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Electron Paramagnetic Resonance Studies of Mn(II)–Mn(II) Interaction in Yeast Inorganic Pyrophosphatase

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Yeast inorganic pyrophosphatase, EC3.6.1.1. (PPase), has two high affinity Mn(II) sites per PPase subunit in the absence of phosphoryl ligands and three high affinity sites in the presence of a phosphoryl ligand such as hydroxymethane bisphosphonate (PCHOHP) [1]. We here present strong evidence from Mn(II) EPR studies that in the presence of PCHOHP 1) the Mn(II) ions in one pair of the divalent metal ion sites are close enough to one another to show significant magnetic (dipolar or exchange) interaction and 2) the Mn(II) ions in another pair of the sites show weaker magnetic interaction. Further, the Mn(II) ions in these latter sites have EPR spectra that are indistinguishable from one another.

EPR Spectra of PPase, Mn(II) and PCHOHP

Q-band (35 GHz) spectra for solutions of PPase and different amounts of Mn(II) in the absence of phosphoryl ligand are shown in Fig. 1(a) and 1(b). Spectrum 1(b) is quite similar to that for $Mn(H_2O)_6^{+2}$ with respect to both g value (1.999 *vs.* 2.001, respectively) and peak to peak line width (17.5 gauss *vs.* 15 gauss, respectively) and is indicative of isotropic and relatively isolated Mn(II) sites. It should be noted, however, that raising the Mn(II) stoichiometry from one to two per subunit actually results in a modest decrease in spectral intensity, probably indicative of a weak Mn(II): Mn(II) magnetic interaction. The concentration of free Mn(II) is negligible in these solutions, since the dissociation constant of Mn(II) from each of two sites on a PPase subunit is equal to 10^{-5} *M* [1]. In Fig. 2, Mn(II) spectra are shown, at increasing Mn(II) to PPase molar ratio, in the presence of the strong competitive inhibitor of PPase, PCHOHP [2]. It is quite clear that addition of PCHOHP leads to much more complex spectra compared to those seen in Fig. 1. The multiplicity of lines clearly distinguishable in Fig. 2a and 2b is similar to that reported by Markham in studies of Mn^{2+} bound to S-adenosylmethionine synthetase [3], and is suggestive of Mn(II)–Mn(II) coupling, although it must be noted that EPR spectra of the same solutions at X-band (9 GHz) did not show the same multiple line EPR spectral patterns, perhaps due to broadening effects, that Markham was able to demonstrate (data not shown). Alternatively, the spectra seen in Fig. 2a and 2b could be accounted for either as a superposition of two spectra with slightly different zero field splittings or by anisotropy in the −1/2 to +1/2 transition. In any event, the broadness of all of the peaks in Fig. 2a, b compared to those seen in Fig. 1 (even the relatively narrow peak at 12,275 gauss has a peak to peak line width of 27 gauss) and the marked decrease in intensity in the low field line at 12,275 gauss as more than one equivalent of Mn(II) is added, provide strong evidence for significantly enhanced Mn(II)–Mn(II) magnetic interaction in the presence of PCHOHP [4].

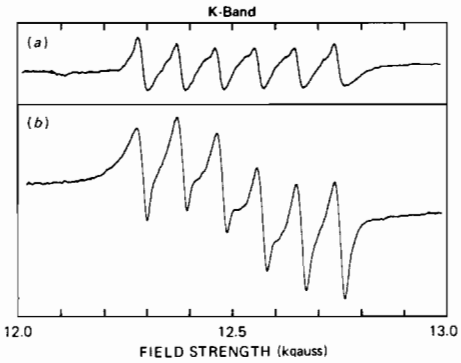


Fig. 1. Q-band spectra of Mn(II)·PPase. (a) 3.0 m*M* in PPase subunits and 1.5 m*M* in Mn(II), (b) 3.0 m*M* in PPase subunits, and 3.0 m*M* Mn(II). Both solutions were 0.1 *M* Tris, 0.1 *M* KCl, pH. 7.2. The spectra were obtained with 6.3 gauss modulation amplitude, 1 KG scans centered at 12.5 KG. Temperature, −6 °C.

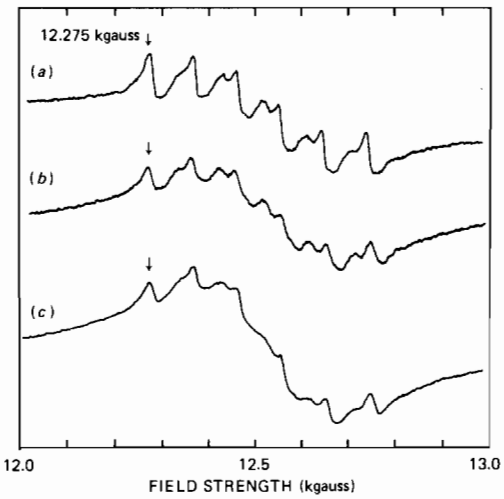


Fig. 2. Q-band spectra of Mn(II)·PPase·PCHOHP complex with increasing Mn(II) equivalents. All solutions were 3.0 m*M* in both PPase subunits and PCHOHP, (a) 3.0 m*M* Mn(II), (b) 4.5 m*M* Mn(II), (c) 6.0 m*M* Mn(II). The solutions contained 0.1 *M* Tris, 0.1 *M* KCl at pH 7.2 The spectra were recorded with 6.3 gauss modulation amplitude, 8×10^4 receiver gain, 1 KG scans centered at 12.5 KG. Temperature, −8 °C. Note the decrease in intensity of the 12,275 gauss line on addition of Mn(II). A field strength of 12.50 KG corresponds to a g value of 2.000.

EPR Spectra of PPase, Mn(II), PCHOHP and Ca^{2+}

Based on our previous binding studies [1] it is evident that in the presence of 1.5–2.0 equivalents of

Mn(II) all three high affinity sites are occupied, but only partially. A difficulty in interpreting our EPR experiments lies in knowing how to assign the observed spectral lines among the Mn(II) bound in the three sites. We sought to simplify this problem of interpretation by introducing a diamagnetic metal ion specifically into one of the three sites. It has been known for some time that Ca^{2+}:inorganic pyrophosphate complex (Ca^{2+}:PPi) binds extremely tightly to PPase ($K_D = 10^{-7}$ *M* [5]) whereas Ca^{2+} has rather poor affinity, much less than that of free Mn(II) [6]. We therefore reasoned that in the presence of PCHOHP and 2 equivalents of Mn(II) per PPase subunit, Ca^{2+} might compete effectively with Mn(II) for the metal ion site corresponding to M^{2+} PPi binding, but not for the two other divalent metal ion sites. The apparent success of this approach is shown clearly in Fig. 3. The addition of Ca^{2+} to a solution of Mn(II), PPase, and PCHOHP results in a suppression of the broad spectral components and in a progressive increase in intensity of a sharp line EPR spectrum. The EPR spectrum resulting from the addition of one equivalent of Ca^{2+} to the Mn(II)·PPase·PCHOHP complex (Fig. 3d) is isotropic in nature, has a peak to peak line width for the low field line of 15 gauss, and shows little or no evidence for a significant magnetic interaction between the Mn(II) ions bound to PPase.

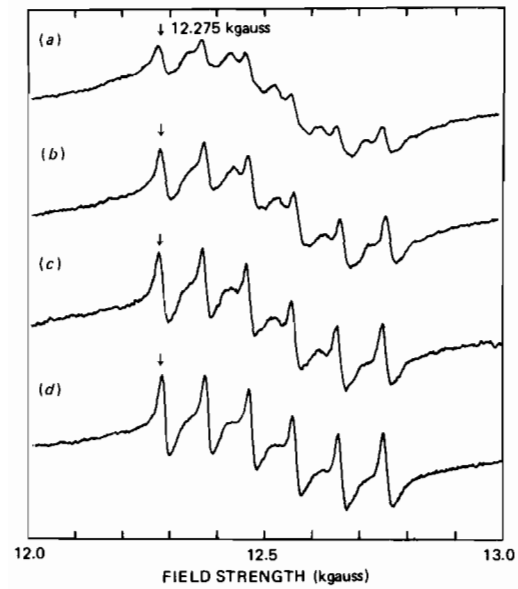


Fig. 3. Q-band spectra of Mn(II)·PPase·PCHOHP·Ca^{2+} complexes with increasing Ca^{2+} equivalents. The solutions were 3.0 m*M* in both PPase subunits and PCHOHP and 4.5 m*M* in Mn(II). (a) 0.5 m*M* Ca^{2+}, (b) 1.5 m*M* Ca^{2+}, (c) 2.5 m*M* Ca^{2+}, (d) 3.5 m*M* in Ca^{2+}. All solutions contained 0.1 *M* Tris, 0.1 *M* KCl at pH 7.2. All spectra were recorded with 6.3 gauss modulation amplitude, 8×10^4 receiver gain, 1 KG scans centered at 12.5 KG. Temperature, –8 °C. Note the increase in intensity of the 12,275 gauss line and the decrease in peak to peak line width with increased mole ratio of Ca^{2+} bound to the complex.

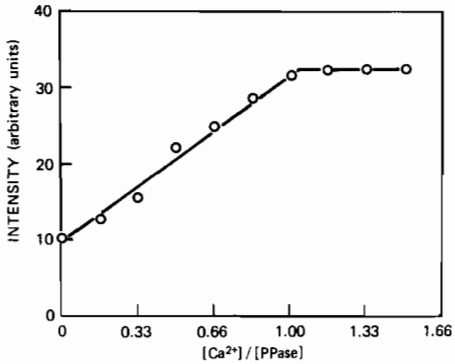
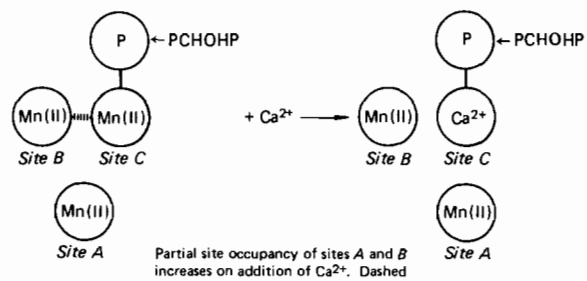


Fig. 4. Titration plot. Change in intensity (arbitrary units) of line at 12,275 gauss of the EPR spectrum (Q-band) in solutions of Mn(II)·PPase·PCHOHP·Ca^{2+} as a function of added Ca^{2+}. To a solution 3.0 m*M* in both PPase and PCHOHP and 4.5 m*M* in Mn(II), aliquots of Ca^{2+} were added and EPR spectra were taken. During the titration all the spectral parameters were kept the same. Temperature, –6 °C.

In addition, this change is completed on addition of one Ca^{2+} per PPase subunit, as shown in Fig. 4.

That the spectrum shown in Fig. 3d represents bound Mn(II) only and has no contribution from free Mn(II) was confirmed by equilibrium dialysis experiments. When a 3 m*M* PPase solution containing 3 m*M* PCHOHP, 3 m*M* $^{45}Ca^{2+}$ and 4.5 m*M* $^{54}Mn(II)$ was dialyzed against 0.1 *M* Tris, 0.1 *M* KCl (pH 7.2), the enzyme solution had the appropriate EPR spectrum. Furthermore, radioactive decay counting of the $^{54}Mn(II)$ and $^{45}Ca^{2+}$ in this solution indicated that one equivalent of Ca^{2+} was bound per PPase subunit along with 1.5 equivalents of Mn(II) ion. By contrast, the dialyzate contained no free $^{45}Ca^{2+}$, negligible amounts of $^{54}Mn(II)$ (10^{-8}*M*), and had no apparent EPR spectrum. We interpret these results in terms of the simple scheme shown below (Scheme 1).

In the absence of phosphoryl ligand, Mn(II) ions populate the A and B sites. These sites are relatively isolated from one another, as mentioned above. In the presence of PCHOHP all three sites, A, B and C, are occupied. The sites B and C are close enough so that the Mn(II) ions occupying them show significant magnetic interaction. Because such interaction can vary with either r^{-3} or with r^{-6} (where r is the distance between the two electronic dipoles), an upper



Partial site occupancy of sites *A* and *B* increases on addition of Ca^{2+}. Dashed line indicates strong dipole-dipole interaction.

Scheme 1.

limit of 7–8 Å can be set for the separation between the Mn(II) ions in these two sites; beyond this limit, the two ions would have only a small (<10%) broadening effect on one another [4]. This result is consistent with preliminary work of Dunaway-Mariano and Villafranca as quoted in Knight *et al.* [7]. The addition of Ca^{2+} displaces Mn(II) from the partially filled site C and the displaced Mn(II) is redistributed amongst the partially filled sites A and B. Since site C is filled with diamagnetic Ca^{2+}, the Mn(II)–Mn(II) magnetic interaction between sites B and C is suppressed and a narrow line spectrum, corresponding to Mn(II) population of sites A and B, is observed. The increased intensity of this spectrum as a function of added Ca^{2+} is reasonable since the population of Mn(II) at these sites is increased by displaced Mn(II) from site C. Finally, from the observed lack of zero field splitting, the results presented in Figs. 1(b) and 3(d) show that the Mn(II) ions in sites A and B have ligand environments with near octahedral symmetry which are indistinguishable from one another, at least as judged by their EPR spectra.

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Iron Coordination in Soybean Lipoxygenase-1

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A model for the coordination of iron in soybean lipoxygenase-1 is proposed on the basis of an exploratory EXAFS study. Comparison of the EXAFS transmission data of lipoxygenase with those of known metalloproteins, *i.e.* the Cu-EXAFS of superoxide dismutase and the Zn-EXAFS of insulin has led us to suggest that the iron in lipoxygenase is, like the metals in superoxide dismutase and insulin, ligated by histidines coordinating with nitrogen of the imidazole ring. The number of imidazoles is estimated at 3 to 4. EPR spectra of the yellow (ferric) enzyme species indicate an axial symmetry for the iron coordination, making 4 imidazoles likely. A model compound, designed on the basis of this proposal is shown to have considerable lipoxygenase activity.

The oxygen binding of soybean lipoxygenase has been studied by fluorescence, magnetic susceptibility measurements and EPR spectroscopy. The fluorescence of the enzyme is partially quenched upon removal of oxygen by flushing with nitrogen. Magnetic susceptibility measurements show no difference between the air-saturated and de-oxygenated enzyme solutions. This can be explained by assuming that either the oxygen is not coordinated to the iron or it is so firmly attached that it is not removed by the deoxygenation procedure. If the latter were the case, anaerobic addition of 1 equivalent of linoleic acid would result in the formation of product hydroperoxides and in the appearance of an EPR signal of the yellow enzyme species. As this does not appear, oxygen coordination to the iron can be ruled out.

In an earlier EPR study, it was established that the yellow (ferric) enzyme species is reduced by the addition of linoleic acid under anaerobic conditions. Kinetic studies with isotope substitution have shown that the proton abstraction from the methylene group in the 1,4-*cis*,*cis*-pentadiene system of the fatty acid substrate is the rate-limiting step in the dioxygenation catalysis. These studies, in combination with those mentioned above, show that the iron atom is involved only in the activation of the substrate to be oxygenated, as in protocatechuate-3,4-dioxygenase, for example, and not in the activation of the oxygen, as many other oxygenases. Unlike the protocatechuate-3,4-dioxygenase, there is a valence change of iron during the catalytic cycle, the ferric enzyme being reduced in the rate-limiting proton abstraction but reoxidized later in the cycle. As the ferric model compound is also capable of reduction of the fatty acid substrate, the abstracted proton may be localized on one of the coordinating imidazoles and transferred back to the fatty acid later on. The mechanism by which the ferrous native enzyme is activated to the ferric species is still under investigation.