Electron Paramagnetic Resonance Study of the Interaction of Yeast Enolase with Activating Metal Ions*

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ABSTRACT: The interactions of Mn^{2+}, Mg^{2+}, and Cu^{2+} with yeast enolase (phosphoenolpyruvate hydratase, EC 4.2.1.11) have been studied with electron paramagnetic resonance. The data at room temperature support quantitatively previous equilibrium dialysis and gel filtration results and confirm the presence of two Mn^{2+} binding sites. Competition experiments showed that the tighter bound Mg^{2+} ion competes against the weaker bound Mn^{2+} ion at low Mg^{2+}/Mn^{2+} ratios, and that Mg^{2+} ion also competes for the tight Mn^{2+} binding sites at high ratios. Additional Mn^{2+} binding sites were detected at 77°K which appear to be situated in the vicinity of the tighter bound Mn^{2+}, the exchange coupling constant is about 0.0086 cm^{-1}.

An analysis was made of those factors which determine the electron paramagnetic resonance line width of metal ions bound to enolase.

Westhead and coworkers (Gawronski and Westhead, 1969; Hanlon and Westhead, 1965, 1969a,b) used difference spectroscopy, equilibrium dialysis, gel filtration, and kinetic methods to demonstrate that phosphoenolpyruvate hydratase (enolase, EC 4.2.1.11) possesses two binding sites for Mg^{2+} and for Mn^{2+} ions. Only one binding site per enolase molecule was detected by earlier magnetic resonance studies (Malmström, 1953; Cohn, 1963). One of the possible sources for the discrepancy could be the degree of contamination by extraneous metal ions. The central objective of this work is to resolve this question of metal ion binding stoichiometry using electron paramagnetic resonance technique.

Electron paramagnetic resonance has been employed extensively in the study of metalloenzymes with impressive results. Some of the notable examples are the determination of heme-plane orientations in acid methemoglobin (Bennet *et al.*, 1957) and the determination of ligand binding stereochemistry in nitrosylhemoglobin (Chien, 1969) and in nitrosylmyoglobin (Chien and Dickinson, 1971). By comparison, the application of electron paramagnetic resonance to the study of metal ion activated enzymes has been much less extensive. One of the reasons may well be attributed to the incomplete understanding of factors governing the line widths of solution electron paramagnetic resonance spectra. Therefore, an ancillary objective of this work is to shed light on those factors which determine the electron paramagnetic resonance line widths of Mn^{2+} and Cu^{2+} ions bound to enolase in solution.

Experimental Section

Materials. Yeast enolase was prepared by the procedure of Westhead and McLain (1964). The enzyme was made metal free by a preliminary dialysis against 10^{-3} M NH_3 during which time the pH of the enzyme solution did not rise above 8.5. This was followed by slow passage through a 1.5 × 25 cm column of mixed-bed ion-exchanger Dowex 501 eluted with deionized water. The enzyme solution was then raised to pH 7.7 ± 0.1 and stored in acid-washed polyethylene containers at a protein concentration of at least 10 mg/ml to prevent inactivation. The enzymes used in this work had specific activities of 290–310 units under standard assay conditions (Westhead and McLain, 1964). The specific activities of occasional samples were checked following electron paramagnetic resonance experiments. In no case was there less than 80% of the original activity even after prolonged experiments at low metal concentrations. Metal salts used were all in the form of their hydrated dichlorides and were Baker Analyzed reagents. KCl was Ultra Pure grade from Alfa Inorganics, Beverly, Mass.

Electron Paramagnetic Resonance Measurements. Electron paramagnetic resonance spectra were obtained with a Varian E-9 spectrometer. Quartz 4-mm outside diameter tubes were used for 77°K measurements. The sample was frozen slowly to avoid shattering of the sample tube. It was found that the frozen enzyme was uniform as indicated by the reproducible electron paramagnetic resonance spectra obtained after repeated freezing and thawing. However, possible concentration gradients caused by slow freezing cannot be discounted entirely.

Quantitative electron paramagnetic resonance measurements on the concentrations of free Mn^{2+} ions were made with a flat aqueous cell at room temperature. Microwave leakage, frequency, and power were maintained constant in these determinations. The concentration was calculated from the first moment of electron paramagnetic resonance spectrum.

Results and Discussion

Interactions of Mn^{2+} and Mg^{2+} with Yeast Enolase. Previous studies by Westhead and coworkers (*loc. cit.*) showed the presence of two Mn^{2+} binding sites and two Mg^{2+} binding sites on enolase. Only one binding site was found by electron paramagentic resonance and nuclear magnetic resonance techniques (Cohn, 1963; Malmström, 1953). The results of this work support the findings of Westhead and coworkers.

At [enolase] = 0.36 mM and [$MnCl_2$] up to 0.41 mM, there were no electron paramagnetic resonance spectra at 298°K. Apparently, Mn^{2+} ions attached to either the strong or the weak binding sites have very broad line widths (Malmström *et al.*, 1958). At [$MnCl_2$] = 0.9 mM there was found 0.41 mM

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TABLE I: Competition of Mn^{2+} and Mg^{2+} Ions for Binding Sites on Yeast Enolase.

			$[Mn^{2+}]$, mM	
[Enolase], mM	$[Mn^{2+}]_0$, mM	$[Mg^{2+}]_0$, mM	Found by Electron Paramagnetic Resonance	Calculated
0.36	0.24	1.0	0	0
0.345	0.405	1.0	0.040	0.048
0.33	0.90	1.0	0.42	0.50
0.24	0.16	7.1	0.12	0.15
0.24	0.29	6.6	0.21	0.24
0.255	0.70	5.3	0.45	0.49

of free Mn^{2+} at room temperature. Assuming that the two Mn^{2+} binding sites have independent stability constants, the calculated free Mn^{2+} concentration of this experiment is 0.59 mM.

One particularly interesting property of yeast enolase is that the tighter bound Mg^{2+} ion competes with the weaker Mn^{2+} ion and the weaker Mg^{2+} site is probably the same as the tighter Mn^{2+} site (Hanlon and Westhead, 1969a). If one designates the Mn^{2+} binding sites as α, β, and γ in order of decreasing affinity, then the observed Mg^{2+} binding is stronger at the β site and weaker at the α site. Hanlon and Westhead (1969a) gave the following dissociation constants in the absence of competing ions: $K_{Mn}{}^{\alpha} = 1.3 \times 10^{-6}$, $K_{Mn}{}^{\beta} = 1.9 \times 10^{-5}$, $K_{Mg}{}^{\alpha} = 4.7 \times 10^{-4}$, and $K_{Mg}{}^{\beta} = 0.96 \times 10^{-5}$ all units of mole $l.^{-1}$. In the presence of Mg^{2+}, the dissociation constants for Mn^{2+} are modified so that: $K_{Mg}{}^{\alpha} = K_{Mg}{}^{\alpha}(1 + [Mg]/K_{Mg}{}^{\alpha})$ and $K_{Mn}{}^{\beta} = K_{Mn}{}^{\beta}(1 + [Mg]/K_{Mg}{}^{\beta})$. Electron paramagnetic resonance measurements were used here to verify these results.

The amounts of unbound Mn^{2+}, determined at various protein, Mg^{2+}, and Mn^{2+} concentrations, are given in Table I, column 4. The amounts calculated with the above dissociation constants are summarized in column 5. The calculations were done stepwise, first for the α site and then for the β site. The agreement seen for the two columns are quite satisfactory. Only small errors are involved in this calculation because of the tenfold difference of the binding constants. The electron paramagnetic resonance measurements are in agreement with the equilibrium dialysis experiments.

Although our electron paramagnetic resonance studies so far do not detect spectral differences between the two Mn sites, the competitive binding pattern shown by Mg and Mn at the two sites allows us to conclude that the two sites are not independent. Enolase appears to be composed of two identical chains (Brewer *et al.*, 1971); if so, we can conclude that there is a strong negative interaction between the metal sites, probably of an allosteric nature. An interesting comparison can be made to the copper–alkaline phosphatase complex (Csopak and Falk, 1970). This enzyme is also composed of two identical chains, and binds 2 moles of Cu^{2+}. However, the electron paramagnetic resonance spectra in this case show distinguishably different patterns for the two copper ions.

Evidence for an Additional Mn^{2+} Binding Site. Because both the strongly and the weakly bound Mn^{2+} have electron paramagnetic resonance line widths too broad for observation at 298°K, measurements were extended to 77°K. In the solid state, the line width can be attributed entirely to the random orientation of the spins when the life time of the ground state is not limited by the direct or Raman relaxation mechanisms (Van Vleck, 1940). Figure 1 is an electron paramagnetic resonance spectrum of Mn^{2+} bound to the α site on enolase. The molar ratio of Mn^{2+} to enolase is 0.58. The hyperfine coupling constant is 87.5 G. At a higher ratio of 1.17, Figure 2 was obtained. Here all of the α sites and a fraction of the β sites are occupied by Mn^{2+}, assuming that the affinities of the sites for Mn are the same at 77 and 298°K. The two figures are essentially the same except for their relative intensities. It should be pointed out that these spectra are also not greatly different from a polycrystalline spectrum of free Mn^{2+} ion. Therefore, free Mn^{2+} ion and Mn^{2+} bound to either α site or β site have similar and small g and A anisotropies and zero-field splittings.

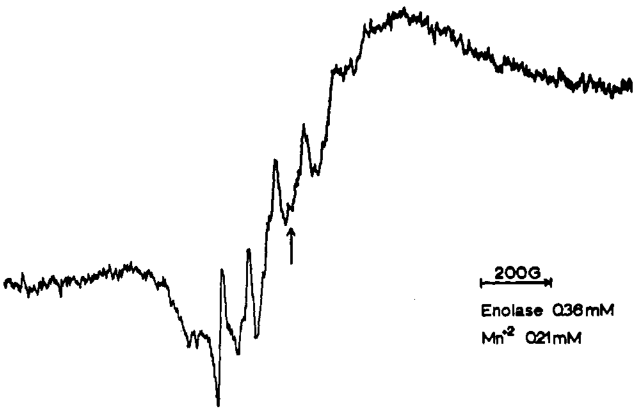

FIGURE 1: Electron paramagnetic resonance spectrum of Mn^{2+} bound to the α site at 77°K. [Enolase] = 0.36 mM, $[Mn^{2+}]$ = 0.21 mM. (Marker is DPPH; receiver gain: 2 × 10^4; modulation amplitude 8.2 G.)

Dramatic changes were observed when $[Mn^{2+}]$/[enolase] exceeds two. Figure 3 is an electron paramagnetic resonance spectrum obtained at $[Mn^{2+}]$/[enolase] = 2.7. At high gain there is resolved 36 components with an average separation of 40.7 G. This type of spectrum was never observed for comparable or higher concentrations of Mn^{2+} ions in the absence of enolase. The spectrum cannot be attributed to various types of noninteracting bound and/or free Mn^{2+} ions. Firstly, since most Mn^{2+} ions have g values near 2.0, this interpretation would require different species with hyperfine coupling con-

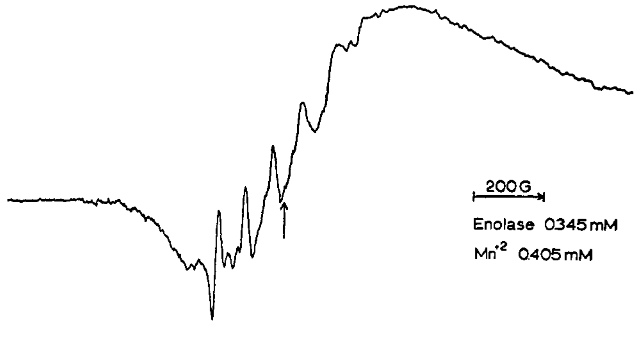

FIGURE 2: Electron paramagnetic resonance spectrum of Mn^{2+} bound to both the α and β sites at 77°K. [Enolase] = 0.345 mM, $[Mn^{2+}]$ = 0.405 mM. (Marker is DPPH; receiver gain: 8 × 10^3; modulation amplitude 8.2 G.)

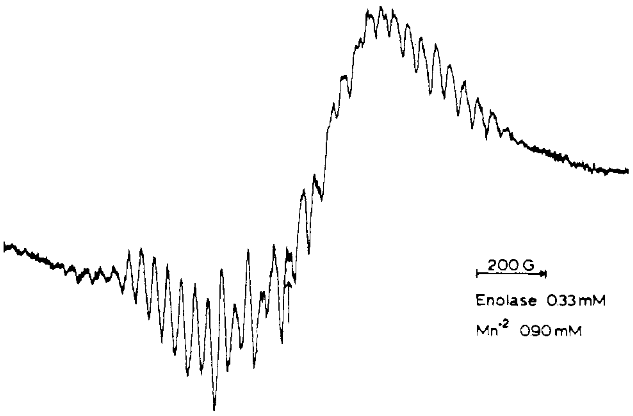

FIGURE 3: Electron paramagnetic resonance spectrum showing exchange coupled pairs of Mn^{2+} ions on enzyme. [Enolase] = 0.33 mM, $[Mn^{2+}]$ = 0.89 mM. (Marker is DPPH; receiver gain: 8 × 10^3; modulation amplitude 3.2 G.)

stants of about 40, 80, 120 G, etc. Secondly, it would be difficult to rationalize the sudden appearance of the 36-line spectrum when the Mn^{2+}/enolase ratio exceeds two. The most probable interpretation is that two Mn^{2+} ions are bound to sites in close proximity to one another and that exchange coupling between them gives rise to the additional electron paramagnetic resonance lines.

Slichter (1955) was the first one to point out that, when there are two interacting paramagnetic centers, the spin Hamiltonian is given by

$$\mathcal{H} = g\beta(S_{1z} + S_{2z})H_0 + a(I_1 \cdot S_1 + I_2 \cdot S_2) + JS_1 \cdot S_2 \quad (1)$$

where a is the electron–nuclear hyperfine coupling constant, J is the electron–electron exchange coupling constant, I is the nuclear angular momentum, S is the electron angular momentum, and subscript z refers to the z component of the angular momentum. The diagonal element when $J \ll a$, is

$$E = g\beta(m_1 + m_2)H_0 + a(m_1M_1 + m_2M_2) \quad (2)$$

where m and M refer to the spin quantum numbers for the electron and nucleus, respectively. At the limit of $J = 0$, there are only $2I + 1$ lines separated by the hyperfine constant a. When $J \neq 0$, the electrons can be considered as forming a system of singlet and triplet with quantum numbers S and m

$$S = 0, 1 \quad m = S, S - 1, \text{--------} -S \quad (3)$$

The diagonal terms of eq 1 is now

$$E = g\beta mH_0 + \frac{J}{2}\left[S(S + 1) - \frac{3}{2}\right] + \frac{a}{2}[m_1M_1 + m_2M_2] \quad (4)$$

In the case of strong exchange, *i.e.*, $J \gg a$, there are $(4I + 1) = 21$ lines with spacing of $a/2$. Forbidden transitions ($\Delta m = \pm 2$) can sometimes be observed under favorable conditions at half of the normal magnetic field.

Finally, when $J \gtrsim 2a$, it can be shown (Sakun *et al.*, 1965) that there would be $(2I + 1)^2 + 2$ lines. For Mn($I = 5/2$) a total of 38 lines in three frequency groups of $(a/2)(M_1 + M_2) + \{J^2 + [(a/2)(M_1 - M_2)]^2\}^{1/2}$, $(a/2)(M_1 + M_2)$, and $(a/2) \times (M_1 + M_2) - \{J^2 + [(a/2)(M_1 + M_2)]^2\}^{1/2}$. The observed spectra (*i.e.*, Figure 3) corresponds to such a case; J is estimated to be about 0.0086 cm^{-1}. The additional binding site which causes the appearances of extra resonance lines will be refered to as γ site.

If there is only one such additional Mn^{2+} binding site on an enolase molecule, it would be important to discover whether the observed exchange coupling is between Mn^{2+} ions on the α and γ sites or those on the β and γ sites. The following experiments show that the Mn^{2+} ions on the α and γ sites are definitely coupled by exchange interaction. When 1 mM of Mg^{2+} was added to a solution containing 0.345 mM of enolase and 0.407 mM of Mn^{2+}, the 36-line electron paramagnetic resonance spectrum was obtained (Figure 3). A similar spectrum was obtained with 0.93 mM of the Mn^{2+} and 0.345 mM of enolase. With these concentrations the data obtained at 298° and the data of Hanlon and Westhead show that no Mn^{2+} ion will be in the β site.

Though the above result confirms the exchange coupling of Mn^{2+} ions bound to α and γ sites, it does not exclude any β–γ interaction. The possibility that there are actually two γ sites per enolase molecule, one on each of the two subunits, is attractive for symmetry reasons.

In a series of metal ion competition experiments, incremental amount of Mg^{2+} ions added to a solution of 0.1 mM of yeast enolase and 0.33 mM of Mn^{2+} and the electron paramagnetic resonance spectra recorded at 77°K. From the relative heights of the low-field lines which are due to exchange interaction and of the midfield lines which becomes prominent when free Mn^{2+} ions are present in large amount, it is estimated that roughly half of the exchange coupled Mn^{2+} ions can be decoupled by Mg^{2+} ions when $[Mg^{2+}]/[Mn^{2+}] = 24$. At these conditions, less than 10% of the Mn^{2+} ions on the α site is displaced by Mn^{2+} ions at room temperature. It is likely that the competition is primarily between Mg^{2+} and Mn^{2+} on the β and γ sites.

The nature of the additional γ binding site or sites is yet unknown. A trivial origin is the adsorption of Mn^{2+} ions on the enzyme surface at low temperatures, another one is the association of Mn^{2+} ions. The latter can be discounted because exchange-coupled Mn^{2+} pairs were not observed in the absence of enolase. We have no data to rule out the first possibility. However, the results on Cu^{2+}–enolase interaction (*vide infra*) suggests that it is unlikely. Cu^{2+} ions are known to have a propensity for dimerization (Boyd *et al.*, 1971, and references cited therein). There was no evidence for exchange-coupled Cu^{2+} ions under conditions where exchange coupled Mn^{2+} ions were detected. Furthermore, electron paramagnetic resonance spectra with exchange-coupled Mn^{2+} become discernible whenever there is a slight excess of Mn^{2+} above that bound to the β site. For example, in the experiment already mentioned above, with [enolase] = 0.345 mM, $[Mn^{2+}]$ = 0.495 mM, and $[Mg^{2+}]$ = 1 mM, the electron paramagnetic resonance spectra showed prominently the characteristics of exchange interaction. In this experiment the maximum amount of free Mn^{2+} ion is only 0.06 mM.

A preferred interpretation is that the γ site is situated in the vicinity of the α site and that it binds Mn^{2+} only weakly but has a definite preference for Mn^{2+} ion over Mg^{2+} ions.

A study of Co^{2+} binding sites on enolase, using H_2O_2 oxidation of Co^{2+} as a probe, has shown that the first 2 equiv of Co^{2+} are not accessible to H_2O_2 oxidation, but that with a slight excess of Co^{2+}, inactivation of the enzyme is extremely efficient. The possibility of an electron-exchange interaction between α and γ cobalt sites, analogous to the reactions observed here between α and γ manganese sites is indicated (Kowolsky and Westhead, in preparation).

TABLE II: Cu^{2+} Resonance Spectra.

Complexing Agents	Molar Ratio[a]	Temp (°K)	g Values			Line Width (G)	Hyperfine Coupling Constants (G)		
			g_{iso}	$g_{\parallel}$	$g_{\perp}$		A_{iso}	$A_{\parallel}$	$A_{\perp}$
None		298	2.162			141			
EDTA	25	298	2.115			174[b]	51.5		
3-PGA[f]	250	298	2.101			126[b]			
2-PGA	25	298	2.130			160[b]			
Enolase	25	298				*c*			
None		77		2.134	2.053	*d*			
EDTA	25	77		2.243	2.078	70[e]		132	
3-PGA	250	77		2.315	2.067	85[e]		116	
2-PGA	25	77		2.260	2.023	60[e]		121	
Enolase	25	77		2.210	2.048	90[e]		151	

[a] Ratio of complexing agent concentration to [Cu^{2+}]. [b] Low signal intensity. [c] Too broad to be recorded. [d] Overlapping parallel and perpendicular lines. [e] Width of perpendicular lines. [f] PGA = phosphoglycerate.

The binding of Mn^{2+} ion on the γ site may be relevant to the inhibition at high Mn^{2+} ion concentrations observed by Hanlon and Westhead (1969b). In this kinetic study it was found that at [enolase] = 1.5×10^{-6} to 4.5×10^{-6} M and a substrate concentration of greater than about 6×10^{-4} M, a decrease in velocity was observed at a concentration of 10^{-5} M Mn^{2+} (total). This observation may be rationalized by simply the result of loss of free Mn^{2+} due to the formation of SM binary complex. However, at much lower concentration of the substrate (2-phosphoglycerate), and at concentration of free Mn^{2+} much above 10^{-5} M, there is also a pronounced inhibition. It is interesting to speculate that this second inhibition mechanism may be caused by the occupation of γ site thus preventing the formation of the EMS ternary complex or the dissociation of the ternary complex to products. More sensitive methods are being developed in this laboratory to detect the γ site under biological conditions.

Electron Paramagnetic Resonance Line Widths of Metal Ions Bound to Enolase. Free Mn^{2+} ion in solution usually give intense electron paramagnetic resonance spectra with well resolved hyperfine components. For example, a Tris buffer solution of 0.33 mM Mn^{2+} gives a spectrum with $g = 2.009$ and $a = 91.5$ G. The average individual hyperfine line width is 24.2 G. In the presence of complexing agents, such as 2-phosphoglycerate and 3-phosphoglycerate, electron paramagnetic resonance lines are excessively broadened. Cohn and Townsend (1954) were also unable to observe electron paramagnetic resonance spectrum of MnEDTA in solution. Mn^{2+} ions bound to enolase also gave no observable spectra.

In a frozen sample the paramagnetic ions are completely immobilized. Most of the solution relaxation mechanisms are inoperative and the electron paramagnetic resonance spectrum is the superposition of resonances of all orientations with respect to the applied field. Such polycrystalline spectra of Mn^{2+} bound to enolase have already been discussed above; those spectra characteristics of Cu^{2+} systems are given in Table II.

Mn^{2+} and Cu^{2+} ions are considered here not only because they are of biological interest but also because they represent examples of two classes of metal ions, those with $S = 1/2$ and those with $S > 1/2$. The electron-spin relaxation mechanisms of these two kinds of metal ions can be quite different.

The basic relaxation theory was developed by Wangness and Bloch (1955) and Redfield (1957) and has been reviewed recently (Lewis and Morgan, 1968; Hudson and Luckhurst, 1968). The ground-state multiplet for complexes of ions with $S > 1/2$ may be split by spin–orbit interaction even in the absence of a magnetic field. The zero-field Hamiltonian appropriate for the first transition elements is given approximately by

$$\mathcal{H} = D\left[S_z^2 - \frac{1}{3}S(S+1)\right] + E[(S_x^2 - S_y^2] + \frac{a}{6}[S_x^4 + S_y^4 + S_z^4] \quad (5)$$

The zero-field splitting may be modulated by changes in orientation of the rapidly tumbling complex in solution or by fluctuation in the magnitude of this splitting or both. This modulation can provide a very efficient relaxation mechanism.

In the case of Mn^{2+} the relaxation times are given (Hudson and Luckhurst, 1968) to be

$$(T_2)^{-1}{}_{5/2,3/2} = \frac{D_{\alpha\beta}:D_{\alpha\beta}}{5}(24J_0 + 48J_1 + 28J_2)$$

$$(T_2)^{-1}{}_{3/2,1/2} = \frac{D_{\alpha\beta}:D_{\alpha\beta}}{5}(6J_0 + 36J_1 + 46J_2) \quad (6)$$

$$(T_2)^{-1}{}_{1/2,1/2} = \frac{D_{\alpha\beta}:D_{\alpha\beta}}{5}(16J_1 + 56J_2)$$

where $D_{\alpha\beta}$ is the Wigner rotation matrix and $D_{\alpha\beta}:D_{\alpha\beta} = (2D^2/3) + 2E^2$, $J_0 = \tau_0$, $J_1 = \tau_0/(1 + \omega^2\tau_0^2)$, and $J_2 = \tau_0/(1 + 4\omega^2\tau_0^2)$. D and E are defined by the spin Hamiltonian (eq 5); the correlation time, τ_0, is given by the Stokes–Einstein equation ($\tau_0 = 4\pi\eta r^3/3kT$).

We consider two limiting situations. In the first case the complex is rapidly tumbling, *i.e.*, $\omega^2\tau_0^2 \ll 1$. Then all transitions are observed with T_2^{-1} equal to $104D_{\alpha\beta}D_{\alpha\beta}\tau_0$. Taking values of $r = 4$ Å for $Mn(H_2O)_6{}^{2+}$, $\eta = 9$ mP, $\tau_0 = 6 \times 10^{-11}$

sec, and assuming a zero-field splitting with only axial terms, one calculates a value of 0.01 cm^{-1} for D from the observed line width of 24 G.

Complexes of Mn^{2+} having no observable room temperature electron paramagnetic resonance spectra must have line widths in excess of 1000 G. Since the correlation time of most complexes is only about four times greater than for free Mn^{2+} ions, the excessive line width must be attributed to increases in D and E values. For these examples the axial zero-field paramater D is calculated by (eq 6) to be greater than 0.03 cm^{-1}. Most Mn^{2+} crystals have D values of this magnitude.

The correlation time of an enzyme with molecular weight of 10^5 is about 10^{-7}–10^{-8} sec. At X-band frequency, $\omega^2\tau_0^2 \gg 1$. Under these conditions, the relaxation times for the $\pm{}^5/_2 \rightarrow \pm{}^3/_2$ and $\pm{}^3/_2 \rightarrow \pm{}^1/_2$ transitions are much shorter than the $\pm{}^1/_2 \rightarrow \pm{}^1/_2$ transitions and therefore determine the line width. T_2^{-1} is found to be $(149\tau_0/10)D_{\alpha\beta}:D_{\alpha\beta}$. For $\tau_0 = 10^{-7}$ sec, even zero-field splitting as small as 0.003 cm^{-1} can broaden individual hyperfine line widths to 1000 G by this mechanism.

The above discussion showed that the electron paramagnetic resonance line width of $S > {}^1/_2$ ions is a sensitive function of zero-field splitting. These ions, when bound to protein molecules will usually have line widths too broad to be detected by virtue of the long correlation times. Even for low molecular weight complexes, T_2^{-1} can be very large except when zero-field splitting is small. Exceptional circumstances are discussed in a later section.

Two relaxation mechanisms are of importance for $S = {}^1/_2$ ions of which Cu^{2+} is an example. The first is due to incomplete averaging of anisotropies and hyperfine tensors coupled to the Brownian motion of the complex (McConnell, 1956). The line width, which is dependent upon the nuclear-spin quantum number M_I is found (Kivelson, 1960; Wilson and Kivelson, 1966) to be

$$T_2^{-1}(M) = A + BM + CM^2 \qquad (7)$$

$$\begin{aligned} A &= \left(\frac{2}{15}J_0 + \frac{J_1}{10}\right)\frac{\beta^2H^2}{\hbar^2}(\mathbf{g}':\mathbf{g}') + \\ &\qquad 4\pi^2\left(\frac{J_0}{20} + \frac{7J_1}{60}\right)I(I+1)(A':A') \\ B &= 2\pi\left(\frac{4J_0}{15} + \frac{J_1}{5}\right)\frac{\beta H}{\hbar}(\mathbf{g}':A') \\ C &= 4\pi^2\left(\frac{J_0}{12} - \frac{J_1}{60}\right)(A':A') \end{aligned} \qquad (8)$$

where $\mathbf{g}':\mathbf{g}' = ({}^3/_2)(g_{\parallel} - g_{\perp})^2$, $\mathbf{g}':A' = ({}^3/_2)(g_{\parallel} - g_{\perp})(A_{\parallel} - A_{\perp})$, and $A':A' = ({}^3/_2)(A_{\parallel} - A_{\perp})^2$ for complexes with axial spin Hamiltonian.

A second relaxation mechanism is due to coupling of the magnetic field, set up by molecular rotation, with the electron spins. The relaxation time for this spin-rotational process is given by

$$(T_2)^{-1} = \frac{9}{\tau_0}[(g_{\parallel} - 2)^2 + 2(g_{\perp} - 2)^2] \qquad (9)$$

The two mechanisms can be readily distinguished when either one or the other dominates. The first process causes asymmetric line broadening which depends on nuclear quantum number and the line widths generally decrease with the increase of temperature. There is no dependence on M_I for the spin-rotational process and the line widths increase with temperature.

In addition to these two processes there are also contributions to line width from chemical exchange, unresolved nuclear hyperfine structure, intermolecular dipolar interaction, and electric field fluctuation mechanism such as the Orbach process.

Because the $\mathbf{g}$ anisotropy is rather small for the Cu^{2+} complexes being considered here, the spin-rotational contribution as calculated according to eq 10 is not significant. Therefore, we consider primarily the first tumbling mechanism. In the limit of $\omega^2\tau_0^2 \ll 1$ the width of the $M_I = {}^3/_2$ line is found to be only about 5 G for free Cu^{2+} ion in solution. The actual situation is rather more complicated and has been carefully reviewed by Lewis and Morgan (1968). In the other limit of $\omega^2\tau_0^2 \gg 1$, such as Cu^{2+} bound to enolase, calculations similar to that given for Mn^{2+} but using eq 8 show clearly line widths much too broad for detection.

Cu^{2+} complexes of EDTA, 2-phosphoglycerate, and 3-phosphoglycerate gave spectra at 298° which have nearly the same widths as those of free Cu(H$_2$O)$_6^{2+}$, but with much reduced intensities (Table II). It appears that complexed Cu^{2+} coexists with solvated Cu^{2+} in these systems and that the rate of exchange is slow compared to the relaxation times. According to this interpretation about 10% of the Cu^{2+} ions is free in the presence of 2-phosphoglycerate at concentrations of Cu^{2+} and 2-phosphoglycerate of 4×10^{-4} M and 1 mM, respectively. Sixty-five per cent of the Cu^{2+} ions is free at [Cu^{2+}] = 4×10^4 and [EDTA] = 1 mM or [3-phosphoglycerate] = 10 mM.

The above discussions by no means have exhausted all sources of line broadening. For example when two Cu^{2+} ions are less than 3 Å apart (Boyd *et al.*, 1971) the exchange interaction is so strong that there is extreme line broadening at 77°K. The ions become diamagnetic if the coupling is antiferromagnetic. Unresolved hyperfine structure is another source of line broadening. For instance the hyperfine splittings of ^{63}Cu and ^{65}Cu are similar and difficult to resolve. If the metal complexes have more than one acid and base forms, the electron paramagnetic resonance spectra will be broadened at some pH.

Aside from those cases where zero-field, g anisotropy, or hyperfine anisotropy is extremely small thus permitting the detection of epr spectra of metal ions bound to enzyme, there are other circumstances whereby protein-bound metal ions may display electron paramagnetic resonance spectra at room temperature. The first is the case when relaxation time is so long that pseudopolycrystalline electron paramagnetic resonance spectra can be observed. A recent but well-documented case appears to be the Mn^{2+}-concanavalin A complex (Reed and Cohn, 1970; Nicholan *et al.*, 1969). This enzyme is believed to be highly associated (Agrawal and Goldstein, 1968). The long relaxation time and the very small zero-field splitting (estimated to be much less than 20 G) both contribute to make the observation of pseudopolycrystalline electron paramagnetic resonance spectra possible. Another possible example is the Cu complex of ceruloplasmin (Vänngärd, 1966; Andreasson and Vänngärd, 1970). The electron paramagnetic resonance spectra obtained at 277 and 108°K were said to have essentially the same characteristics.

A second case is when the metal ions are bound to that portion of protein which has a great deal of freedom of rotation. Then the local correlation time could be orders of magnitude shorter than that of the whole protein molecule.

Whereas, no example of metal ions bound in this way is known to the authors, numerous examples have been reported for nitroxide spin labels. For example, Stone *et al.* (1965) showed that there exist at least two distinct types of binding of the nitroxide derivative to bovine serum albumin; one to sites where the nitroxide group is free to tumble quite rapidly and has short correlation time, and a second to sites where the nitroxide group has much less freedom of motion and is probably tumbling at the same rate as the entire bovine serum albumin molecule.

Finally there is the case of rapid exchange between metal ions bound to enzyme and free metal ions in solution with characteristic correlation times of τ_b and γ_f and relaxation times of T_{2b} and T_{2f}. Only rapid and intermediate exchange rates are of interest in this discussion. If the t_b is the time a given metal ion remained bound and t_f is the time when it is free, then for intermediate rates of exchange, the effective transverse relaxation time T_2' is

$$\begin{aligned}(T_{2f}')^{-1} &= (T_{2f})^{-1} + (t_f)^{-1}\\ (T_{2b}')^{-1} &= (T_{2b})^{-1} + (t_b)^{-1}\end{aligned} \qquad (10)$$

The line width of the free metal-ion resonance will be broadened and its position will be shifted. This can be misconstrued as signals of bound metal ions if the actual bound metal ion resonance has excessively broad line width. In the limit of rapid exchange, the two resonances collapse into one with

$$(T_2^*)^{-1} = \left(\frac{t_b}{t_f + t_b}\right)(T_{2b})^{-1} + \left(\frac{t_f}{t_f + t_b}\right)(T_{2f})^{-1} \qquad (11)$$

Electron paramagnetic resonance signals can be observed if the short relaxation time of the bound metal ion is compensated by short t_b.

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