Evidence for a spin-coupled binuclear iron unit at the active site of the purple acid phosphatase from beef spleen

(EPR spectrometry/magnetic susceptibility/zinc substitution/phosphate inhibition)

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Communicated by Richard H. Holm, May 13, 1982

ABSTRACT The purple acid phosphatase from beef spleen, which contains two iron atoms per molecule, is EPR silent in its native (oxidized) purple form. Treatment with mild reducing agents results in conversion to a pink, enzymatically active form, which exhibits an unusual EPR signal centered at \( g = 1.77 \); double integration of the EPR spectrum gives one spin per two iron atoms. A similar EPR spectrum is observed for enzyme reduced anaerobically by one electron, using sodium dithionite. Variable-temperature magnetic susceptibility measurements show that the oxidized and reduced proteins are both antiferromagnetically coupled systems, with \( S = 0 \) and \( \frac{1}{2} \) ground states, respectively. Replacement of one of the iron atoms by zinc produces an FeZn enzyme with full catalytic activity. The FeZn enzyme exhibits a highly temperature-dependent \( g = 4.3 \) EPR signal, and magnetic susceptibility data are consistent with an \( S = \frac{1}{2} \) paramagnet. Treatment of the FeZn enzyme with phosphate, a competitive inhibitor, results in sharpening of the EPR spectrum; double integration at 77 K gives one spin per iron. These results strongly suggest the presence of a spin-coupled bimetallic unit at the active site of the enzyme.

Purple metalloproteins with phosphatase activity have now been isolated from mammalian, plant, and microbial sources (1). All but that from Micrococcus sodonensis (2) are similar in being basic glycoproteins with optimal activity at low pH, and all exhibit an intense absorption band at 510–550 nm (\( e = 4,000 \) \( M^{-1} \text{cm}^{-1} \)) with a shoulder at 310–320 nm. Despite these obvious similarities, there exists substantial disagreement with regard to the metal content. Thus, the protein from beef spleen (3, 4) is reported to contain two iron atoms (5) and that from porcine ureterine fluids either one (6) or two (5) iron atoms. The protein from kidney beans apparently contains iron as well (7), although the stoichiometry has not been determined. In contrast, the apparently analogous sweet potato enzyme is reported to contain a single manganese atom (8), and preparations of purple phosphatase from soybeans (9, 10), spinach leaves (11), and cultured rice plant cells (12) are all reported to contain manganese in unspecified amounts. In addition to the ambiguity regarding the identity of the chromophore, there is not even general agreement regarding the function of these proteins, although all have specific activities for phosphate ester hydrolysis comparable to other known phosphatases. A role in iron transport has been proposed for the purple acid phosphatase from porcine ureterine fluid (13).

The protein most studied by physical techniques is that from porcine ureterine fluid. It is apparently very similar to that from beef spleen in molecular weight, amino acid composition, and limiting specific activity for \( p \)-nitrophenylphosphate hydrolysis (5, 6, 14). Both exist in purple (\( \lambda_{\text{max}} \approx 505 \text{ nm} \)) and pink (\( \lambda_{\text{max}} \approx 505 \text{ nm} \)) forms; the pink form is produced by treatment of the native purple form with mild reducing agents (1, 14). Resonance Raman spectra of the purple form of the porcine enzyme (15) show evidence for coordination of tyrosine phenolate groups [in agreement with similar studies on the sweet potato enzyme (8, 16)]. Low-temperature EPR spectra of the porcine enzyme exhibit a novel \( g' = 1.74 \) signal, which together with magnetic susceptibility results was interpreted in terms of mononuclear ferric iron in an unusual \( S = \frac{1}{2} \) spin state in both the purple and pink forms (17).

In an attempt to resolve the questions of the nature of the chromophore and its role in catalysis, we have examined the purple acid phosphatase from beef spleen in some detail. We describe herein EPR spectra and magnetic susceptibility data on the native (purple) form, the reduced (pink) form, and a form in which half the iron has been replaced by zinc, with complete retention of enzymatic activity. Together with our earlier studies, the present results strongly suggest that the enzyme contains a binuclear spin-coupled iron unit at the active site.

MATERIALS AND METHODS

The purple acid phosphatase from beef spleen was prepared and assayed as described (1). All preparations used in this work had specific activity \( \geq 1,200 \text{ units/mg} \) and absorbance ratio \( A_{500}/A_{280} \leq 16 \). The FeZn form of the enzyme, in which half the iron content has been replaced by zinc, was prepared as described by Zerner and co-workers (18) for the porcine ureterine fluid enzyme. Metal contents were determined by using inductively coupled plasma emission spectroscopy on a Jarrell-Ash model 955 Plasma Atomcomp, iron contents were also analyzed by using a colorimetric method as a check. Protein concentration was determined from the absorbance at 280 nm, using a value of 1.59 for the absorbance of a 1 mg/ml solution in a 1-cm cuvette. This value is based upon a dry weight determination (5).

Anaerobic titrations with sodium dithionite were performed in the double septum seal apparatus previously described (19). Dithionite solutions were standardized by anaerobic titration with excess methyl viologen, monitored spectrophotometrically (20); samples were transferred anaerobically to EPR tubes and frozen within 2 min.

EPR spectra were run on a Bruker ER200D spectrometer equipped with an Oxford liquid helium cryostat or on a Varian E-4 spectrometer equipped with a liquid nitrogen Dewar flask as a sample holder. The EPR spectra were quantitated by double integration versus a 1 mM copper(II) EDTA standard, using the \( g \) value corrections of Aasa and Vännberg (21). Magnetic susceptibility data over the temperature range 4.5–250 K were obtained on an SHE Corporation superconducting quantum

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interference device (SQUID) susceptometer, using frozen samples (40 µl, 1.5–3.0 mM Fe). The raw data were corrected for slight residual paramagnetism of the sample holder and fit to a simple two-term expression: \( x'_d(\text{obs}) = x'_d + x'_0 \), in which \( x'_0 \) (the molar paramagnetic susceptibility) was assumed to have a simple Curie-law temperature dependence, and \( x'_d \) (the diamagnetic susceptibility) was assumed to be temperature independent. Separate runs on buffer blanks gave values of \( x'_d \) within 5% of those obtained in the fitting procedure.

RESULTS AND DISCUSSION

Native Protein. Analytical results from both our laboratory (1) and Zerner’s (5) indicate that the native purple phosphatase from beef spleen contains 2 ± 0.1 iron atoms per molecule. The enzyme as isolated (purple; \( \lambda_{\text{max}} = 550 \text{ nm} \)) is relatively inactive, but treatment with mild reducing agents converts it to a highly active form (pink; \( \lambda_{\text{max}} = 505 \text{ nm} \)) with no change in metal content. Treatment with saturating concentrations of phosphate, a competitive inhibitor (\( K_i = 3.6 \text{ mM} \)), results in reversion to the purple form even in the presence of excess reductant (1). The transition responsible for the visible absorption band has been identified as tyrosine phenolate-to-metal charge transfer in both the porcine uterine fluid (15) and sweet potato (8, 16) enzymes. Resonance Raman spectra of both purple and pink forms of the beef spleen enzyme (unpublished data) are virtually identical to those of the corresponding forms of the porcine uterine fluid protein (15), suggesting a similar assignment for the former.

In order to gain more information about the nature of the metal chromophore, we have examined the low-temperature EPR spectra of the purple, pink, and phosphate-inhibited forms of the enzyme. Except for a small and variable signal at \( g = 4.3 \) (corresponding to ≤5% of the total Fe concentration by double integration), the oxidized (purple) form of the enzyme is EPR silent (Fig. 1, spectrum A) under all conditions examined, as reported previously (1). In contrast, the reduced (pink) form of the enzyme shows a complex signal centered at \( g = 1.77 \) (Fig. 1, spectrum B). The signal is very temperature sensitive, broadening rapidly above 12 K, possibly accounting for our previous failure to observe it on a less sensitive instrument. The signal at \( g = 4.3 \) is of variable intensity and is absent in samples prepared by (slow) reduction with dithioerythritol alone; it is apparently due to slight oxidation of the ferrous iron in the Fe\(^{2+}\)/ascorbate mixture used to simulate assay conditions and ensure rapid reduction of the enzyme. Addition of 10 mM phosphate to the enzyme solution containing excess Fe\(^{2+}\)/ascorbate (Fig. 1, spectrum C) or excess dithioerythritol (not shown) results in immediate loss of the \( g = 1.77 \) signal, consistent with the optical spectral changes reported previously (1).

The high-field region of spectrum B in Fig. 1 is shown enlarged in Fig. 2, spectrum A. Double integration of the spectrum gives 0.9 ± 0.1 spin per molecule (i.e., one spin per two Fe). This signal is very similar to that reported for the purple and pink forms of the porcine uterine fluid enzyme (17), both in overall appearance and in \( g \) values (1.92, 1.77, and 1.63 vs. 1.93, 1.74, and 1.59 for the principal features). Careful examination shows that the spectrum of the bovine enzyme is due to at least two species, as found for the porcine enzyme (17); the small feature at \( g = 1.85 \) is clearly part of a second signal.

Because the purple–pink conversion in the porcine enzyme has been previously attributed to reduction of one or more disulfide links and a concomitant change in conformation about the iron (14), and because we observe no EPR signal for the oxidized form of the beef spleen enzyme, we have chosen to investigate the stoichiometry of the reduction more carefully. A titration in which 0.5-equivalent aliquots of sodium dithionite solution were added anaerobically to the purple form of the

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**Fig. 1.** EPR spectra at 4.5 K of beef spleen purple acid phosphatase. Spectrum A, oxidized (purple) form; spectrum B, reduced (pink) form produced by treating oxidized enzyme with 1.0 mM (NH\(_4\))\(_2\)Fe(SO\(_4\))\(_2\) and 100 mM ascorbate, pH 5.0; spectrum C, sample prepared as for B followed by addition of 10 mM sodium phosphate, pH 5.0. All samples contained 0.23 mM protein (9 mg/ml) in sodium acetate buffer, pH 5.0. Spectra were obtained on a Bruker ER200D spectrometer under the following conditions: microwave frequency, 9.47 GHz; microwave power, 10 mW; modulation frequency, 100 Hz; modulation amplitude, 10 G; time constant, 0.2 sec; field set, 2,200 G; scan range, 4,000 G; scan time, 200 sec; instrument gain, 5 × 10\(^5\) (A) or 2.5 × 10\(^5\) (B, C).

**Fig. 2.** EPR spectra at 4.2 K. Spectrum A, pink form of beef spleen acid phosphatase produced by Fe\(^{2+}\)/ascorbate treatment (same sample as in Fig. 1, spectrum B); spectrum B, oxidized enzyme (0.23 mM) plus 1 equiv of Na\(_2\)S\(_2\)O\(_4\) in 0.073 M Tris-HCl buffer, pH 7. Conditions of EPR spectrometry as in Fig. 1, except: microwave power, 5 mW (A) or 2.5 mW (B); field set, 4,000 G; scan range, 2,000 G; instrument gain, 4 × 10\(^5\) (A) or 6.4 × 10\(^5\) (B).
enzyme showed maximal formation of the $g = 1.77$ EPR signal at 1 equivalent; this was accompanied by a change in color from purple to pink. Addition of excess dithionite resulted in rapid bleaching of the protein (complete at $\approx 1$ additional equivalent) and loss of the EPR spectrum. The EPR spectrum of the one-electron reduced (by dithionite) protein is shown in Fig. 2, spectrum B. It is similar, but not identical, to that obtained upon reduction with $Fe^{2+}$/ascorbate. The principal differences are the relative intensities of the two low-field peaks and the intensity of the trough between the crossover point and the high-field peak, as well as small changes in the $g$ values of the crossover point and the high-field peak. These differences are consistent with a change in the relative populations of the two species presumably present, and may be due either to the difference in reducing agents or to the higher pH used to prevent decomposition of dithionite during the titration. Double integration of the signal in spectrum B of Fig. 2 gives $0.8 \pm 0.1$ spin per Fe, in contrast to published data on the porcine enzyme, which gives a ratio of 1 spin per Fe. Regardless of the method of reduction, our EPR results are most consistent with formulation of the oxidized (purple) enzyme as containing an antiferromagnetically coupled $[Fe(III)]_2$ unit ($S = \text{integer}$), and the active reduced (pink) enzyme as containing a spin-coupled mixed-valence $Fe(III)$-$Fe(II)$ unit, net spin $S = \frac{1}{2}$.

Recent results on hemerythrin, an apparently unrelated protein, lend support to our formulation. Thus, two different "semi-met" mixed-valence $Fe(III)$-$Fe(II)$ forms of hemerythrin with EPR spectra similar to those described above have been observed (22). One-electron reduction of methemerythrin and one-electron oxidation of deoxyhemerythrin yield species exhibiting rhombic ($g = 1.95, 1.87, 1.67$) and axial ($g = 1.95, 1.72$) EPR spectra, respectively. The overall shape, $g$ values, and temperature dependence of the semi-met hemerythrin signals are very similar to those of the signals observed in the purple acid phosphatases from beef spleen (this work) and porcine uterine fluid (17), strongly suggesting the existence of a related binuclear unit in the latter two proteins. The apparent heterogeneity of the EPR spectra of the purple phosphatases may well be due to the existence of two spectral forms, as has been observed with hemerythrin.

Because the lack of an observable EPR spectrum in the oxidized (purple) acid phosphatase could be due to a number of factors, including unusual relaxation properties, net integral spin $S > 0$, or net diamagnetism, and because of the unusual nature of the spin-coupled ($S = \frac{1}{2}$) unit proposed for the reduced (pink) enzyme, we have obtained complementary magnetic susceptibility data on frozen solutions of both forms of the protein. The results are shown in Fig. 3 A and B for the oxidized (purple) and dithioerythritol-reduced (pink) forms, respectively. The oxidized form is effectively diamagnetic over the temperature range (4.5-250 K) examined. The paramagnetic susceptibility observed is less than 11% of that expected even for an $S = \frac{1}{2}$ (per protein molecule) paramagnet and may be accounted for by the presence of 2.5% high-spin $Fe(III)$ ($S = \frac{5}{2}$) contaminant. In contrast, the reduced EPR-active form exhibits a paramagnetic susceptibility slightly greater than that expected for a spin-only Curie law $S = \frac{1}{2}$ paramagnet per protein molecule. Analysis of the slope of a $\chi$ vs. $1/T$ plot gives a value of $\mu_{eff} = 2.2 \pm 0.2$ $\mu_B$ (Bohr magneton) per 2 Fe, suggesting substantial orbital contribution to the magnetic moment. There is no detectable deviation from linearity in the $\chi$ vs. $1/T$ plots (not shown) for either oxidized or reduced forms up to 250 K, indicating a strong antiferromagnetic interaction in both cases. (We estimate $-2J = \approx 100$ cm$^{-1}$ for each.) The magnitude of the coupling observed in the oxidized enzyme is similar to that reported for oxhemerythrin ($J = -77$ cm$^{-1}$) and metahemerythrin ($J = -134$ cm$^{-1}$) (23).

**FeZn Protein.** Zerner has shown that it is possible to selectively remove a single Fe atom from the porcine uterine enzyme and replace it with Zn with 100% restoration of activity (18). We have found that this is also true for the beef spleen enzyme, although detectable scrambling of iron to give small amounts (<10%) of holoprotein containing two Fe atoms occurs during the reconstitution (unpublished results). Our metal analyses reproducibly give values of $1.0 \pm 0.1$ Zn and $1.0 \pm 0.1$ Fe atom per molecule for the FeZn protein. We find that the enzymatic activity of the FeZn protein is identical in all respects (including $V_{max}$, $K_m$ for p-nitrophenylphosphate, and $K_i$ for phosphate, p-aminobenzyolphosphonate, and fluoride) to that of the native enzyme, except that activation by mild reductants is not required for activity (unpublished results). The optical spectrum of the purple form of the FeZn protein ($\lambda_{max} = 550$ nm, $\varepsilon = 2,100$ M$^{-1}$ cm$^{-1}$) is not perturbed by mild reductants but is abolished by stoichiometric amounts of sodium dithionite. These results strongly suggest that the functional unit in both the FeZn and the FeZn forms of the enzyme is the 40,000 molecular weight protein (1, 5) containing one Fe(III) and one Fe(II) or Zn(II) ion.

If the native protein does indeed contain antiferromagnetically coupled iron atoms, this coupling obviously must be absent in the FeZn protein, in which a Zn$^{2+}$ has presumably replaced one of the iron atoms in the spin-coupled unit. One would thus expect to see magnetic behavior typical of isolated Fe(III) or...
Fe(II) ions. Low-temperature ($T < 30$ K) EPR spectra (not shown) show a broad $g = 4.3$ signal that is easily saturated (above 0.25 mW at 13 K) and broadens rapidly above 30 K. This is qualitatively characteristic of high-spin Fe(III) in a rhombic environment, but the peculiar relaxation properties make quantitation by double integration impossible. Addition of saturating concentrations of phosphate results in dramatic sharpening of the signal and an apparent increase in the spin-lattice relaxation time, such that the signal is readily observed at 77 K (cf. Fig. 4). This suggests strongly that phosphate binds near, if not at, the iron site. Double integration of the spectrum obtained in the presence of phosphate gives $0.9 \pm 0.1$ spin per Fe atom.

Low-temperature magnetic susceptibility studies on the FeZn protein are also consistent with the presence of an $S = \frac{3}{2}$ paramagnets (Fig. 3C). Simple Curie-law behavior consistent with a temperature-independent magnetic moment of $\mu_{\text{eff}} = 6.0 \pm 0.2 \mu_B$ is observed.

Conclusions. The results presented above demonstrate that the native purple acid phosphatase from beef spleen contains a novel spin-coupled binuclear iron unit that can exist in both the diferric and ferric-ferrous oxidation states. EPR and magnetic data are consistent with overall $S = 0$ and $S = 1/2$ ground states in the oxidized (purple) and one-electron reduced (pink) forms, respectively. The strength of the interaction is most consistent with a linear or nearly linear Fe-L-Fe structure, in which L is probably a monatomic bridging ligand such as oxide. Such units are known to result in strong antiferromagnetic coupling (24–27). It must be noted, however, that no evidence for such a bridge is observed in the structure of metazidohemerythrin (25), which contains two iron atoms joined by three bridging ligands (two carboxylates and either a water molecule or a hydroxide or oxide ion) and which exhibits strong magnetic coupling ($J = -134$ cm$^{-1}$) between the ferric ions in the metaxo form (23). An analogous structure for the binuclear core of the bovine spleen purple phosphatase is also consistent with the magnetic and EPR data presented herein. Replacement of one of the iron atoms by zinc results in abolition of the magnetic coupling, as expected, and produces a species exhibiting a strongly temperature-dependent $g = 4.3$ EPR signal due to high-spin Fe(III). Complementary $^{57}$Fe Mössbauer studies are required to fully describe the electronic structure of these systems.

Phosphate, a competitive inhibitor of phosphate ester hydrolysis, strongly perturbs the EPR spectrum of the FeZn protein and appears to decrease the oxidation-reduction potential of the binuclear iron unit, resulting in reversion of the optical and EPR spectra to those characteristic of the oxidized native protein. These results strongly suggest that phosphate (and, by inference, substrate) binds near or at the bimetallic site. At the moment, there is no evidence that would differentiate between binding of phosphate and substrate to (or near) the nonexchangeable (ferric) or the exchangeable (ferrous or zinc) metal ions. Electron nuclear double resonance (ENDOR) and pulsed EPR spectrometry experiments utilizing $^{67}$Zn-substituted enzyme and $^{31}$P phosphatase, as well as Fe and Zn K-edge extended x-ray absorption fine structure (EXAFS) spectroscopy experiments on phosphate- and arsenate-inhibited FeZn enzyme should allow us to distinguish between these alternatives.

Some comments regarding the relationship of the beef spleen enzyme to the enzymes from porcine uterine fluid and from sweet potatoes are in order. Qualitatively, the EPR and magnetic properties we describe are very similar to those reported for the porcine enzyme (17). The major differences arise in the quantitation of spins versus iron concentration and in the observation of essentially identical properties for both purple and pink forms of the latter. The similarity between the EPR spectra of the purple phosphatases and of the semi-met forms of hemerythrin (22) seems to us to be strong circumstantial evidence supporting a binuclear, mixed-valence formulation in the former. Finally, the purple acid phosphatases from sweet potato and certain other plant sources appear to be very similar to those from mammalian sources in their chemical and spectroscopic properties, yet they are reported to contain manganese, identified as Mn(III) in the sweet potato enzyme (8, 16). In view of the fact that manganese is a common contaminant in proteins derived from plant materials, it is unfortunate that no definitive evidence has been presented to show that these proteins do not, in fact, contain iron in addition to manganese.

We thank M. Johnson for expert technical assistance; she was supported in part by a National Institutes of Health Minority High School Student Research Apprentice Program. We gratefully acknowledge the assistance of S. R. Tonsager, M. B. Martin, and the Analytical Toxicology Section of the Animal Health Diagnostic Lab in the elemental analyses. This work was supported in part by funds from the Michigan State University Agricultural Experiment Station for purchase of the plasma emission spectrometer. This work was also supported in part by a Cottrell Research Grant from the Research Corporation, by Grant GM 28636 from the National Institutes of Health, and by Grants CHE-79-04810 and DMR-80-12899 from the National Science Foundation for purchase of the EPR spectrometer and susceptor, respectively. J. C. D. was a Sotho Graduate Fellow, 1981–1982. B. A. A. was an A. P. Sloan Foundation Fellow, 1981–1983.