Electrochemical Properties of the Diiron Core of Uteroferrin and Its Anion Complexes†

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ABSTRACT: The reduction potentials (E_m) of the purple acid phosphatase from porcine uterus, uteroferrin (Uf), and its phosphate, arsenate, and molybdate complexes were determined by coulometric methods at various pH values. The midpoint potential of Uf at the pH value for optimal enzyme activity (pH 5) was found to be +367 mV versus a normal hydrogen electrode (NHE), while at pH 6.01 Uf exhibits a reduction potential of +306 mV. At pH 6.01 molybdate was found to shift the potential of Uf more positive by 192 mV, while phosphate and arsenate shift the potential of Uf more negative by 193 and 89 mV, respectively. These shifts are consistent with the different susceptibilities of Uf to aerobic oxidation in the presence of these anions. Comparison of the reduction potential of Uf at pH 7.0 with those reported for other dinuclear non-heme iron enzymes and various (μ-oxo)diiron model complexes suggest that the potential of Uf is too positive to be consistent with a μ-oxo-bridge in Uf_o. The pH dependence of the reduction potentials of Uf (60 mV/pH unit) and the fact that the electron transfer rate increases with decreasing pH indicate a concomitant participation of a proton during the oxidation–reduction process. This process was assigned to the protonation of a terminally bound hydroxide ligand at the Fe(II) center upon reduction of Uf_o. Structural implications provided by the electrochemical data indicate that molybdate affects the dinuclear core in a manner that differs from that of phosphate and arsenate. This observation is consistent with previous spectroscopic and biochemical studies. Several possible anion binding modes are discussed and related to our recently proposed model for the interaction of anions with Uf_r [David, S. S., & Que, L., Jr. (1990) *J. Am. Chem. Soc. 112*, 6455–6463].

An important class of dinuclear iron proteins, which include hemerythrin (Hr),[1] ribonucleotide reductase (RRB2), methane monooxygenase (MMO), and the purple acid phosphatases (PAP) (Que & True, 1990; Sanders-Loehr, 1989), has recently emerged. The phosphatase enzymes, which are postulated to participate in cell regulation mechanisms involving phosphorylated proteins (Schindelmeister et al., 1987), hydrolyze phosphate esters under acidic pH conditions in vitro (Antanaitis & Aisen, 1983). Uteroferrin (Uf), a purple acid phosphatase from porcine uterus, consists of a single polypeptide chain (35 000 daltons) that binds 2 mol of iron/mol of enzyme (Antanaitis et al., 1980, 1983).

The dinuclear iron center of Uf can be obtained in either of two oxidation states: a reduced Fe(III)Fe(II) form (Uf_r) and an oxidized Fe(III)Fe(III) form (Uf_o) (Chen et al., 1973; Campell et al., 1978). Uf_o is purple ($\lambda_{max} \sim 550$ nm, $\epsilon = 4000$ M^{-1} cm^{-1}), EPR silent, and enzymatically inactive, while Uf_r is pink ($\lambda_{max} \sim 510$ nm, $\epsilon = 4000$ M^{-1} cm^{-1}) and exhibits acid phosphatase activity (Keough et al., 1983; Schlosnagle et al., 1974, 1976; Antanaitis & Aisen, 1982). Uf_r also displays EPR signals with g values of 1.94, 1.76, and 1.56 that are consistent with an antiferromagnetically coupled high-spin Fe(III)Fe(II) center (Antanaitis et al., 1983; Averill et al., 1987). On the basis of SQUID magnetization studies (Day et al., 1988), the antiferromagnetic coupling ($-2J$) between the iron centers is weak, on the order of 20 cm^{-1}, which is consistent with NMR (Lauffer et al., 1983) and EPR (Antanaitis et al., 1983) estimates.

SQUID magnetization measurements on PAP_o's estimate the antiferromagnetic coupling to be very strong, on the order of 200–300 cm^{-1} (Averill et al., 1987; Sinn et al., 1983), while Evan's susceptibility measurements suggest a value >80 cm^{-1} (Lauffer et al., 1983). These data suggest the presence of an oxo bridge. However, EXAFS (Kauzlarich et al., 1986; Que & Scarrow, 1988) and resonance Raman studies (Averill et al., 1987) were unable to demonstrate the existence of such a bridge. Furthermore, a recent magnetic susceptibility study of PAP_o from bovine spleen reported the antiferromagnetic coupling to be weak ($-2J = \sim 30$ cm^{-1}) (Gehring et al., 1990), which indicates the lack of a μ-oxo bridge. The discrepancy in bridge type of PAP_o clearly warrants further investigation.

The coordination environment of the dinuclear core of Uf is not completely established. A comparison of the EXAFS analyses of the histidine-rich metHrN_3 (r_{av} for Fe–O,N = 2.13 Å) and the oxygen-rich metRRB2 (r_{av} for Fe–O,N = 2.05 Å) (Scarrow et al., 1987) with Uf_oPO_4 (r_{av} for Fe–O,N = 2.03 Å) (Que & Scarrow, 1988) indicates that the dinuclear core of Uf likely resides in an oxygen-rich environment. Resonance

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[1] Abbreviations: bipy, 2,2′-bipyridine; BPMP, 2,6-bis[[bis(2-pyridylmethyl)amino]methyl]-4-methylphenol; Co(bipy)$_3^{2+}$, tris(2,2′-bipyridyl)cobalt(II); DCP, 2,6-dichlorophenylindophenol; DMFc, 1,1-dimethylferrocene; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetatic acid; FO, fraction oxidized; FR, fraction reduced; HBpz$_3$, hydrotris(pyrazolyl)borate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethenesulfonic acid; HMFc, hydroxymethylferrocene; Hr, hemerythrin; Me$_3$TACN, 1,4,7-trimethyl-1,4,7-triazacyclononane; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MMO, methane monooxygenase; N5, *N*-(hydroxyethyl)-*N*,*N*′,*N*′-tris(2-benzimidazolylmethyl)-1,2-diaminoethane; NHE, normal hydrogen electrode; OAc, acetate; OBz, benzoate; OPr, propionate; PAP, purple acid phosphatases; RRB2, ribonucleotide reductase; Ru(NH$_3$)$_6^{2+}$, hexaammineruthenium(II); TPA, tris(2-pyridylmethyl)-amine; Tris, tris(hydroxymethyl)aminomethane; Uf, uteroferrin.

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Raman (Antanaitis et al., 1982; Averill et al., 1987), NMR (Lauffer et al., 1983; Scarrow et al., 1990), and pulsed EPR (Antanaitis et al. 1985) measurements have identified a histidine ligand coordinated at each metal site and a tyrosine at the redox-inactive Fe(III) center. The weak antiferromagnetic coupling observed for Uf_r indicates a hydroxo group bridges the two metal centers.

Several tetraoxo anions have been shown to bind Uf and inhibit phosphatase activity. The vast majority of these studies have focused on phosphate, the substrate analogue and the product of phosphatase activity. However, comparative data involving arsenate and molybdate binding have begun to emerge. Phosphate and arsenate are weak competitive inhibitors of Uf_r with K_i's of 12 and 1.3 mM, respectively, at pH 4.9 (David & Que, 1990; Pyrz et al., 1986). They both potentiate the aerobic oxidation of the dinuclear cluster and form tightly bound Uf_o complexes. On the other hand, molybdate is a potent inhibitor of Uf_r with a K_i of 4 μM (David & Que, 1990) and affords a complex that is air stable.

The oxidation–reduction chemistry of uteroferrin suggests the possibility of a novel redox-linked regulation of enzyme activity. The two forms of Uf can be interconverted by using reductants such as β-mercaptoethanol and ascorbate or oxidants such as hydrogen peroxide and ferricyanide (Debrunner et al., 1983). In this paper, the reduction potentials of Uf and its phosphate, arsenate, and molybdate complexes are reported at various pH values.

Experimental Procedures

Materials

Uteroferrin and Its Anion Complexes. Uteroferrin (Uf) was purified and its anion complexes were obtained as previously described (David & Que, 1990; Basha et al., 1980; Scarrow et al., 1990). The concentrations of enzyme solutions were determined by using a molar extinction coefficient of 4000 M^{-1} cm^{-1} at 510 nm for Uf_r, 505 nm for Uf_r·AsO_4, 536 nm for Uf_r·PO_4, 525 nm for Uf_r·MoO_4, and 550 nm for Uf_o, Uf_o·PO_4, and Uf_o·AsO_4.

Buffers and Mediators. Buffers used for the pH dependence studies were 0.1 M glycine and 0.2 M NaCl, pH = 3.15; 0.1 M acetate and 0.2 M NaCl, pH = 4.01–5.01; 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) and 0.2 M NaCl, pH = 6.01; 0.025 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and 0.3 M NaCl, pH = 6.95; and 0.02 M tris(hydroxymethyl)aminomethane (Tris) and 0.3 M NaCl, pH = 7.91. The pH values of each solution were measured before and after each experiment. All of the mediators used had E_m values vs NHE that had been previously measured by cyclic voltammetry. The mediators were hydroxymethylferrocene (HMFc) (+434 mV for pH = 3.00–5.00), 1,1-dimethylferrocene (DMFc) (+303 mV for pH = 3.00–6.01), $Co(2,2'\text{-bipy})_3^{2+}$ (+273 mV for pH = 5.00–8.00), horse heart cytochrome *c* (+243 mV for pH = 7.00, +260 mV for pH = 8.00), $Ru(NH_3)_6^{2+}$ (+50 mV for pH = 6.01), and 2,6-dichlorophenylindophenol (DCP) (+217 mV for pH = 7.00). The concentrations of the mediators were 100–200 μM except for DMFc, which was used as a saturated solution.

Methods

Microcoulometry. The basic coulometric techniques used have previously been described (Watt, 1979; Spence et al., 1982). The three-electrode cell of 2–3-mL capacity is a modification of the cell described by Watt (Figure 1). The working electrode was platinum gauze, while a silver–silver chloride wire, contained in a fritted compartment that was

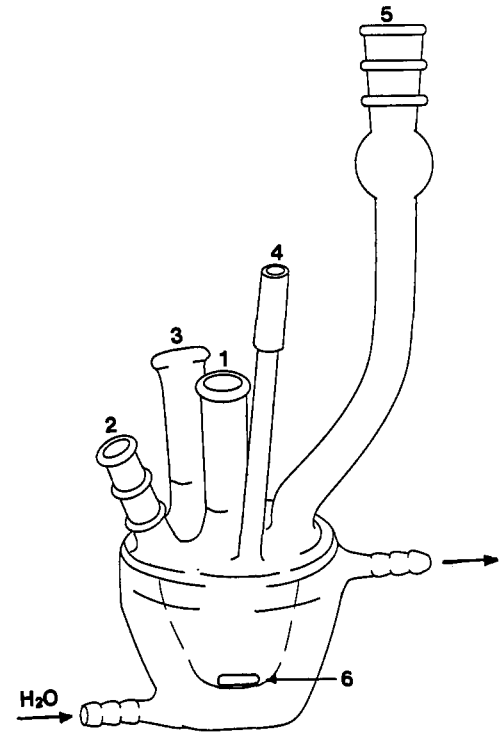


FIGURE 1: Cell used for the microcoulometric titrations. Openings are for (1) platinum gauze working electrode, (2) Ag/AgCl reference electrode, (3) Ag/AgCl auxiliary electrode, (4) gastight syringe for enzyme sample delivery, and (5) main connection to Ar line. (6) Stir bar.

plugged with porous vycor and filled with 0.1 M KCl solution, served as the reference electrode. The auxiliary electrodes were made of silver wire in a fritted compartment. All of the microcoulometry experiments were performed on a BAS 100 electrochemical analyzer with each reduction potential reported versus NHE. Both the enzyme (~80 μL) and the mediator solutions were prepared under anaerobic conditions.

For a typical microcoulometry experiment ~2 nmol of protein was injected into ~2 mL of solution that contained the appropriate mediator at a controlled potential for each titration point. The injected material (0.7–1.0 μM) was reduced at the controlled potential and the current was integrated. A total of 7–10 data points per experiment were taken, which required ~14–20 nmol of protein. The concentrations of Uf_o and Uf_r were calculated at each point in the titration experiment. For experiments done in the reductive direction, $[Uf_r]$ was calculated by measuring the number of coulombs transferred and then multiplying by Faraday's constant to give the moles of electrons for a one-electron transfer. This value was then divided by the volume of enzyme added to the solution. The total enzyme concentration $[Uf_T]$ and the volume added at each point were accuratley known and allowed the $[Uf_o]$ to be calculated by difference.

For Uf there is a large potential difference between the first and second electron transfer; the one-electron-reduced form is thus stable over a wide potential range. In these experiments, the potential was set at a value that allowed only the first electron transfer process to take place.

The potentials of Uf and its anion complexes at all pH values were measured in one direction only because of the convenience of sample preparation. The catalytically active Uf_r is reasonably stable for extended periods of time under aerobic conditions, as is Uf_r·MoO_4, while Uf_o·PO_4 and Uf_o·AsO_4 are considerably more stable then their reduced counterparts. Furthermore, Uf_o prepared by H_2O_2 oxidation was shown by Mössbauer spectroscopy to be a mixture of different forms

Table I: Midpoint Potentials of Uteroferrin at Various pH Values[a]

pH	3.15	4.05	5.00	6.01	6.95	7.91
E_m	+457	+434	+367	+306	+242	+181

[a] E_m values are given in millivolts versus a normal hydrogen electrode (NHE).

(Sage et al., 1989); we deemed this situation to be inappropriate for electrochemical studies. The data obtained for Uf and each of its anion complexes fit a theoretical curve described by a reversible one-electron transfer process. The redox reactions thus appear to be reversible. In many of the published applications of microcoulometry experiments, the reactions are run only in a single direction (Spence et al., 1982; Barber et al., 1988).

Data Analysis. The coulometric data recorded by the BAS 100 electrochemical analyzer were transferred to a Macintosh SE computer in order to facilitate the simulations with *Excel* software. Midpoint potentials were obtained by comparison with the theoretical $n = 1$ Nernstian equation, which is expressed in terms of the fraction of oxidized enzyme (FO) and reduced enzyme (FR):

$$FO = \frac{[Uf_o]}{[Uf_T]} = \frac{1}{1 + \exp(-F/RT)(E - E_m)} \quad (1)$$

$$FR = \frac{[Uf_r]}{[Uf_T]} = \frac{1}{1 + \exp(+F/RT)(E - E_m)} \quad (2)$$

The total enzyme concentration is given by $[Uf_T]$ and the remaining parameters have their standard meanings. Previous error estimates of midpoint potentials determined by microcoulometric techniques indicated an accuracy of approximately ±10 mV (Spence et al., 1982).

Results

Midpoint Potentials of Uteroferrin. Organic dyes such as DCP (E_m +260 mV for pH = 4.90) and phenazine methosulfate (E_m = +210 mV pH = 4.90) were used as the mediators in previous coulometric titrations of Uf monitored spectroelectrochemically (Soltysik et al., 1987). The equilibrium between Uf and these mediators was found to be slow with non-Nernstian slopes. This behavior is likely due to a poor match between mediator and Uf midpoint potentials.

In the present work, coulometric measurements were carried out with several inorganic mediators from pH 3.15 to 8.00, a pH range in which the enzyme was found to be stable (David & Que, 1990). These data are summarized in Table I. A coulometric titration of Uf performed in the oxidation mode at pH 5.00, which corresponds to the pH value for optimal enzyme activity, is shown in Figure 2. The midpoint potential under these conditions was determined to be +367 mV vs NHE. The midpoint potentials vary as a function of pH with a value of 60 mV/pH unit (Figure 3). This clearly indicates a concomitant participation of a proton during the oxidation–reduction of Uf. It should also be noted that the presence of DCP improved the electron transfer between cytochrome *c* and the electrode in titrations at pH values 7.00 and 8.00. Direct communication of cytochrome *c* with the platinum electrode was found to be poor.

Analysis of the current (i) vs time (t) curve can give rise to an estimation of the kinetic characteristics of the electrolysis system (Watt, 1979). By maintainig the solution volume, stirring rate, and electrode surface area identical for different titrations, a plot of i vs t allows the relative rates of the mediator–enzyme interactions to be compared. Figure 4 shows characteristic i vs t plots at the respective midpoint potentials.

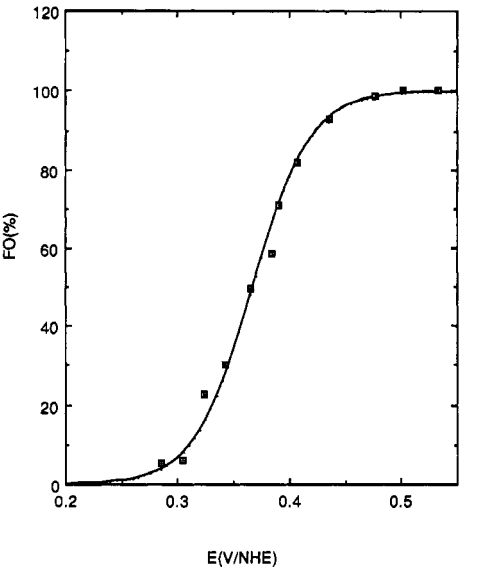


FIGURE 2: Microcoulometric titration of 0.187 mM Uf at pH 5.00. Simulation of the fraction oxidized (FO) with E_m = +367 mV (solid curve). The individual data points are shown (squares).

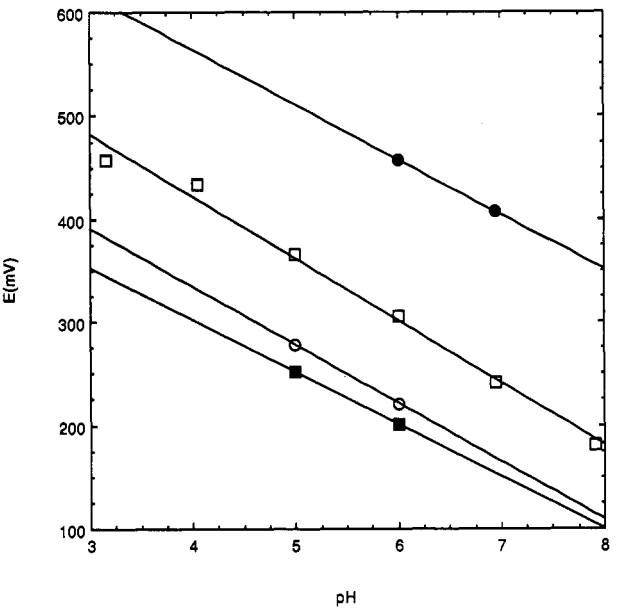


FIGURE 3: Plots of the reduction potentials of native Uf (open squares) and $E_{m(XO_4)}$ values of Uf·PO_4 (solid squares), Uf·AsO_4 (open circles), and Uf·MoO_4 (solid circles) as a function of pH.

At a pH value of 3.15, 1.02 μM of Uf_r is oxidized by 75 μM HDFc and about 20 μM DMFc (saturated) within 4 min. At a pH value of 5.00, 0.914 μM of Uf_r is oxidized by 97.3 μM $Co(bipy)_3^{2+}$ and about 18 μM within 15 min. In these two cases, the amounts of both the enzyme and dyes were nearly identical, but the time required for complete oxidation at pH 5.00 was 4 times longer. In addition, with a similar integration area, a nearly straight line is obtained at pH 3.15 as compared to the nonlinear curve obtained at pH 5.00. A slower electron transfer rate is indicated in the latter case (Watt, 1979). The interaction of HDFc and DMFc with Uf_r at pH 3.15 was more rapid than the interaction of DMFc and $Co(bipy)_3^{2+}$ with Uf_r at pH 5.00.

The observed rate of electron transfer between Uf_r and its mediators appears to decrease as the pH is increased from 3.15 to 7.91. There are two possible explanations for this observation. The actual electron transfer between Uf_r and its mediators decreases with increasing pH, or the different mediators employed at the various pH values studied influence

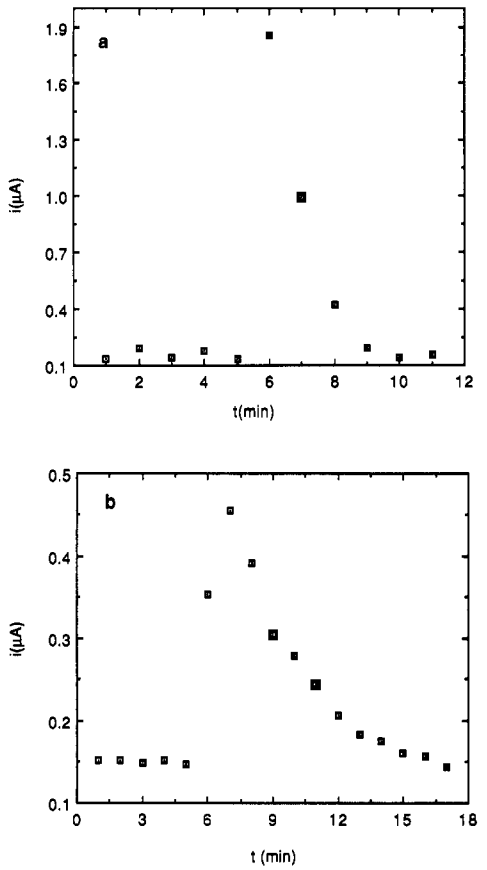


FIGURE 4: Current (i) vs time (t) curves of Uf titrations. Conditions: (a) pH = 3.15, [Uf] = 0.2 mM, equilibrium potential = +458 mV, FO = 51%, dyes HMF and DMFc: (b) pH = 5.00, [Uf] = 0.187 mM, equilibrium potential = +366 mV, FO = 48.9%, dyes DMFc and $Co(bipy)_3^{2+}$.

the electron transfer rate of the enzyme. Electron transfer processes between mediators and enzymes are generally considered to occur through an outer-sphere mechanism in which the driving force is related to the midpoint potentials of the mediators and enzymes.

Midpoint Potentials of Anion Binding Complexes. The binding of anions such as phosphate, arsenate, and molybdate to Uf results in midpoint potential shifts. The anion binding reactions are considered to be as follows:

$$\begin{array}{ccc} Uf_o + XO_4 & \overset{K_{io}}{\rightleftharpoons} & Uf_oXO_4 \\ E_m \updownarrow & & \updownarrow E_{m(UfXO_4)} \\ Uf_r + XO_4 & \underset{K_{ir}}{\rightleftharpoons} & Uf_rXO_4 \end{array}$$

The anion dissociation constants for the oxidized and reduced forms of Uf are K_{io} and K_{ir}, respectively. The anions are abbreviated by XO_4 where X = P, As, and Mo. E_m is the midpoint potential for free Uf, while $E_{m(XO_4)}$ is the midpoint potential of Uf in the presence of a given XO_4 concentration and is therefore a function of $[XO_4]$. $E_{m(UfXO_4)}$ is the midpoint potential of Uf that is saturated with anion and is therefore not a function of $[XO_4]$. We consider $E_{m(UfXO_4)}$ as the midpoint potential of the anion binding complexes of Uf.

At a given anion concentration and with the previously determined dissociation constant K_{ir} (or K_{io}) (see references in Table II), the experimentally determined midpoint potential $E_{m(XO_4)}$ can provide K_{io} (or K_{ir}) from (Clark, 1960)

$$E_{m(XO_4)} = E_m + 0.059 \log \frac{(1 + [XO_4]/K_{ir})}{(1 + [XO_4]/K_{io})} \quad (3)$$

Table II: Reduction Potentials and K_is of Uteroferrin–Anion Complexes[a]

	pH	K_{io}	K_{ir}	$E_{m(XO_4)}$	$E_{m(UfXO_4)}$
$Uf{\cdot}PO_4$	5.00	0.019	12[b]	+252[c]	+183
	6.01	0.030	25[b]	+201[c]	+135
	6.01	0.013	25[b]	+119[d]	+113
$Uf{\cdot}AsO_4$	4.98	0.033	1.3[e]	+278[f]	+262
	6.01	0.062	2.0	+221[f]	+217
$Uf{\cdot}MoO_4$	6.01	3	0.03	+457[g]	+498
	6.95			+407[g]	

[a] E_m values are given in millivolts vs NHE; K_i values are millimolar. [b] Pyrz et al. (1986). [c] The data were obtained with 2 mM free phosphate present. [d] The data were obtained with 100 mM free phosphate present. [e] David & Que (1990). [f] The data were obtained with 11 mM free arsenate present. [g] The data were obtained with 2.44 mM free molybdate present.

The calculated midpoint potential $E_{m(UfXO_4)}$ can thus be obtained from

$$E_{m(UfXO_4)} = E_m + 0.059 \log (K_{io}/K_{ir}) \quad (4)$$

Previous reports have shown that phosphate binds weakly to Uf_r (K_{ir} = 25 mM at pH 6.00) (Pyrz et al., 1986) and very tightly to Uf_o (Keough et al., 1982). The coulometric reduction titrations of $Uf_o{\cdot}PO_4$ performed at pH values of 5.00 and 6.01 in the presence of 2 mM excess phosphate clearly indicate that the $E_{m(PO_4)}$ is pH dependent with a change of 51 mV between pH 5 and 6 (Figure 3). At pH 6.01 the $E_{m(PO_4)}$ value is +201 mV and the K_{io} was calculated to be 0.03 mM from eq 1 with the literature value for K_{ir}. The $E_{m(UfPO_4)}$ value determined from eq 1 was calculated to be +135 mV; however, at 2 mM free phosphate only 7% of Uf_r was bound by phosphate. This may have caused a larger than acceptable error in the determination of $E_{m(UfPO_4)}$. To minimize this possible error, the titration at pH 6.01 was also carried out with 100 mM free phosphate present in the mediator solution. Under these conditions, ~80% of Uf_r was bound by phosphate. $E_{m(PO_4)}$ was found to be +119 mV, which provides a K_{io} of 0.013 mM and an $E_{m(UfPO_4)}$ of +113 mV. This value differs from the $E_{m(UfPO_4)}$ value determined in the presence of 2 mM phosphate by 22 mV (Table II). Thus the phosphate anion shifts the midpoint potential of Uf more negative by 193 mV (Table II). From these data, the ratio of the dissociation constants was calculated to be 5.2×10^{-4}, indicating that phosphate binds about 2×10^3 times more tightly to Uf_o than to Uf_r (Table II).

Arsenate also binds weakly to Uf_r (K_{ir} = 2 mM at pH 6.00). The coulometric titrations were performed in the reductive mode on $Uf_o{\cdot}AsO_4$ similarly to those performed on $Uf_o{\cdot}PO_4$. These titrations were done at pH 6.01 and 4.98 in the presence of excess arsenate (11 mM), which requires ~80% of Uf_r to be bound by arsenate at both pH 4.98 and 6.01. $E_{m(AsO_4)}$ is also pH dependent with a change in potential of 45 mV between pH 5 and 6. At pH 6.01 the $E_{m(AsO_4)}$ value is +221 mV and K_{io} was determined to be 0.062 mM from eq 1 with the literature value for K_{ir}. The $E_{m(UfAsO_4)}$ value, determined from eq 2, was calculated to be +217 mV. Thus arsenate shifts the midpoint potential of Uf negatively by 89 mV at pH 6.01. From these data, the ratio of the dissociation constants was calculated to be 3.1×10^{-2}, indicating that arsenate binds about 0.3×10^2 times more tightly to Uf_o than to Uf_r. Kinetically, the rate of electron transfer is quite similar to that observed for phosphate.

The coulometric titration of $Uf_r{\cdot}MoO_4$ was performed in the oxidative direction in the presence of 2.44 mM free molybdate in the mediator solution at pH 6.01 and 6.95. Figure 3 shows that the $E_{m(MoO_4)}$ of the $Uf_r{\cdot}MoO_4$ solution is

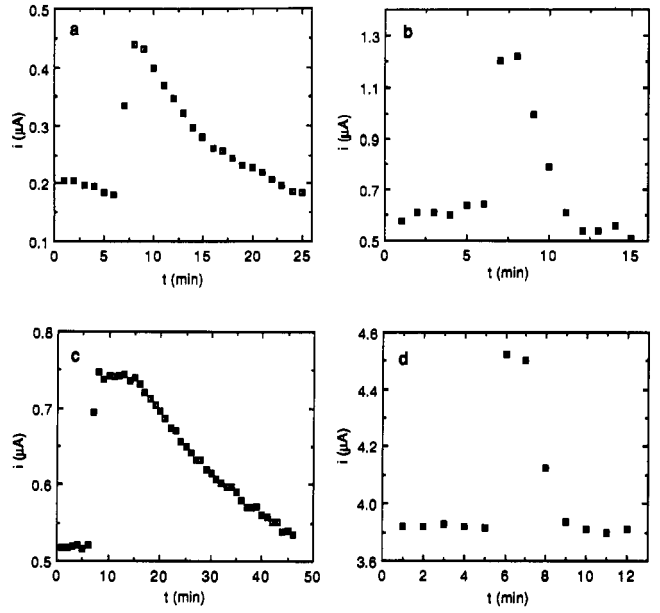


FIGURE 5: Current (i) vs time (t) curves of Uf titrations at pH 6.01. Conditions: (a) [Uf] = 0.294 mM, FO = 83%, dyes HMF and DMFc. (b) [Uf_r·MoO_4] = 0.544 mM, FO = 84.3%, dye HMF and DMFc. (c) [Uf_o·PO_4] = 0.179 mM in the presence of 2 mM free phosphate, FR = 68.9%, dyes cytochrome c and $Co(bipy)_3^{2+}$. (d) [Uf_o·PO_4] = 0.139 mM in the presence of 100 mM free phosphate, FR = 50.6%, dyes cytochrome c, $Ru(NH_3)_6^{2+}$, and $Co(bipy)_3^{2+}$.

also pH dependent with a change in potential of 50 mV between pH 5 and 6. Molybdate was found to bind Uf_r very tightly (K_{ir} = 30 μM at pH 6.01) and the $E_{m(MoO_4)}$ values obtained under these conditions were 151 mV more positive than that for free Uf ($E_{m(MoO_4)}$ = +457 mV at pH 6.01). This large potential shift indicates that molybdate binds much less tightly to Uf_o than to Uf_r. The K_{io} for molybdate binding was calculated to be 3 mM from eq 1, which provides an $E_{m(UfMoO_4)}$ value of +498 mV.

From the data summarized in Table II, it is immediately apparent that phosphate binding and arsenate binding of Uf_r destabilize the enzyme toward oxidation by −19 and −10 kJ, respectively, while molybdate has a stabilizing effect by 14 kJ. For phosphate, the rate of electron transfer between Uf·PO_4 and its mediators seem to increase as the free phosphate concentration increases (Figure 5c,d). Moreover, the electron transfer rate of Uf is faster in the presence of each anion, which implies that these anions affect the Fe(II) center in some way that facilitates electron transfer (Figure 5b).

Discussion

Purple acid phosphatases catalyze non-redox-active hydrolytic reactions; however, the oxidation state of the diiron active site dramatically affects the catalytic activity. The reduced (or mixed valence) form is enzymatically active, while oxidation to the diferric form yields an essentially inactive species. It has been proposed that the redox chemistry associated with these enzymes may be utilized in the regulation of phosphatase activity (Vincent & Averill, 1990). Indeed, while the native reduced enzyme is relatively air stable, phosphate, the product of enzymatic reaction, potentiates its aerobic conversion to inactive enzyme and may act as a switch to shut off phosphatase activity. Thus we have characterized the redox chemistry of Uf and several of its anion complexes by electrochemical methods.

The Fe(III)Fe(III)/Fe(III)Fe(II) reduction potential of Uf was determined to be +367 mV vs NHE at pH 5.00 by using coulometric techniques. This potential is consistent with a number of its redox properties. Uf_r autoxidizes slowly in the presence of air; however, this oxidation is rapid upon the addition of hydrogen peroxide but relatively slow with ferricyanide (Antanaitis & Aisen, 1982). The difference in behavior may be ascribed to the comparable potentials of Uf and ferricyanide (E_m = +430 mV at pH = 8.2 vs NHE).

The binding of various tetraoxo anions to Uf_r elicits marked spectroscopic and chemical changes on the enzyme (David & Que, 1990). Both phosphate and arsenate are weak competitive inhibitors (K_i's in the millimolar range) and render Uf_r more susceptible to aerobic oxidation. Molybdate, on the other hand, is a potent inhibitor (K_i in the micromolar range) and stabilizes Uf in its reduced state. The different susceptibilities of the anion complexes of Uf_r to oxidation are reflected in the reduction potentials of these enzyme–anion complexes. Molybdate shifts the potential of Uf more positive by 192 mV at pH 6.01 and effectively shuts off autoxidation. Phosphate and arsenate shift the Uf potential more negative by 193 and 89 mV, respectively, at pH 6.01. The larger shift observed for phosphate is consistent with the greater susceptibility of the Uf_r·PO_4 complex to autoxidation.

The electrochemical data for uteroferrin can be compared with the reduction potentials of other non-heme iron proteins of known structure (Table III) and also with those of appropriate synthetic complexes to gain insight into structural details of the diiron site (Table IV). As would be expected, the E_m values become more negative as the number of oxyanion ligands on the iron centers increases. Thus transferrin, with its $Fe(Tyr)_2(His)(Asp)(CO_3)$ active site (Anderson et al., 1989), has the most negative potential (Kretchmar et al., 1988). Two other proteins with mononuclear iron sites, namely, the fer-

Table III: Reduction Potentials for Fe(III)/Fe(II) Couples of Relevant Iron-Containing Enzymes[a]

enzyme	E_m	pH	ref
ferroquinone center (photosystem II)	+370	7.5	*b*
methane monooxygenase	≤+350	7.0	*c*
myohemerythrin [$(semimet)_o$/deoxy]	+350	8.2	*d*
hemerythrin [$(semimet)_o$/deoxy]	+310	8.2	*d*
iron superoxide dismutase	+270	7.0	*e*
uteroferrin	+242	7.0	*f*
	+181	7.9	*f*
hemerythrin [met/$(semimet)_R$]	+110	8.2	*d*
myohemerythrin [met/$(semimet)_R$]	+70	8.2	*d*
ribonucleotide reductase B2 subunit	≥−110	7.5	*g*
transferrin	−520	7.4	*h*

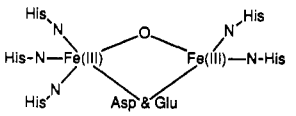
[a] E_m values are given in millivolts vs NHE. [b] Petrouleas & Diner (1986). [c] Woodland et al. (1986); Fox et al. (1989). [d] Armstrong et al. (1983). [e] Barrette et al. (1983). [f] This work. [g] Lam et al. (1990). [h] Kretchmar et al. (1988).

Table IV: Reduction Potentials for High-Spin Fe(III)/Fe(II) Couples of Relevant Model Complexes[a]

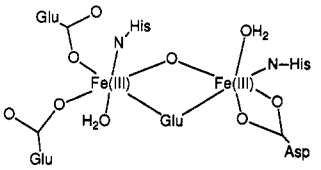
no.	complex	E_m	solvent	site	ref
	$[Fe_2BPMP\{O_2P(OPh)_2\}_2]^{3+/2+}$	+995	CH_3CN	FeN_3O_3	*b*
	$[Fe_2BPMP(OPr)_2]^{3+/2+}$	+932	CH_3CN	FeN_3O_3	*c*
	$[Fe_2BPMP(OAc)_2]^{3+/2+}$	+920	CH_3CN	FeN_3O_2	*d*
	$Fe(H_2O)_6^{3+/2+}$	+770	H_2O	FeO_6	
1	$[N5FeOFeCl_3]^{+/0}$	+348	CH_3CN	FeN_5O	*e*
2	$[Fe_2O(TPA)_2OAc]^{3+/2+}$	+180	CH_3CN	FeN_4O_2	*f*
	$FeEDTA^-$	+130	H_2O	FeN_2O_4	*g*
3	$[Fe_2O(Me_3TACN)_2(OAc)_2]^{2+/+}$	−130	CH_3CN	FeN_3O_3	*h*
	$[Fe_2O(HBpz_3)_2\{O_2P(Ph)_2\}_2]^{0/-}$	−430	CH_3CN	FeN_3O_3	*i*
4	$[Fe_2O(HBpz_3)_2(OAc)_2]^{0/-}$	−470	CH_3CN	FeN_3O_3	*j*
	$[Fe_2O(HBpz_3)_2\{O_2P(OPh)_2\}_2]^{0/-}$	−760	CH_3CN	FeN_3O_3	*i*

[a] E_m values are given in millivolts vs NHE. [b] Schepers et al. (1990). [c] Suzuki et al. (1987). [d] Borovik et al. (1989). [e] Gomez-Romero et al. (1989). [f] Que and True (1990). [g] Grinstead (1960). [h] Hartman et al. (1987). [i] Turowski et al. (1990). [j] Armstrong et al. (1984).

metHr



RRB2met



Ufr

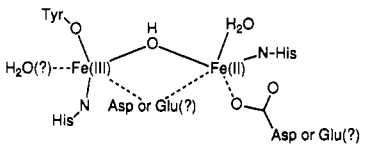


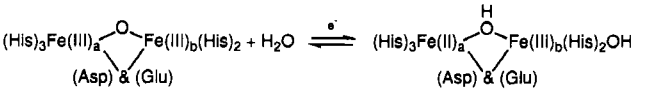
FIGURE 6: Structural representations of the crystallographically defined methemerythrin and metribonucleotide reductase B2 protein and proposed structural representation of uteroferrin, where dashed lines indicate possible ligands.

roquinone center of photosystem II (Petrouleas & Diner, 1986) and iron superoxide dismutase (Barrette et al., 1983), have significantly more positive potentials than transferrin since their coordination environments consist of $Fe(His)_4(Glu)$ (Deisenhofer et al., 1985) and $Fe(His)_3(Asp)$ sites (Stoddard et al., 1990; Stallings et al., 1983; Ringe et al., 1983), respectively.

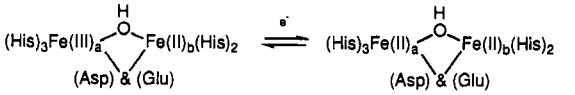
A similar trend can be recognized among diiron oxo proteins and their synthetic analogues (Tables III and IV). For synthetic Fe–O–Fe complexes, it is clear that the Fe(III)Fe(III)/Fe(III)Fe(II) potential depends on the number of anionic ligands bound to the iron center that is reduced. Complex **1** (Gomez-Romero et al., 1989), with its FeN_5O site, is the most positive at +348 mV vs NHE, followed by **2** (Que & True, 1990) with its FeN_4O_2 site (+180 mV) and then **3** (Hartman et al., 1987) with its FeN_3O_3 site (−130 mV). Although **3** and **4** (Armstrong et al., 1984) both have FeN_3O_3 sites, **4** exhibits a more negative potential due to the negatively charged $HBpz_3$ ligand. It should be noted that the potentials for synthetic μ-phenoxo complexes of BPMP are more positive relative to the μ-oxo bridged complexes.

The Fe(III)Fe(III)/Fe(III)Fe(II) potential of metHr is +110 mV vs NHE (Armstrong et al., 1983). MetHr has a crystallographically determined (μ-oxo)bis(μ-carboxylato)-diiron(III) unit with five terminal histidine ligands (Stenkamp et al., 1984) (Figure 6) and would be expected to exhibit a potential close to those of **3** and/or **4**. The difference may arise from the fact that the electrochemistry of the synthetic complexes was performed in acetonitrile, where protonation of the oxo bridge upon reduction is unlikely. Such a concomitant protonation of the oxo bridge upon one-electron reduction would be expected to engender a positive potential shift to the values found for **3** and **4**. Several observations indicate that the redox interconversion between metHr and $(semimet)_RHr$ entails this type of change transforming the Fe_a center from an $Fe(III)(\mu\text{-}O)(His)_3(Asp)(Glu)$ site in metHr to an $Fe(II)(\mu\text{-}OH)(His)_3(Asp)(Glu)$ site in $(semimet)_RHr$ (Scheme I) (McCormick & Solomon, 1990; Scarrow et al., 1987;

Scheme I



Scheme II



Maroney et al., 1986). Similarly, the even more positive value of the $(semimet)_oHr$/deoxyHr couple (+310 mV) results from the presence of a hydroxo bridge in both forms of the redox pair (Scheme II).

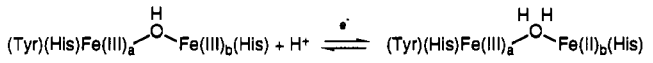
Though the redox chemistry of RRB2 is not well characterized, the reduction potential for the diferric enzyme was estimated to be in the vicinity of −110 mV vs NHE on the basis of the observation that Nile Blue is the dye which exhibits the highest potential that can still mediate the reduction of the diferric site (Lam et al., 1990). This more negative value for RRB2 relative to that of metHr is consistent with the X-ray crystallographic results, which established the presence of an oxo-bridged diiron site with four carboxylate ligands (Figure 6) (Nordlund et al., 1990).

The Fe(III)Fe(III)/Fe(III)Fe(II) potential for the diiron unit in MMO is estimated to be +350 mV vs NHE (Woodland et al., 1986; Fox et al., 1989). If an oxo bridge were present in the diiron unit, an active site with mostly nitrogen ligands would be expected. However, EXAFS studies (Ericson et al., 1988) indicate that, like RRB2, the MMO diiron site is dominated by oxygen-containing ligands. Furthermore, the visible spectrum of oxidized MMO is featureless (Fox et al., 1989) and its 20 K Mössbauer spectrum indicates a diiron unit that is weakly antiferromagnetically coupled (Fox et al., 1988). The accumulated data thus appear to be inconsistent with the presence of a (μ-oxo)diferric unit and are more consistent with a μ-hydroxo bridge in the diferric enzyme.

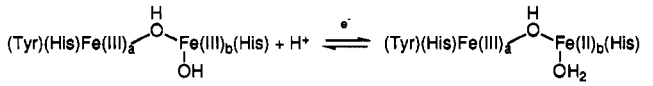
The presence of an oxo bridge in the diferric form of PAPs has been a matter of controversy. SQUID susceptibility measurements on oxidized PAPs estimate $-2J$ to be 200–300 cm^{-1} (Averill et al., 1987; Sinn et al., 1983), in support of an oxo bridge, but EXAFS (Kauzlarich et al., 1986, Que & Scarrow, 1988), resonance Raman (Averill et al., 1987), and a more recent magnetic susceptibility study (Gehring et al., 1990) disagree with this conclusion. From NMR (Scarrow et al., 1990) and EXAFS studies (Kauzlarich et al., 1986, Que & Scarrow, 1988), purple acid phosphatases also appear to contain an oxygen-rich environment. The reduction potential of Uf_o would therefore be expected to be similar to that of RRB2 if an oxo bridge were present in Uf_o (Table III). However, the reduction potential of Uf_o lies between the MMO and $(semimet)_oHr$/deoxyHr couples on the positive side and the metHr/$(semimet)_RHr$ couple on the negative side. These data, taken together with the accumulated spectroscopic and magnetic data, suggest the absence of an oxo bridge in the diferric active site of Uf_o.

The marked pH dependence on the reduction potential of Uf and the fact that the electron transfer rate increases with decreasing pH indicates that a proton participates in the oxidation–reduction process of the redox-active diiron center. Two possibilities for this interaction are possible. First, the hydroxo bridge of Uf_o may be protonated upon reduction (Scheme III). However, a protonation of this type is unlikely, since SQUID magnetization (Day et al., 1988) and EPR studies (David & Que, 1990) indicate that the antiferro-

Scheme III



Scheme IV



magnetic coupling of the diiron centers in Uf_r is greater than would be expected for a water bridge. Furthermore, upon phosphate binding the antiferromagnetic coupling between the iron centers decreases dramatically (Day et al., 1988; David & Que, 1990) suggesting that a proton from the phosphate inhibitor interacts with the hydroxo bridge. Such an interaction would not be possible if a water bridge were already present in Uf_r. The more likely alternative is the protonation of a terminally bound hydroxide upon reduction of Uf_o (Scheme IV). ENDOR and ESEEM studies (Antanaitis et al., 1985; Doi et al., 1988) revealed solvent-exchangeable features, some of which were assigned to coordinated water molecules.

Recently, we proposed a model for the interaction of anions with Uf_r (David & Que, 1990) wherein phosphate and arsenate coordinate to the Fe(III) site and interact wiht the hydroxo bridge while molybdate bridges the dinuclear center. It is clear from our electrochemical data that questions remain regarding anion binding that may not be entirely met by our previous model. Like earlier spectroscopic and biochemical results, our electrochemical data indicate that molybdate affects the dinuclear core in a manner that differs from that of phosphate or arsenate. Presumably this difference implies a distinct mode of binding for molybdate. At present, there is insufficient information from model compounds in particular to allow a more definitive model to be proposed. However, such a model must account for the following observations: (1) the negative shifts in the reduction potential caused by phosphate or arsenate binding and the positive shift caused by molybdate binding, (2) the weakening of the antiferromagnetic coupling between the iron centers upon phosphate or arsenate binding as a result of hydrogen bonding (or proton transfer) to the hydroxo bridge (David & Que, 1990), and (3) the fact that phosphate coordinates to the Fe(III) center (David & Que, 1990) and molybdate interacts with the diiron center (Antanaitis, et al. 1985; Doi, et al. 1988).

The dilemmma posed by phosphate and arsenate binding is now to increase the electron density at the Fe(II) site and at the same time protonate the hydroxo bridge. A bridging phosphate is certainly conceivable in the case of Uf given the number of synthetic diiron complexes available with phosphate bridges. (Norman et al., 1990; Turowski et al., 1990; Drüeke et al., 1989). However, if phosphate or arsenate does indeed bridge the diiron center, how would molybdate coordination differ to engender its much greater binding affinity? The interaction of a proton from phosphate or arsenate with the hydroxo bridge would also be hard to envision if these anions were briding. Alternatively, phosphate and arsenate may only bind the Fe(III) center and the anion required at the Fe(II) site may be a terminal hydroxide derived from the ionization of a bound water molecule upon protonation of the hydroxo bridge. A deprotonation of this type is consistent with our electrochemical results and previously reported spectroscopic data. Furthermore, the participation of a terminal hydroxide in the enzymatic mechanisms of phosphatases has been previously implicated (Coleman & Chlebowski, 1979). However, the question still remains as to how molybdate causes a positive shift in the reduction potential of Uf. Further clarification of this phenomenon and the various anion binding modes in general will undoubtedly emerge from Mössbauer and EXAFS studies as well as the synthesis of appropriate model compounds.

Registry No. PAP, 9001-77-8; Fe, 7439-89-6.

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