temperature studies it is concluded that the longitudinal relaxation rate of phosphate in the complex is not limited by the phosphate exchange rate while transverse relaxation is exchange limited. The activation energy for transverse relaxation is E_a = 8.0 Kcal/mole.

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