# Evidence for a Binuclear Iron Site in Pig Allantoic Fluid Acid Phosphatase (Uteroferrin)

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Pig allantoic fluid acid phosphatase (also called progesterone-induced glycoprotein and uteroferrin) is in iron-containing protein which closely resembles an acid phosphatase from beef spleen in molecular size, visible absorption spectrum and catalytic properties [1–3]. Both enzymes exist in two readily interconvertible redox forms: a violet, oxidized form which is catalytically inactive, and a pink reduced form which is active.

Antanaitis *et al.* [4] showed that at low temperatures ($T \leqslant 20$ K), the major EPR signal seen in samples of the resting pig enzyme (largely the violet form) has $g' = 1.74$, with principal $g$ values of $g_x = 1.922$, $g_y = 1.736$ and $g_z = 1.582$. Reduction of the protein to the pink form caused no appreciable change in the spectrum. Antanaitis and Aisen [5] and Davis and Averill [6] have recently reported that the beef spleen enzyme in the reduced form has a very similar low temperature EPR spectrum to that of the pig enzyme. Antanaitis and Aisen [5] have suggested that earlier failures to observe the $g' = 1.74$ signal in the beef enzyme were due to the presence of tightly bound phosphate in the isolated enzyme, especially in samples prepared using hydroxylapatite chromatography. The low temperature EPR spectra of the iron-containing acid phosphatases are similar to those of semimethemerythrins [7] which appear to contain one Fe(II) and one Fe(III) atom bridged by an oxygen atom.

In our hands, the enzymes prepared from pig allantoic fluid and beef spleen both contain two iron atoms per molecule of 40,000 daltons. Davis *et al.* [3] have confirmed that the beef enzyme contains two iron atoms per molecule. However, Roberts and Aisen and their coworkers [2, 4, 8] have consistently measured one iron atom per molecule of pig enzyme prepared from either allantoic fluid or uterine secretions. Antanaitis *et al.* [4] determined the magnetic susceptibility of violet uteroferrin as a function of temperature, and interpreted their results as supporting the presence of the big majority of the iron in a mononuclear low spin complex. The close similarity of the EPR spectra of the beef and pig enzymes with those of the semimethemerythrins, which contain a binuclear iron centre, argues against a mononuclear iron centre in the pig enzyme. We now report the results of our study of the magnetic susceptibility of pig allantoic fluid acid phosphatase.

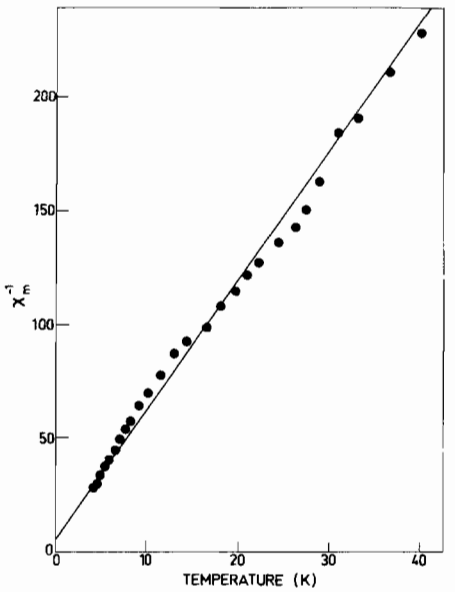


Fig. 1. Magnetic susceptibility of the violet form of pig allantoic fluid acid phosphatase in 0.1 *M* acetate buffer as a function of temperature ([E] = $8.54 \times 10^{-4}$ *M*; [Fe] = 1.70 m*M*; units of $\chi_m$, emu/g-atom Fe). The line is calculated for the best fit of the data to the Curie–Weiss equation $\chi = C/(T - \theta) + D$, where D is the diamagnetic term; C = 0.175 cgs units; $\theta = -0.883$; D = zero.

## Experimental

Pig allantoic fluid acid phosphatase was purified to homogeneity from the allantoic fluid of 65-day pregnant sows by methods already described [1] and dialysed against 0.1 *M* acetate buffer, pH 4.9, containing 0.1 *M* EDTA, followed by the same buffer without EDTA. The enzyme had a $\lambda_{max}$ of 550 nm, indicating that it was very largely (≥90%) in the violet form, and an $A_{280}/A_{550}$ of 15.1, indicating a high state of

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purity. The protein concentration was calculated using an $A^{1\%}_{1\,cm}$ at 280 nm of 14.2, and a molecular weight of 40 000 [1]. The concentration of iron in the samples was determined by atomic absorption spectroscopy. Magnetic properties of the enzyme were determined from 4-50 K using a SQUID magnetometer.

## Results and Discussion

Figure 1 shows the effect of temperature on the molar magnetic susceptibility, $\chi_m$, of the violet form of pig allantoic fluid acid phosphatase. From these data, a Curie constant, C, of 1.75 cgs units/g-atom of iron in the sample is obtained. This corresponds to a magnetic moment, $\mu$, of 1.18 BM. Similar results were obtained with a second more dilute sample of enzyme ([E] = $1.67 \times 10^{-4}$ *M*; [F] = $3.05 \times 10^{-4}$ *M*).

An EPR spectrum at 77 K of the concentrated enzyme sample used in the magnetic susceptibility measurements showed a $g' = 4.3$ signal characteristic of high spin Fe(III). All EPR spectra determined on pig allantoic fluid acid phosphatase have shown a $g' = 4.3$ signal. Antanaitis *et al.* [4] showed by integration of low temperature EPR spectra that the $g' = 4.3$ signal is accounted for by only a small % of the total iron in the samples (7%, 8% and 1–2% in three results quoted). Similarly, beef spleen acid phosphatase shows a small and variable signal at $g' = 4.3$ which accounts for ≤5% of the total Fe [6]. The $g' = 4.3$ signal has therefore been attributed to a small amount of high spin Fe(III) present as an impurity *e.g.* in denatured enzyme. Using a value of 5.9 BM for the magnetic moment of high spin Fe(III), it can be shown that the measured magnetic susceptibility (Fig. 1) would be accounted for by the presence of 4.0% of the total iron in the sample as high spin Fe(III), with the remaining iron being diamagnetic.

Models for the iron complex in pig allantoic fluid acid phosphatase which involve mononuclear iron centres seem to be ruled out by the following arguments. High spin Fe(II) and high spin Fe(III) may be immediately discarded because of the very low observed paramagnetism. Low spin Fe(II), as in $[Fe(CN)_6]^{4-}$, would be diamagnetic, but it is difficult to envisage protein ligands which would cause the spin pairing. Further, the visible absorption [1] and resonance Raman spectra [9, 10] argue strongly for the existence of Fe(III)–phenolate bonds. Low spin Fe(III) also seems highly unlikely given (i) the nature of the possible ligands; (ii) the residual unpaired electron which would be expected to show greater paramagnetism than is measured; and (iii) the difference between the observed temperature dependence (Fig. 1) and that expected for low spin Fe(III) due to spin–orbit coupling.

The low paramagnetism observed could be explained by the existence of a binuclear iron centre in which the two iron atoms are strongly and antiferromagnetically coupled. The most likely model for the violet (oxidized) form of the enzyme is an iron centre with two high spin ferric iron atoms, bridged by an oxygen atom, *viz.* Fe(III)–O–Fe(III). Such a binuclear iron centre has been found in many small molecule complexes which have strong coupling ($-J \sim 100\ cm^{-1}$) and no paramagnetism at low temperatures [11]. It has also been proposed as the iron centre in methemerythrins [12]. A similar iron centre with two high spin Fe(II) atoms, Fe(II)–O–Fe(II), is unlikely to be present in the oxidized enzyme, and conflicts with the evidence mentioned above for the existence of Fe(III)–phenolate bonds. The partially reduced form, Fe(II)–O–Fe(III) (both high spin), does not explain the low paramagnetism since one unpaired electron remains no matter how strong the coupling, and since spin-orbit coupling would increase the observed magnetic moment.

In the favoured model, Fe(III)–O–Fe(III), a bridging oxygen atom is proposed. Based on studies with model bridged complexes, other possible bridges, such as a phosphate molecule, seem unlikely to give sufficiently strong coupling to explain the experimental data. A bridging sulfur atom can be ruled out since the enzyme contains no detectable inorganic sulfide (J. de Jersey and B. Zerner, unpublished results).

Given a strongly coupled Fe(III)–O–Fe(III) centre in the violet oxidized enzyme, it is logical to propose an Fe(II)–O–Fe(III) coupled centre, with one unpaired electron, for the pink reduced form of the enzyme. The main evidence against these propositions is the observation by Aisen and his coworkers that the low temperature EPR spectra of the violet and pink forms of the pig enzyme are the same [4, 8]. The strongly coupled Fe(III)–O–Fe(III) centre proposed here for the violet form should be EPR silent, whereas the Fe(II)–O–Fe(III) centre proposed for the pink form could well produce the low temperature EPR spectrum observed, as evidenced by the EPR spectrum of semimethemerythrins. One possible explanation for this discrepancy may be the presence of a small % (say 5–10%) of the active pink form of the enzyme in the samples of the violet enzyme used in Aisen's EPR experiments. In our hands, rigorous oxidation is required to decrease the acid phosphatase activity, and hence the amount of the pink form of the enzyme, to a negligible level. In this context, it is worth noting that oxidation of the pig enzyme with $H_2O_2$ abolishes the $g' = 1.74$ EPR signal [Ref. 8, and B. Zerner and J. de Jersey, unpublished results]. A second possibility, supported by their analytical results,

is that the enzyme isolated by Aisen, Roberts and their coworkers is different in that it contains less than the full complement of two iron atoms per molecule.

Davis and Averill [6] have recently published results on beef spleen acid phosphatase which support the structures proposed above for the iron centres in the oxidized and reduced forms of the pig enzyme. The violet form of the beef enzyme is EPR silent; one-electron reduction of the enzyme with dithionite gives the pink form of the enzyme which has a low temperature EPR spectrum very similar to that of the pig enzyme; the oxidized form of the enzyme is essentially diamagnetic at low temperatures, whereas the reduced enzyme has a magnetic susceptibility consistent with the presence of a single unpaired electron.

Finally, Antanaitis and Aisen [5] have shown that beef spleen acid phosphatase as isolated contains an approximately stoichiometric amount of tightly bound phosphate. We have recently shown that the oxidized forms of both beef spleen and pig allantoic fluid acid phosphatases bind phosphate very tightly with 1:1 stoichiometry, while phosphate binds only weakly to the reduced forms of the enzymes [13]. It seems probable that the experiments performed by Davis and Averill [6] on the oxidized form of the beef enzyme were actually carried out on the 1:1 enzyme–phosphate complex. The presence of some phosphate in the samples of pig enzyme used in this work cannot be excluded. However, activity measurements on the enzyme after the first step of the purification procedure (CM-cellulose chromatography) with and without prior activation showed the enzyme to have ~75% of the maximum activity without activation. Since the enzyme had no subsequent contact with phosphate, the maximum amount of bound phosphate was 0.25 mol per mol of enzyme.

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