Enzyme Turnover. Reactions were performed in 50 mM Tris, pH 7.4, containing 1.1 μM P-450$_{cam}$, 1.79 μM putidaredoxin, 1.71 μM putidaredoxin reductase, 225 μM NADH, 20 mM KCl, 450 μM substrate, and, in the appropriate cases, 200 μg of catalase (2500 EU). Reactions were initiated with substrate in a final volume of 2.8 mL. Reactions were performed in a cuvette equipped with a Clark oxygen electrode, allowing NADH and O_2 consumption to be monitored simultaneously. NADH was quantitated by the change in absorbance at 340 nm, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. O_2 consumption was calculated by using a full scale deflection as a change in O_2 concentration of 213.8 μM. H_2O_2 was determined as the difference in O_2 consumption in the presence and absence of catalase.[28] After reaction was complete, a known amount of standard was added, either 2-*exo*-norborneol or 3-*endo*-bromocamphor. The mixture was extracted 3 times with an equal volume of $CHCl_3$. The organic extract was concentrated under a slow stream of N_2 and analyzed by GC on a 15-m megabore DB-17 column programmed at 70 °C for 4 min followed by temperature ramp to 200 °C at 7 °C/min. The amount of hydroxynorcamphor was determined by integration of peak areas with a HP 3390A reporting integrator.

Mass spectral data were obtained on VG 7070E mass spectrometer in line with an HP 5700 gas chromatograph equipped with a 30-m DB-5 capillary column. The mass spectrometer was interfaced with an 11250 VG multispec data system.

(28) Nordbloom, G. D.; Coon, M. J. *Arch. Biochem. Biophys.* **1977**, *180*, 343.

Acknowledgment. Gas chromatographic mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, which is supported in part by a grant from the National Institute of General Medical Sciences, GM27029. Research was supported by a grant to S.G.S., NIH GM31756. We are grateful to Jean Lewis for her expert typing of this manuscript.

Spectroscopic and Magnetic Studies of the Purple Acid Phosphatase from Bovine Spleen

Bruce A. Averill,*[1a,b] James C. Davis,[1c] Sudhir Burman,[1d] Teresa Zirino,[1a] Joann Sanders-Loehr,[1e] Thomas M. Loehr,[1e] J. Timothy Sage,[1f] and Peter G. Debrunner[1g]

Contribution from the Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901, and Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999. *Received June 26, 1986*

Abstract: The properties of the purple acid phosphatase from bovine spleen have been examined by optical, EPR, ^{57}Fe Mössbauer, and resonance Raman spectroscopy and by variable temperature magnetic susceptibility measurements. Anaerobic titrations monitored by optical and low-temperature EPR spectra show that conversion of the purple, enzymatically inactive form to the enzymatically active pink form is a one-electron process. The pink form exhibits a $g' = 1.77$ EPR spectrum due to a pH-dependent mixture of two rhombic species, with an apparent pK_a of ca. 4.4. The temperature dependence of the EPR signal of the pink form over the range 6-20 K is consistent with a weak antiferromagnetic coupling ($-2J = 11 \pm 2$ cm^{-1}) between an $S = 2$ Fe(II) and an $S = {}^5/_2$ Fe(III). Bulk magnetic susceptibility studies of a lyophilized sample of the purple, oxidized enzyme are consistent with the presence of a strongly antiferromagnetically coupled binuclear high-spin ferric system, with $-2J \geq 300$ cm^{-1}. Natural abundance ^{57}Fe Mössbauer spectra of the purple form confirm the coupled diferric site and indicate that the iron atoms are in relatively low symmetry environments. Resonance Raman spectra demonstrate the presence of tyrosyl phenolate ligands to iron in both the purple and pink forms, but experiments with $H_2{}^{18}O$ gave no direct evidence of a μ-oxo ligand. Integration of these data with previous spectroscopic results suggests the presence in the oxidized enzyme of a binuclear iron center bridged by a μ-oxo and possible additional ligands such as carboxylates. In the oxidized form, one ferric iron is coordinated by two tyrosinates, while the other iron is probably coordinated to a phosphate. In the reduced form, the phosphate is lost from the new ferrous site, the ferric site retains the tyrosinate ligands, and the putative bridging oxo group is probably protonated. Histidine imidazoles and other as yet unidentified ligands complete the coordination of the iron atoms.

The purple acid phosphatases[2] constitute an apparently diverse group of metalloenzymes involved in regulation of the levels of phosphate and phosphorylated metabolites in a wide range of organisms. Despite superficial spectral similarities (all exhibit an absorption band at 500-550 nm with $\epsilon \sim 2000$-4000 M^{-1} cm^{-1}), it now appears as if three distinct types of chromophore may be present in these enzymes. The purple acid phosphatases from plants are reported to contain either Mn(III) (the sweet potato enzyme[3]) or Fe(III) and Zn(II) (the kidney bean enzyme[4]). In contrast, those purple acid phosphatases obtained to date from mammalian sources, including porcine uterine fluid,[5] bovine spleen,[6] rat spleen,[7] and human spleen,[8] have been shown to contain two iron atoms. Several other mammalian enzymes[9-13] exhibit

(1) (a) Department of Chemistry, University of Virginia, Charlottesville, VA 22901; (b) Alfred P. Sloan Foundation Fellow, 1981-1985. (c) Department of Chemistry, Michigan State University, East Lansing, MI 48824. Current address: SOHIO Research Center, 4440 Warrensville Rd., Cleveland, OH 44128. (d) Current address: Department of Biochemistry, Cornell Medical Center, 1300 New York Ave., New York, NY 10021. (e) Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, 19600 N.W. Von Neumann Dr., Beaverton, OR 97006-1999. (f) Current address: Department of Physics, Northeastern University, 360 Huntington Ave., Boston, MA 02115. (g) Department of Physics, 1110 W. Green St., University of Illinois, Urbana, IL 61801.

(2) Antanaitis, B. C.; Aisen, P. *Adv. Inorg. Biochem.* **1983**, *5*, 111-136.

(3) (a) Sugiura, Y.; Kawabe, H.; Tanaka, H.; Fujimoto, S.; Ohara, A. *J Biol. Chem.* **1981**, *256*, 10664-10670. (b) Sugiura, Y.; Kawabe, H.; Tanaka, H. *J. Am. Chem. Soc.* **1980**, *102*, 6581-6582.

(4) Beck, J. L.; McConachie, L. A.; Summors, A. C.; Arnold, W. N.; de Jersey, J.; Zerner, B. *Biochim. Biophys. Acta* **1986**, *869*, 61-68.

(5) Antanaitis, B. C.; Aisen, P.; Lilienthal, H. R. *J. Biol. Chem.* **1983**, *258*, 3166-3172.

(6) Davis, J. C.; Averill, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4623-4627.

(7) Hara, A.; Sawada, H.; Kato, T.; Nakayama, T.; Yamamoto, H.; Matsumoto, Y. *J. Biochem. (Tokyo)* **1984**, *95*, 67-74.

(8) Ketcham, C. M.; Baumbach, G. A.; Bazer, F. W.; Roberts, R. M. *J. Biol. Chem.* **1985**, *260*, 5768-5776.

(9) Lam, K.-W.; Lai, L. C.; Burkart, P. T.; Yam, L. T. *J. Biol. Chem.* **1977**, *252*, 3371-3373.

(10) Robinson, D. B.; Glew, R. H. *J. Biol. Chem.* **1980**, *255*, 5864-5870.

0002-7863/87/1509-3760$01.50/0

significant similarities to the diiron enzymes, in that they are basic glycoproteins of mol wt ca. 40000 and are not inhibited by tartrate.

The obvious biological and medical importance of the mammalian enzymes makes their active site chemistry of substantial interest. To date, only the enzymes from bovine spleen and porcine uterine fluids (also referred to as "uteroferrin") have been examined in any detail. After a period of controversy,[2,5,6] the following key points are generally agreed upon for both enzymes. The inactive, oxidized, purple enzyme (λ_{max} = 550 nm) contains two high-spin Fe^{3+} ions magnetically coupled via one or more as yet unidentified bridging ligands (presumably a μ-oxo) to give an $S = 0$ ground state.[5,6,14,15] Treatment with mild reductants results in conversion to the enzymatically active, pink form (λ_{max} = 505–510 nm), which contains an antiferromagnetically coupled mixed-valence Fe^{3+}–Fe^{2+} unit[5,6,15,16] that exhibits an unusual EPR signal at $g' = 1.74$ (uteroferrin) or 1.77 (bovine spleen enzyme).[5,6,15,17] Resonance Raman spectra of the purple and pink forms of the porcine enzyme[18] demonstrate the presence of tyrosine ligands to iron and identify the visible absorption band as a tyrosinate-to-iron(III) charge-transfer transition. Both ^1H NMR[16] and electron spin echo[19] studies on the reduced porcine enzyme are consistent with coordination of at least one imidazole N to Fe. The oxidized form of the enzymes as isolated contains 1 mol of tightly bound phosphate[17,20,22] that is lost upon reduction. Treatment of the pink form with excess phosphate[21,24] results in loss of enzymatic activity and the $g' = 1.74$ EPR signal, a shift in λ_{max} to 540–545 nm, and eventual conversion to the oxidized, purple form with incorporation of phosphate, although reported time dependences of these phenomena vary with the system[21,22] and the $g' = 1.74$ EPR signal disappears under anaerobic conditions.[23] A variety of oxyanions perturb the $g' = 1.74$ EPR signal, apparently by binding at or near the iron center.[23] Finally, treatment with strong reductants results in reduction of both iron atoms, one of which is lost rapidly.[25] The resulting apo and half-apo forms have been used to prepare enzyme substituted with ^{57}Fe for Mössbauer studies[15] and enzyme in which one Fe is replaced by Zn,[6,25,26] Ni,[25] Cu,[26,27] or Hg.[27] The Fe–Zn enzyme is reported to exhibit activity virtually identical with that of the native enzyme,[6,26,27] suggesting possible similarities to the kidney bean Fe–Zn enzyme.[4]

In order to characterize the binuclear iron center of the purple phosphatases more fully and to permit comparison to other enzymes known to contain binuclear oxo-bridged iron centers, including ribonucleotide reductase[28] and the structurally characterized hemerythrin,[29,30] we have carried out an extensive series of spectroscopic and magnetic measurements on the bovine spleen enzyme. Some of these results, including preliminary magnetic susceptibility[2] and X-ray absorption (EXAFS and XANES)[31] studies, have been reported earlier. The present paper describes the results of anaerobic titrations and EPR, magnetic susceptibility, resonance Raman, and natural abundance ^{57}Fe Mössbauer studies on the bovine spleen enzyme.

Experimental Section

Enzyme Samples. The purple acid phosphatase (FePase) from bovine spleen was purified and assayed as described previously.[24] Reduced (pink) FePase was prepared by treating the native (purple) enzyme (ca. 2–5 mg/mL) with 100 mM ascorbate and 6 mM ferrous ammonium sulfate in 50 mM NaOAc buffer, pH 5.0, followed by desalting and buffer exchange on Sephadex G-25 (1.6 × 25 cm). For the variable pH studies, the sample pH was adjusted by adding aliquots of 1 M, 5 M, or glacial HOAc (low pH) or 1 M NaOH or 1 M Tris buffer, pH 7.5 (high pH). Samples were allowed to stand at 0 °C for 30 min prior to freezing to ensure complete pH equilibration; no difference in EPR spectra was observed for selected samples that were thawed, allowed to stand at 0° or 25 °C for >1 h, and refrozen. EPR samples were frozen either slowly by immersion in liquid N_2 or rapidly by immersion in isopentane maintained just above its freezing point; no significant differences in EPR spectra were observed that were attributable to the method used for freezing samples. Concentrated solutions (≤5 mM) of FePase for Mössbauer and resonance Raman spectroscopy were obtained by ultrafiltration (Amicon 8MC/PM10 membrane or Centricon). D_2O or $H_2{}^{18}O$ exchange was accomplished by dilution of a concentrated FePase sample (100 μL) with 200–250 μL of D_2O or $H_2{}^{18}O$ (95% ^{18}O) containing 50 mM NaOAc, pH 5.0, followed by concentration (Amicon Centricon). This process was repeated twice, resulting in a final solution that was >95% D_2O or $H_2{}^{18}O$. To maximize the probability of ^{18}O exchange of a putative μ-oxo group, reduced (pink) FePase was incubated in $H_2{}^{18}O$ overnight at 4 °C and reoxidized with a 5-fold excess of phosphate in $H_2{}^{18}O$, pH 5.0.[22] The sample of oxidized FePase used for magnetic susceptibility was prepared by lyophilization of a solution containing 17 mg of FePase in 1 mM $(NH_4)OAc$ buffer, pH 5.0; optical spectra and enzymatic activity showed ≥95% recovery of enzyme after magnetic measurements. A sample of 17 mg of bovine serum albumin treated analogously was used as a blank.

Titrations of oxidized FePase (1.5 mM in 50 mM NaOAc, pH 5.0) with methyl viologen radical cation ($MV^{\bullet+}$) were carried out in the double-septum-seal (dss) apparatus previously described[32] and monitored by optical spectroscopy and low-temperature EPR spectroscopy. Methyl viologen in 10 mM Tris·HCl, pH 7.0, was reduced with slightly less than stoichiometric amounts of $Na_2S_2O_4$, and the concentration of $MV^{\bullet+}$ was determined by measuring the absorbance at 600 nm.[33] Reductive titrations of FePase (0–2 electrons/mol) were accomplished by transferring microliter quantities of $MV^{\bullet+}$ solution into degassed enzyme solutions in dss cuvettes via gas-tight Hamilton syringes. The enzyme solution was carefully mixed between additions. Visible spectra of samples with ≤1.2 equiv of $MV^{\bullet+}$ added per mol were stable for at least 5 min, but samples containing >1.2 equiv of $MV^{\bullet+}$ per mol showed rapid protein precipitation. EPR samples were prepared analogously in dss tonometers to which an EPR tube was attached via a $\overline{\Psi}$ $^7/_{25}$ joint. The sample was transferred to the tube and frozen anaerobically within 2 min of $MV^{\bullet+}$ addition.

Physical Methods. Sample pH was determined with a Radiometer Model PHM82 pH meter and a combination electrode. Measurements in D_2O were corrected via pD = pH + 0.4. Visible and UV spectra were obtained on a Cary 219 or 17 spectrophotometer. EPR spectra were run on either a Bruker ER 200D spectrometer equipped with an Oxford liquid He cryostat or a Varian 109 spectrometer equipped with an Air

(11) Anderson, T. R.; Toverud, S. U. *Calcif. Tissue Int.* **1982**, *34*, 54–58.
(12) Anderson, T. R.; Toverud, S. U.; Price, J. B.; Hamrick, D.; Hogan, D.; Braswell, L. *Arch. Oral Biol.* **1982**, *27*, 255–259.
(13) Fujimoto, S.; Urata, Y.; Nakagawa, T., Ohara, A. *J. Biochem. (Tokyo)* **1984**, *96*, 1079–1088.
(14) (a) Sinn, E.; O'Connor, C. J.; de Jersey, J.; Zerner, B. *Inorg. Chim. Acta* **1983**, *78*, L13–L15. (b) Mockler, G. M.; de Jersey, J.; Zerner, B.; O'Connor, C. J.; Sinn, E. *J. Am. Chem. Soc.*, **1983**, *105*, 1891–1893.
(15) Debrunner, P. G.; Hendrich, M. P.; de Jersey, J.; Keough, D. T.; Sage, J. T.; Zerner, B. *Biochim. Biophys. Acta* **1983**, *745*, 103–106.
(16) Lauffer, R. B.; Antanaitis, B. C.; Aisen, P.; Que, L., Jr. *J. Biol. Chem.* **1983**, *258*, 14212–14218.
(17) Antanaitis, B. C.; Aisen, P. *J. Biol. Chem.* **1982**, *257*, 5330–5332.
(18) Antanaitis, B. C.; Strekas, T.; Aisen, P. *J. Biol. Chem.* **1982**, *257*, 3766–3770.
(19) Antanaitis, B. C.; Peisach, J.; Mims, W. B.; Aisen, P. *J. Biol. Chem.* **1985**, *260*, 4572–4574.
(20) Antanaitis, B. C.; Aisen, P. *J. Biol. Chem.* **1984**, *259*, 2066–2069.
(21) Keough, D. T.; Beck, J. L.; de Jersey, J.; Zerner, B. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 1643–1648.
(22) Burman, S.; Davis, J. C.; Weber, M. J.; Averill, B. A. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 490–497.
(23) Antanaitis, B. C.; Aisen, P. *J. Biol. Chem.* **1985**, *260*, 751–756.
(24) Davis, J. C.; Lin, S. S.; Averill, B. A. *Biochemistry* **1981**, *20*, 4062–4067.
(25) Keough, D. T.; Dionysius, D. A.; de Jersey, J.; Zerner, B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 600–605.
(26) Beck, J. L.; Keough, D. T.; de Jersey, J.; Zerner, B. *Biochim. Biophys. Acta* **1984**, *791*, 357–363.
(27) Buhi, W. C.; Gray, W. J.; Mansfield, E. A.; Chun, P. W.; Ducsay, C. A.; Bazer, F. W.; Roberts, R. M. *Biochim. Biophys. Acta* **1982**, *701*, 32–38.
(28) Sjöberg, B.-M.; Loehr, T. M.; Sanders-Loehr, J. *Biochemistry* **1982**, *21*, 96–102.
(29) (a) Elam, W. T.; Stern, E. A.; McCallum, J. D.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1982**, *104*, 6369–6373. (b) Hendrickson, W. A.; Co, M. S.; Smith, J. L.; Hodgson, K. O.; Klippenstein, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6255–6259.
(30) (a) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H.; McCallum, J. D.; Sanders-Loehr, J. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 713–716. (b) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Inorg. Biochem.* **1983**, *19*, 247–253. (c) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Am. Chem. Soc.* **1984**, *106*, 618–622.
(31) Kauzlarich, S. M.; Teo, B. K.; Zirino, T.; Burman, S.; Davis, J. C.; Averill, B. A. *Inorg. Chem.* **1986**, *25*, 2781–2785.
(32) Averill, B. A.; Bale, J. R.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1978**, *100*, 3034–3043.
(33) Erbes, D. L.; Burris, R. H. *Biochim. Biophys. Acta* **1978**, *525*, 45–54.

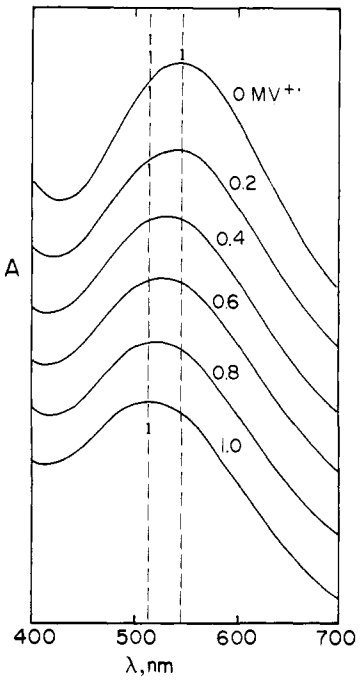

Figure 1. Optical spectra of purple (oxidized) bovine spleen FePase (10 mg/mL in 50 mM NaOAC, pH 5.0) titrated with indicated amounts of methyl viologen radical cation ($MV^{\bullet+}$). Successive spectra are offset by 0.1 A.

Products liquid He cryostat. The EPR spectra were quantitated by double integration of signal-averaged scans with a Nicolet Model 1180 computer interfaced with the Bruker spectrometer with commercially available software or by manual double integration[34] of scans obtained on the Varian spectrometer. A 1 mM Cu^{2+}-EDTA or $CuSO_4$ solution was used as a standard. The g-value corrections of Aasa and Vänngard[35] were used to correct the integrated areas to the Cu^{2+} standards. The variable temperature studies were performed on the Varian spectrometer. Sample temperatures were checked by a calibrated carbon resistor inserted into a dummy EPR tube in the cryostat.

The Mössbauer spectra were measured at the University of Illinois with a constant acceleration instrument. A concentrated solution (≈150 μL) of FePase (2 mM in 50 mM NaOAc, pH 5.0) was loaded into a 0.5-in. Delrin container and transferred to a Janis Research Corp. variable temperature cryostat. The spectra were least-squares fitted to Lorentzian lines.[36] Isomer shifts are referenced to metallic Fe at 298 K.

Magnetic susceptibilities were determined with an SHE Corp. SQUID magnetometer. The raw data were corrected for a slight paramagnetism due to the sample holder and fitted to a two-term expression: $\chi_m(\text{obsd}) = \chi_p + \chi_d$, where χ_p (the molar paramagnetic susceptibility) was assumed to have a Curie law temperature dependence and χ_d (the diamagnetic susceptibility) was assumed to be temperature independent. The bovine serum albumin blank gave a value of χ_d within 5% of that obtained via the data fitting procedure.

Resonance Raman spectra were obtained on 2–5-mM samples of purple and pink FePase prepared as described above. Sample integrity was monitored by the intensity of the Raman spectrum during repeated scans and by measurement of optical spectra and enzyme activity determinations upon dilution after the runs. Raman spectra were recorded on a computerized Jarrell-Ash spectrophotometer[40] equipped with a cooled RCA C31034A photomultiplier tube and an Ortec Model 9302 amplifier-discriminator. Samples were illuminated with selected lines from Spectra-Physics Model 164-01 Kr ion and 164-05 Ar ion lasers.

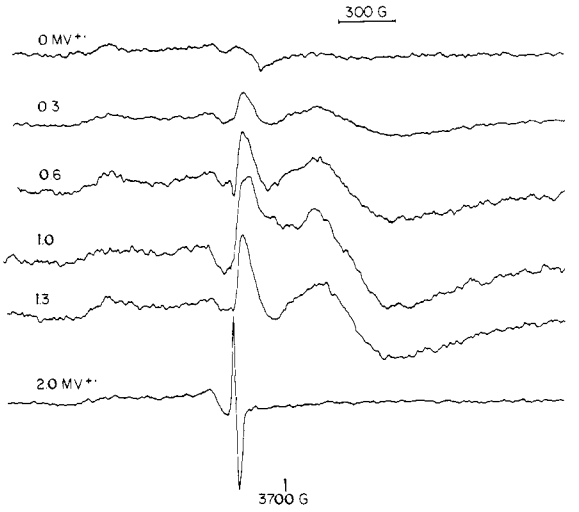

Figure 2. EPR spectra of purple (oxidized) FePase (10 mg/mL in 50 mM NaOAc, pH 5.0) titrated with indicated amounts of methyl viologen radical cation ($MV^{\bullet+}$). Spectra were obtained on a Bruker ER 200D spectrometer under the following conditions: temperature, 4.5 K; microwave frequency, 9.47 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude 10 G; time constant, 0.2 s; field set, 3700 G; scan range, 1000 G; scan time, 200 s; instrument gain, 2.5×10^5.

Table I. Results of Double Integration of EPR Spectra[a] and Position of Visible Absorption Maximum for Bovine Spleen FePase Titrated Anaerobically with Methyl Viologen Radical Cation ($MV^{\bullet+}$)

$MV^{\bullet+}$/enzyme	EPR (spin/mol)	λ_{max} (nm)
0	0	550
0.3	0.26	538
0.6	0.56	527
1.0	0.97	510
1.3	0.74	*b*
2.0	0	*b*

[a] Conditions of EPR spectrometry as in Figure 2. [b] Obscured by turbidity due to protein precipitation.

Results and Discussion

Anaerobic Titrations. The purple to pink conversion in the porcine enzyme was originally attributed to a reduction of one or more disulfide links resulting in a conformational change about the iron,[37] despite earlier reports that no sulfhydryls could be modified in either the purple or pink forms of the bovine spleen enzyme.[38] The presence in the pink form of bovine FePase of a $g' = 1.77$ EPR signal[6,17] similar to that observed for semimethemerythrin[39] and the absence of a detectable EPR signal in the purple form[6,32] strongly suggest that reduction is occurring at the iron atom(s), as indicated by ^{57}Fe Mössbauer data on the porcine enzyme.[15] In order to establish the stoichiometry of the reduction and to confirm the lack of involvement of disulfide residues, we have carried out anaerobic reductive titrations of purple FePase.

An anaerobic titration of the oxidized enzyme with $MV^{\bullet+}$, monitored by optical spectroscopy over the range 400–700 nm, is shown in Figure 1. The visible absorption shifts from 550 nm for the oxidized, purple enzyme to 510 nm for the reduced, pink enzyme; 1.1 equiv of $MV^{\bullet+}$/mol is required for complete conversion to the pink form. Addition of ≥1.2 equiv of $MV^{\bullet+}$/mol resulted in turbidity caused by precipitation of protein, which obscured the spectrum. The pink color (determined visually) was almost entirely eliminated at 2 equiv of $MV^{\bullet+}$/mol. At 2.2 equiv of $MV^{\bullet+}$/mol, a distinct blue color due to the presence of excess $MV^{\bullet+}$ was apparent. Results of a similar titration monitored by low-temperature EPR spectrometry are shown in Figure 2. It is clear that the $g' = 1.77$ EPR signal reaches a maximal intensity at 1.0–1.2 equiv of $MV^{\bullet+}$/mol and disappears completely at 2.0 equiv of $MV^{\bullet+}$/mol. The results of double integration of the EPR spectra in Figure 2 and λ_{max} of the optical spectra in Figure 1 are

(34) Ayscough, P. B. *Electron Spin Resonance in Chemistry*; Methuen & Co., Ltd.: London, 1967; pp 442–443.
(35) Aasa, R.; Vänngard, T. *J. Magn. Reson.* **1975**, *19*, 308–315.
(36) Chrisman, B. L.; Tumolillo, T. A. *Computer Phys. Commun.* **1971**, *2*, 322–330.
(37) Schlosnagle, D. C.; Sander, E. G.; Bazer, F. W.; Roberts, R. M. *J. Biol. Chem.* **1976**, *251*, 4680–4685.
(38) Sabel, K.-G.; Glomset, J.; Porath, J. *Biochim. Biophys. Acta* **1961**, *50*, 135–140.
(39) Muhoberac, B. B.; Wharton, D. C.; Babcock, L. M.; Harrington, P. C.; Wilkins, R. G. *Biochim. Biophys. Acta* **1980**, *626*, 337–345.
(40) Loehr, T. M.; Keyes, W. E.; Pincus, P. A. *Anal. Biochem.* **1979**, *96*, 456–463.

given in Table I. Both the EPR and optical data clearly show that conversion from the purple to pink form is a one-electron process centered at the iron atoms and rule out any possibility of additional involvement of a disulfide/sulfhydryl redox couple.

pH Dependence of the EPR Spectrum. Virtually all EPR spectra reported for the porcine[5,41] and bovine[6,17] purple phosphatases show evidence for the presence of at least two species in variable proportions. This behavior has been attributed to a mixture of aggregated and monodisperse enzyme in the case of uteroferrin[41b] and is similar to results reported for semimethemerythrin,[39] for which either a rhombic or an axial EPR signal is observed under different conditions. In order to ascertain the chemical basis for the existence of two distinct species, we have examined the pH dependence of the EPR spectrum of bovine spleen FePase.

As shown in Figure 3, the EPR spectrum exhibits a strong pH dependence. At pH <3.5, the spectrum is attributable to a rhombic species with apparent g values of 1.94, 1.78, and 1.65. At pH >5, the major component of the spectrum is a new rhombic signal with apparent g values of 1.85, 1.73, and 1.58. The pink form of bovine FePase is unstable at pH >5.5; the pH 5.42 spectrum represents an approximately 90:10 mixture of the high and low pH forms. Traces of the high pH form are visible in the pH 3.10 spectrum. The other feature that is observed at lower field in the spectra of Figures 2 and 3 and which increases in intensity with increasing pH is attributable to trace amounts (<5%) of Cu^{2+}.[41a] The spectra shown in Figure 3 strongly suggest the presence of an ionizable group with a pK_a of approximately 4.4, which has a dramatic effect on the EPR spectrum. A plot of relative intensity of the two components vs. pH is shown in Figure 3 (bottom) and is in reasonable agreement with that predicted for a simple one-proton ionization (solid line) via the Henderson–Hasselbalch equation. The exact pK_a depends strongly on the composition of the solution. For example, addition of 50 mM ascorbate causes an apparent shift of the pK_a to lower pH by 1–1.5 pH units. Use of positively charged buffers (pyridine or aniline hydrochloride, pH 3–5.5) resulted in spectra similar to those obtained with acetate buffer (aniline) or spectra in which the signals overlapped more extensively and the ratio of the species did not change appreciably with pH (pyridine). Addition of 0.5 M KCl did not have a significant effect on the spectra in any case. Optical spectra showed no significant change over the pH range 3–5.5 (data not shown).

The lack of dependence on ionic strength, together with the high isoelectric point[42] and resulting large positive charge of the protein in the pH range examined, makes it unlikely that the heterogeneity observed is due to aggregated vs. monodisperse enzyme molecules. It is clear that the EPR spectrum is best described as being due to a mixture of two species: a high-pH form and a low-pH form that are probably related by ionization of a single group on the protein. The identity of the ionizable group is uncertain at present.

Temperature Dependence of the EPR Spectrum. The magnetic properties of the pink forms of uteroferrin and the bovine spleen FePase have been investigated by several methods. Direct magnetic susceptibility measurements as a function of temperature on uteroferrin[41a] and bovine spleen FePase[6] are consistent with a relatively strong antiferromagnetic coupling ($-2J \approx 100$ cm^{-1}) between a high-spin Fe(III) and a high-spin Fe(II) to give a net $S = {}^1/_2$ ground state. Analysis of the temperature dependence of isotropically shifted proton NMR resonances[16] and of the intensity of the EPR spectrum[5] over the temperature ranges 0–50 °C and 1–18 K, respectively, suggests a much weaker coupling, with $-2J \approx 20$–30 cm^{-1} for reduced uteroferrin. The only reported results favoring a relatively strong coupling in the pink form of the uterine or bovine FePase are bulk magnetic susceptibility data,[6,41a] which are expected to be quite sensitive to small amounts of adventitious high-spin Fe(II) generated during reduction.

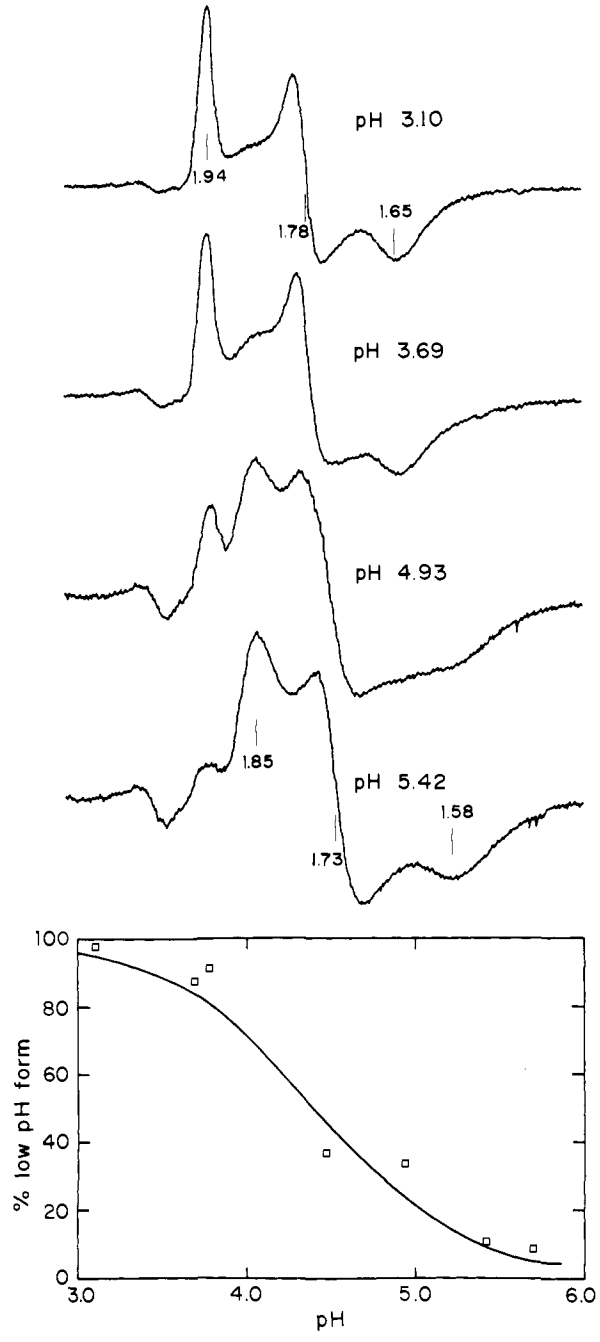

Figure 3. (Top) EPR spectra of pink (reduced) bovine spleen FePase (25 mg/mL) as a function of pH. Spectra were obtained on a Varian 109 spectrometer under the following conditions: temperature, 6–7 K; microwave frequency, 9.07 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 0.128 s; field set, 3500 G; scan range, 2000 G; scan time, 4 min; instrument gain, 6.3×10^3. (Bottom) Plot of relative intensity of low pH form (open squares) vs. pH. Relative intensities were obtained by summing the high and low pH extreme spectra in varying ratios to fit the observed spectra. The solid line is that calculated for a single ionizable group with $pK_a = 4.4$.

Accordingly, we have examined the temperature dependence of the EPR spectrum of bovine spleen FePase, which can give direct information on the magnetic properties of the binuclear center.

As reported earlier[6] and as observed for pink uteroferrin,[5,41a] the EPR spectrum of bovine spleen FePase is highly temperature dependent, being observable only at $T \leq 30$ K. From 6–20 K, the apparent line width does not vary significantly, while above 20 K rapid relaxation and resulting lifetime broadening are important. If the observed EPR signal is due to the $S = {}^1/_2$ ground state of a weakly antiferromagnetically coupled system, then appreciable population of higher excited states with increasing

(41) (a) Antanaitis, B. C.; Aisen, P.; Lilienthal, H. R.; Roberts, R. M.; Bazer, F. W. *J. Biol. Chem.* **1980**, *255*, 11204–11209. (b) Antanaitis, B. C.; Aisen, P. *J. Biol. Chem.* **1982**, *257*, 1855–1859.

(42) pI = 9.7 for uteroferrin: Chen, T. T.; Bazer, F. W.; Cetorelli, J. J.; Pollard, W. E.; Roberts, R. M. *J. Biol. Chem.* **1973**, *248*, 8560–8566.

Table II. Mössbauer Parameters for the Phosphate Complexes of Oxidized Bovine Spleen FePase and of Uteroferrin (UF_o·PO_4)[a]

	T (K)	δ^A	$\Delta E_Q{}^A$	Γ^A	δ^B	$\Delta E_Q{}^B$	Γ^B	ref
FePase	4.2	0.51 (2)	1.03 (5)	0.30 (3)	0.54 (2)	1.36 (5)	0.28 (3)	*b*
FePase	100	0.47 (2)	0.99 (5)	0.45 (3)	0.53 (2)	1.32 (5)	0.32 (3)	*b*
UF_o·PO_4	4.2	0.52	1.02	0.27	0.55	1.38	0.25	*c*

[a] Isomer shifts δ (relative to the centroid of metallic iron at 300 K), quadrupole splittings ΔE_Q and line widths Γ(fwhm) in units of Doppler shift (mm/s) are based on least-squares fits of the spectra to four Lorentzians of equal areas. Unrestricted four-line fits reduced χ^2 by less than 4%. Numbers in parentheses are uncertainties in the last significant digit. [b] This work. [c] Reference 49.

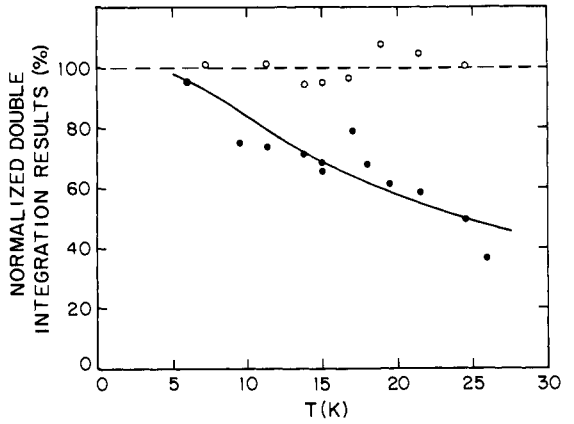

Figure 4. Plot of normalized double integrated intensities (intensity × T) of the EPR spectrum of the pink form of bovine spleen FePase as a function of temperature (solid circles). Open circles are data for a $CuSO_4$ standard. The solid line is a curve showing the population of the $S = {}^1/_2$ ground state of an $S_1 = {}^5/_2$, $S_2 = 2$ antiferromagnetically coupled system for $-2J = 11$ cm^{-1} and $\hat{H} = -2JS_1 \cdot S_2$.

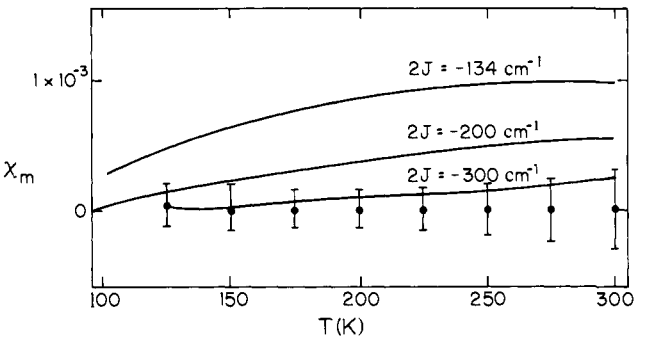

Figure 5. Magnetic susceptibility data as a function of temperature for a 17-mg lyophilized sample of the purple form of bovine spleen FePase (solid circles). Error bars shown are estimated uncertainties in susceptibility. The other three lines are computer-generated χ vs. T curves for an $S_1 = S_2 = {}^5/_2$ antiferromagnetically coupled system with $\hat{H} = -2JS_1 \cdot S_2$ and $-2J = 134$ cm^{-1}, 200 cm^{-1}, and 300 cm^{-1}.

temperature should occur. This will result in a decrease in the intensity of the $g' = 1.77$ EPR signal, as shown for bovine spleen FePase in Figure 4. Also shown in Figure 4 are the normalized data (double integration times T) for a $CuSO_4$ standard and a calculated curve showing the ground-state population as a function of temperature for an antiferromagnetically coupled $S_1 = {}^5/_2$, $S_2 = 2$ system[43] with $-2J = 11$ cm^{-1} and $\hat{H} = -2JS_1 \cdot S_2$ (the separation between the ground and first excited state ($S = {}^3/_2$) is $3J$). The agreement between the calculated curve and the experimental data over the range 6–20 K strongly suggests that the exchange coupling constant is $-2J = 11 \pm 2$ cm^{-1}.[44] The rapid decrease in integrated intensities above 20 K is due to severe broadening of the signal. This result is in reasonable agreement with the values of $-2J \approx 14$ and 20 cm^{-1} obtained for uteroferrin from analysis of the temperature dependence of the EPR[5] and NMR[16] spectra, respectively. The bulk of the available evidence thus suggests that the magnetic coupling between iron atoms in the binuclear center of the pink form of the purple acid phosphatase is an order of magnitude weaker than in the oxidized, purple form, possibly due to protonation of a bridging oxo group upon reduction.[16,30a,31]

Magnetic Susceptibility. Previous studies on the magnetic properties of the oxidized (purple) form of uteroferrin[14b,16] and bovine spleen FePase[6] have only been able to set lower limits of $-2J > 160$ and > 80 cm^{-1}, respectively, for the strength of the magnetic exchange interaction. Because the only direct magnetic susceptibility studies reported[5,6] have utilized dilute (1–2 mM) enzyme, we have reexamined the temperature dependence of the magnetic susceptibility of bovine spleen FePase using a lyophilized sample and a SQUID magnetometer. The low-temperature results (not shown) are similar to those previously reported,[6] with a paramagnetic contribution that can be accounted for by the presence of 1–2% high-spin Fe^{3+}; the bulk of the iron is effectively diamagnetic. The high-temperature data, however, permit quantitative assessment of the strength of the antiferromagnetic coupling and are shown in Figure 5 for $T = 100$–300 K, along with curves calculated[43] for $-2J = 134$, 200, and 300 cm^{-1}. The value of $-2J = 300$ cm^{-1} is the smallest that yields a curve that cannot be distinguished from the experimental data; this sets a lower limit of $-2J \geq 300$ cm^{-1} for the coupling constant in the purple form of bovine spleen FePase.

As has been pointed out for uteroferrin,[16] a coupling constant of this magnitude is most compatible with a μ-oxo bridge, the presence of which has been established for hemerythrin[29] and ribonucleotide reductase.[28] The only magnetic susceptibility results available are on methemerythrin and oxyhemerythrin of *Phascolopsis gouldii*, for which values of $-2J = 268$ and 154 cm^{-1} are reported, respectively.[45] These values are somewhat larger than those reported for simple binuclear Fe(III) complexes with μ-oxo bridges, for which $-2J$ ranges from ca. 180 to 210 cm^{-1}.[46,47] Results on a triply bridged model compound for hemerythrin[48a] suggest that the higher values of the exchange constant may be due to the presence of bridging groups in addition to the μ-oxo. Alternatively, a recent report suggests that unsymmetrical ferric dimers with a single bridging oxo group can exhibit exchange couplings as large as $-2J = 250$ cm^{-1}.[48b] The present magnetic results are thus consistent with the presence of either a multiply bridged or an unsymmetrical singly bridged binuclear Fe(III) center in oxidized bovine spleen FePase.

Mössbauer Data. ^{57}Fe Mössbauer spectroscopy provides a direct probe of the electronic and chemical environment of all of the iron

(43) (a) Mabbs, F. E.; Machin, D. J. *Magnetism and Transition Metal Complexes*; Chapman and Hall: London, 1973; pp 170–203. (b) Wojciechowski, W. *Inorg. Chim. Acta* **1967**, *1*, 324–328.

(44) In principle, one should be able to verify this by observing and quantitating the EPR signal from the $S = {}^3/_2$ level that is being populated with increasing temperature. In practice, the combination of inherent line width and relaxation broadening at higher temperature make the $S = {}^3/_2$ EPR difficult to detect: cf. nitrogenase MoFe protein[44a] and Fe protein.[44b-d] (a) Münck, E.; Rhodes, H.; Orme-Johnson, W. H.; Davis, L. C.; Brill, W. J.; Shah, V. K. *Biochim. Biophys. Acta* **1975**, *400*, 32–53. (b) Lindahl, P. A.; Day, E. P.; Kent, T. A.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 11160–11173. (c) Hagen, W. R.; Eady, R. R.; Dunham, W. R.; Haaker, H. *FEBS Lett.* **1985**, *189*, 250–254. (d) Watt, G. D.; McDonald, J. W. *Biochemistry* **1985**, *24*, 7226–7231.

(45) Dawson, J. W.; Gray, H. B.; Hoenig, H. E.; Rossman, G. R.; Schredder, J. M.; Wang, R.-H. *Biochemistry* **1972**, *11*, 461–465.

(46) Murray, K. S. *Coord. Chem. Rev.* **1974**, *12*, 1–35.

(47) Moss, T. H.; Lilienthal, H. R.; Moleski, C.; Smythe, G. A.; McDaniel, M. C.; Caughey, W. S. *J. Chem. Soc. Chem. Commun.* **1972**, 263–264.

(48) (a) Armstrong, W. H.; Spool, A.; Papaefthymiou, G. C.; Frankel, R. B.; Lippard, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 3653–3667. (b) Gomez-Romero, P.; DeFotis, G.; Jameson, G. B. *J. Am. Chem. Soc.* **1986**, *108*, 851–853.

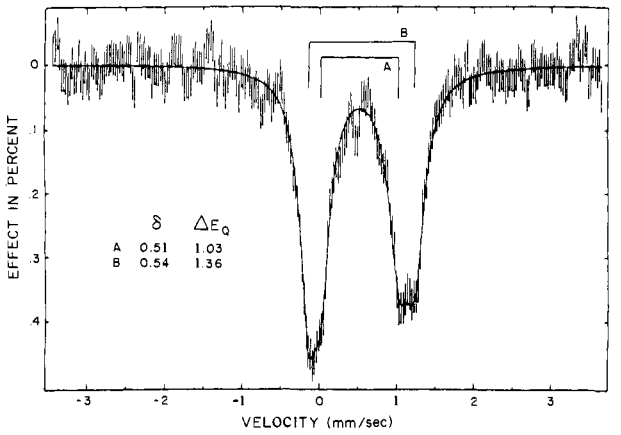

Figure 6. ^{57}Fe Mössbauer spectrum at 4.2 K of purple (oxidized) bovine spleen FePase (2 mM in 50 mM NaOAc, pH 5.0). The solid line is a fit to the data with use of the parameters in Table II. Data aquisition time was 47 h.

present in a sample. Enrichment with ^{57}Fe is necessary if magnetically split spectra are to be analyzed; to date, however, the instability of the apo enzyme has prevented us from obtaining significant amounts of bovine spleen FePase enriched in ^{57}Fe. Here we present an analysis of the Mössbauer spectra recorded for an unenriched sample of the phosphate complex of oxidized bovine spleen FePase (Figure 6). The spectra are fitted adequately by two overlapping quadrupole doublets of equal areas; no other iron sites are evident. Table II lists the fit parameters for spectra taken at 4.2 and at 100 K as well as the corresponding values for the phosphate complex of oxidized uteroferrin.[49] The line width at 4.2 K slightly exceeds the minimum instrumental line width of $\Gamma_{min} = 0.24$ mm/s, indicating some heterogeneity in the iron sites (as is typically found in iron proteins).

The isomer shifts and quadrupole splittings characterize both sites as high-spin ferric; the absence of magnetic hyperfine splitting at 4.2 K is consistent with a spin-coupled, diamagnetic ground state, as indicated by the magnetic susceptibility data and the lack of an EPR signal. It is obvious from Table II that the Mössbauer parameters of bovine FePase are remarkably close to the corresponding uteroferrin values; the parameters of both sites in these systems differ considerably from those of phosphate-free oxidized uteroferrin.[15] Previous results[22,31] strongly suggest that phosphate binds to one of the iron atoms, and a concomitant change in the electric field gradient at the iron is therefore not surprising.

Spectra taken at 100 K are broadened considerably by the application of a 0.17 T magnetic field. Quantitative analysis of these spectra has not yet been attempted, but we note that similar observations for oxidized uteroferrin and its phosphate complex have been related to the thermal population of an $S = 1$ excited state,[15,49] which could give rise to magnetic interactions. If the exchange coupling is indeed of the order $-2J \geq 300$ cm^{-1}, as discussed above, then a ca. 4% population of the $S = 1$ state is expected at 100 K.

These observations further support the existence of substantial similarity between the binuclear iron centers of bovine spleen FePase and uteroferrin. In addition, the Mössbauer parameters of the iron sites in these purple acid phosphatases are very similar to those reported for azidomethemerythrin[50a] and methemerythrin.[50b] All have relatively large quadrupole splittings for high-spin ferric iron, suggesting major distortions from effective octahedral geometry.

Resonance Raman Spectra. Metalloproteins containing tyrosinate residues as ligands are readily identified by the presence

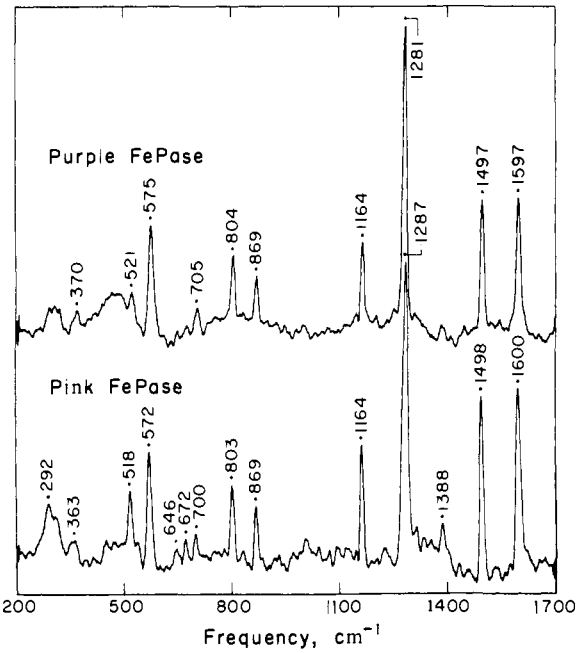

Figure 7. Resonance Raman spectra of 5 mM purple (top) and 2.7 mM pink (bottom) forms of bovine spleen FePase in 10 mM NaOAc buffer, pH 5.0 at 5 °C. Sample temperature was maintained by placing the capillary in the cold-finger of an ice-filled Dewar.[28] Data were collected with use of 514.5-nm excitation, 100-mW incident power, and 140° back-scattering geometry. The spectra shown are an average of 3 scans (purple) and 8 scans (pink) taken at a scan rate of 1 cm^{-1}/s and a slit width of 4–5 cm^{-1} and then subjected to a 13-point smooth.

of four resonance-enhanced tyrosine ring modes between 1650 and 1150 cm^{-1} upon excitation into the visible absorption band. The presence of these features serves as a fingerprint for tyrosine ligands and identifies the absorption band as arising from phenolate-to-metal charge transfer.[51] Resonance Raman spectra using visible excitation have been reported for uteroferrin.[18,52] In addition to the four tyrosinate ring modes at 1603, 1503, 1285, and 1168 cm^{-1}, low-frequency modes at 872, 805, and 575 cm^{-1} were observed for the purple form.[18] The only significant changes observed upon reduction to the pink form were a shift of the 1285-cm^{-1} band to 1293 cm^{-1}, and a decrease in intensity of the 872-cm^{-1} feature.[18] The 805- and 872-cm^{-1} bands were assigned to a metal-coordinated tyrosyl Fermi doublet and the 575 cm^{-1} feature to a combination mode with a significant Fe–O stretching contribution. In order to characterize the binuclear center of bovine spleen FePase as fully as possible, we have measured high-quality resonance Raman spectra of the purple and pink forms.

As shown in Figure 7, the resonance Raman spectra of bovine spleen FePase are similar to those reported earlier for uteroferrin. Although the exact peak positions are not identical in the two proteins, the general features of the spectra and the shift of the 1281-cm^{-1} band to 1287 cm^{-1} upon reduction correspond closely to the reported data for uteroferrin. The remarkable similarity in the spectral properties of the two suggests a common chromophore in both proteins. In the excitation profile of the 1281-, 804-, 575-, and 521-cm^{-1} bands (Figure 8), the intensities appear to follow the visible absorption band, providing further evidence that all four vibrations are due to the Fe(III)–tyrosinate chromophore. The fact that the intensity of both the Tyr→Fe(III) charge transfer band and the Fe–tyrosinate resonance Raman peaks remains unchanged upon reduction suggests that only one of the two iron atoms is tyrosine coordinated and that this iron remains in the ferric state in pink FePase. Similar conclusions have been drawn from the NMR spectrum of pink uteroferrin.[16]

(49) Pyrz, J. W.; Sage, J. T.; Debrunner, P. G.; Que, L., Jr. *J. Biol. Chem.* **1986**, *261*, 11015–11020.
(50) (a) Clark, P. E.; Webb, J. *Biochemistry* **1981**, *20*, 4628–4632. (b) Okamura, M. Y.; Klotz, I. M.; Johnson, C. E.; Winter, M. R. C.; Williams, R. J. P. *Biochemistry* **1969**, *8*, 1951–1958. (c) Kurtz, D. M., Jr.; Sage, J. T.; Hendrich, M.; Debrunner, P. G.; Lukai, G. S. *J. Biol. Chem.* **1983**, *258*, 2115–2117.
(51) Que, L., Jr. *Coord. Chem. Rev.* **1983**, *50*, 73–108.
(52) Gaber, B. P.; Sheridan, J. P.; Bazer, F. W.; Roberts, R. M. *J. Biol. Chem.* **1979**, *254*, 8340–8342.

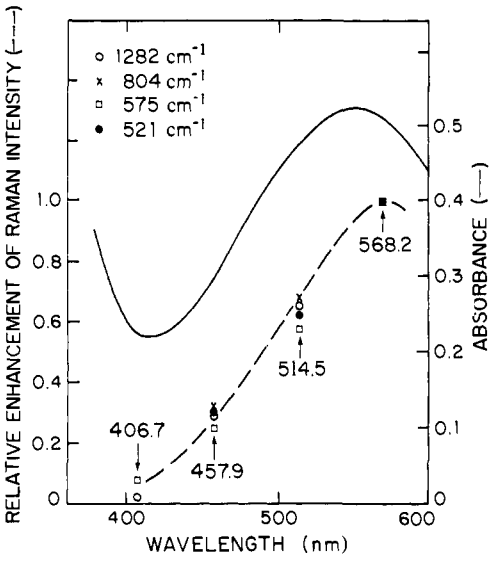

Figure 8. Excitation profile (dashed line) for four peaks in the Raman spectrum of the purple form of bovine spleen FePase, compared to the absorption spectrum (solid line). The 1.7 mM protein sample in a glass capillary was maintained at 5 °C by a stream of cooled nitrogen. Data were collected in a 90° scattering geometry. Enhancement was calculated from the height of the sample peak relative to the height of the 927-cm^{-1} acetate peak (0.05 M) and normalized to a value of 1.0 for 568.2-nm excitation.

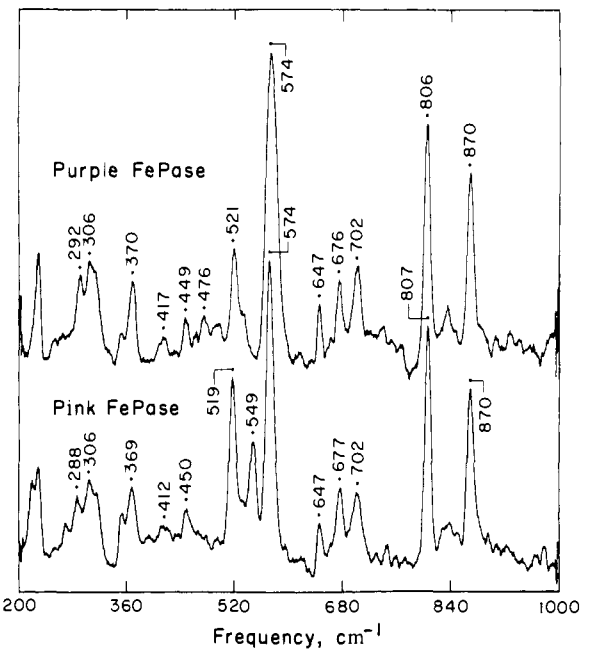

Figure 9. Low-frequency resonance Raman spectra of 5 mM purple (top) and 2.7 mM pink (bottom) forms of bovine spleen FePase in 10 mM NaOAc buffer, pH 5.0, at 15 K. Samples were pipetted directly onto a gold-plated copper cold finger of a closed-cycle helium refrigerator (Air Products Displex), which was then cooled to ≈225 K under nitrogen, evacuated, and finally cooled to 15 K. Data were collected with use of 514.5-nm excitation, 100-mW incident power, and 140° back-scattering geometry. The spectra shown are an average of 5 scans (purple) and 9 scans (pink) taken at a scan rate of 2 cm^{-1}/s and a slit width of 6 cm^{-1} and then subjected to a 17-point smooth. The feature at 549 cm^{-1} is observed only with the pink form, but its intensity varies among different samples (e.g., Figure 7, bottom).

In addition to the tyrosinate modes discussed above, the spectra in Figure 7 show the presence of a number of previously unreported features at lower energies. Optimal low-frequency spectra were obtained at 15 K by use of a He Displex, which gives considerably less interference from the 400–500-cm^{-1} glass band (prominent in Figure 7, top). Figure 9 shows well-resolved features at 702, 676, 647, 450, 417, 370, and 292 cm^{-1} for the purple form of the enzyme; all appear to have a similar enhancement profile to the tyrosine modes. The peak at 417 cm^{-1} may well be the ν_{16a} fundamental, heretofore predicted but unobserved in Fe(III)–tyrosinate proteins.[18] This peak is at the proper energy to be responsible for the Fermi resonance coupling of an ≈830-cm^{-1} internal tyrosine ring mode with $2\nu_{16}$, whose configuration interaction leads to the splitting of the bands now observed at 804 and 869 cm^{-1}. These three features are equally apparent in the low-frequency spectrum of pink FePase (Figure 9, bottom) and in more recent spectra of pink uteroferrin.[53]

The only major difference between the purple and pink forms is the ≈2-fold increase in the intensity of the 521-cm^{-1} peak. A similar selective increase in the intensity of the ≈520-cm^{-1} peak has been observed in pink uteroferrin relative to purple uteroferrin (prepared in the absence of phosphate).[53] Since this is the only mode whose intensity is sensitive to the oxidation state of the adjacent iron atom (which is presumably not coordinated to tyrosine), it may involve a contribution from a bridging ligand. In view of the lack of ^{18}O-dependence that would be expected for an oxo or hydroxo bridge vibration (see below), a possible assignment is the O–C–O bend of a bridging carboxylate coupled to an Fe–tyrosinate vibration. A carboxylate deformation mode has been tentatively identified by resonance Raman spectroscopy at 530 cm^{-1} in methemerythrin[54] and at 581 cm^{-1} in the μ-oxo, di-μ-acetato model compound $\{[Fe(TACN)(OAc)]_2O\}^{2+}$.[55]

In an attempt to assign some of these peaks to specific vibrations, the Raman spectra of both purple and pink FePase in $H_2{}^{18}O$ and D_2O were obtained. Of particular interest was the possibility of observing an Fe–O–Fe symmetric stretch in the purple state. Previous work on oxyhemerythrin[56] and ribonucleotide reductase[28] has shown that this vibrational mode occurs at ≈500 cm^{-1}, shifts ≈15 cm^{-1} to lower energy with ^{18}O in the bridge, and is most strongly enhanced with near-UV excitation. The corresponding feature in azidomethemerythrin[56] has maximal intensity with visible excitation, reflecting a change in the location of the oxo→Fe(III) charge transfer band responsible for resonance enhancement. The ^{18}O-containing samples of acid phosphatase were prepared by overnight equilibration of pink enzyme with isotopically labeled water, followed by aerobic oxidation with phosphate.[22] This procedure is based on that used to obtain ^{18}O-exchanged hemerythrin[53] and is intended to maximize the likelihood of isotope incorporation via the more labile reduced enzyme.

The low-frequency Raman spectra of purple and pink FePase obtained with visible excitation revealed no significant and reproducible shifts in peak positions with either D_2O or $H_2{}^{18}O$. Irradiation of liquid (0 °C) or frozen (90 K) samples with laser light of wavelength less than 400 nm resulted in rapid and complete bleaching of the sample, and no Raman spectra were observed. Use of the He Displex (sample at 15 K) permitted near-UV irradiation without sample bleaching. No new features were observed, however, and the peaks which were observed were considerably less enhanced than those in Figure 7 with visible excitation. Thus, we must conclude that none of these peaks can be assigned to either the Fe–O–Fe symmetric stretch or to an Fe–imidazole vibration, which typically occurs near 300 cm^{-1} and shows an ≈2-cm^{-1} decrease upon deuteriation.[57] Although there is suggestive evidence for the presence of histidine ligands in uteroferrin,[16,19] the resonance Raman spectra of both proteins are clearly dominated by the tyrosinate ligands. Furthermore, there are no significant changes in the Raman spectrum of pink FePase between pH 3.0 and 5.5, despite the marked alteration in the EPR

(53) Doi, K.; Aisen, P.; Sanders-Loehr, J.; Loehr, T. M., unpublished results.
(54) Shiemke, A. K. Ph.D. Dissertation, Oregon Graduate Center, 1986.
(55) Spool, A.; Williams, I. D.; Lippard, S. J. *Inorg. Chem.* **1985**, *24*, 2156–2162.
(56) Shiemke, A. K.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1984**, *106*, 4951–4956.
(57) Larrabee, J. A.; Spiro, T. G.; Ferris, N. S.; Woodruff, W. H.; Maltese, W. A.; Kerr, M. S. *J. Am. Chem. Soc.* **1977**, *99*, 1979–1980.

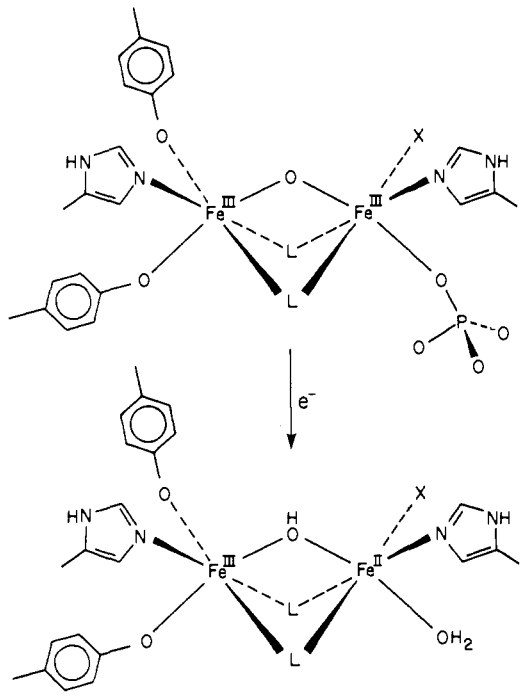

Figure 10. Proposed model for the binuclear iron site of purple and pink bovine spleen FePase and uteroferrin, based on this work and that cited in the text. L indicates possible bridging ligands in addition to a μ-oxo; X indicates unidentified ligand.

spectrum over this same pH range.

It has been found that the resonance enhancement of the Fe–O–Fe symmetric stretch can vary by several orders of magnitude relative to a sulfate internal standard (981-cm^{-1} symmetric stretch), depending on the bridging geometry and on the nature of the nonbridging ligands.[58] With excitation in the visible region, relative scattering factors of ≈200–1200 are observed for hemerythrin, ribonucleotide reductase, and $\{[Fe(HBpz_3)(OAc)]_2O\}$. In contrast, factors of only ≈15–40 are observed for $\{[Fe(TACN)(OAc)]_2O\}^{2+}$, $\{[Fe(N\text{-propsal})_2]_2O\}$, and $\{[Fe(HEDTA)]_2O\}^{2-}$. The high scattering factors appear to correlate with triply bridged structures having an unsaturated nitrogen ligand trans to the μ-oxo bridge. Under the conditions in Figure 9, the 575-cm^{-1} peak of purple FePase has an enhancement of 225 relative to a sulfate internal standard. We estimate that the data in Figure 9 are of sufficient quality to allow detection of an Fe–O–Fe vibration with a scattering factor of ≈40. Thus, if an Fe–O–Fe center is present in purple FePase and has undergone exchange with $H_2{}^{18}O$, it can be only weakly resonance-enhanced. This would imply that the putative oxo group is not trans to a histidine ligand.

(58) Wheeler, D.; Shiemke, A. K.; Averill, B. A.; Loehr, T. M.; Sanders-Loehr, J., unpublished results.

Conclusions. Anaerobic titrations monitored by optical spectroscopy and low-temperature EPR spectrometry have shown that conversion of the purple form of the bovine spleen purple acid phosphatase to the enzymatically active pink form is a one-electron process. The heterogeneity of the $g' = 1.77$ EPR spectrum of the pink form is due to a pH-dependent mixture of two species with different rhombic EPR signals, probably due to the ionization of a single group on the protein. The temperature dependence of the EPR signal of the pink form over the range 6–20 K is consistent with a weak antiferromagnetic coupling between an $S = {}^5/_2$ Fe(III) and an $S = 2$ Fe(II), with $-2J = 11 \pm 2$ cm^{-1}. Bulk magnetic susceptibility studies of the purple (oxidized) form indicate a strongly antiferromagnetically coupled binuclear high-spin ferric system with $-2J \geq 300$ cm^{-1}. Mössbauer spectra of the phosphate complex of the purple form confirm the coupled diferric site and indicate that the iron atoms are in relatively low symmetry environments. Resonance Raman spectra demonstrate the presence of tyrosyl phenolate ligands to iron in both the purple and pink forms, but experiments with $H_2{}^{18}O$ gave no direct evidence for the presence of a μ-oxo bridging ligand. Integration of the above data with previously reported NMR[16] and pulsed EPR[19] studies on uteroferrin and EXAFS studies[31] on the bovine spleen purple phosphatase results in the structural model shown in Figure 10.

Although there is no direct evidence for a bridging μ-oxo group or μ-hydroxo group in the purple and pink form, respectively, they are by far the most obvious choices to provide magnetic coupling of the magnitudes observed. The presence of additional bridging ligands L (Figure 10) is more tenuous and is proposed primarily from the similarity of some of the chemical and spectroscopic properties of FePase to those of hemerythrin. These are the following: (i) the ability to form a stable mixed-valence species, (ii) an iron–iron separation of less than 3.3 Å according to EXAFS,[31] and (iii) the unusually large antiferromagnetic coupling in the purple form. Interaction of phosphate with one of the iron atoms is supported by the phosphate-induced oxidation of pink to purple enzyme[24] and by preliminary EXAFS results.[31] Because phosphate is a competitive inhibitor of phosphate ester hydrolysis, this strongly suggests that at least one iron atom of the binuclear core participates in substrate hydrolysis. Further studies are in progress to determine the mechanism of catalysis of a simple hydrolytic reaction by a protein containing a mixed-valence binuclear iron center.

Acknowledgment. We thank Gerald T. Babcock and Robert Ingle for preliminary resonance Raman spectra of the purple phosphatase at Michigan State University and Andrew K. Shiemke for assistance with the Raman spectra at the Oregon Graduate Center. This work was supported by grants from the National Institutes of Health (GM 32117 and GM 28636 to B.A.A., GM 18865 to T.M.L. and J.S.L., and GM 16406 to P.G.D.). Funds for purchase of much of the instrumentation used in enzyme purification were provided by a Cottrell Research Grant from the Research Corporation.