Interaction of Porcine Uterine Fluid Purple Acid Phosphatase with Vanadate and Vanadyl Cation†

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ABSTRACT: Uteroferrin, the purple acid phosphatase from porcine uterine fluid, is noncompetitively inhibited by vanadate in a time-dependent manner under both aerobic and anaerobic conditions. This time-dependent inhibition is observed only with the diiron enzyme and is absent when the FeZn enzyme is used. The observations are attributed to the sequential formation of two uteroferrin–vanadium complexes. The first complex forms rapidly and reversibly, while the second complex forms slowly and results in the production of catalytically inactive oxidized uteroferrin and V(IV), which is observed by EPR. The redox reaction can be reversed by treatment of the oxidized enzyme first with (V(IV)) and then EDTA to generate a catalytically active uteroferrin. Multiple inhibition kinetics suggests that vanadate is mutually exclusive with molybdate, tungstate, and vanadyl cation. The binding site for each of these anions is distinct from the site to which the competitive inhibitors phosphate and arsenate bind. The time-dependent inhibition by vanadate of uteroferrin containing the diiron core represents a new type of mechanism by which vanadium can interact with proteins and gives additional insight into the binding of anions to uteroferrin.

Anion interactions with the purple acid phosphatases (PAPs) are particularly important for understanding the detailed mechanism of phosphate ester hydrolysis (Vincent & Averill 1991; Doi et al., 1987). PAPs are distinguished from other phosphatases because PAP contains a diiron center that can exist in two oxidation states: a catalytically active Fe(II)–Fe(III) form and an inactive Fe(III)–Fe(III) form. Phosphate is a weak competitive inhibitor ($K_i \sim 6$–14 mM) of reduced uteroferrin (Uf_r), the PAP from porcine uterine fluid (Baumbach et al., 1986; Pyrz et al., 1986). Under anaerobic conditions, this interaction is reversible, and removal of the phosphate leads to recovery of the phosphatase activity. In the presence of oxygen, phosphate facilitates the oxidation of uteroferrin (Uf_o). The phosphate is tightly bound in this form and cannot be removed by gel filtration or dialysis (Keough et al., 1982). Arsenate, a phosphate analog, exhibits similar inhibition properties ($K_i \sim 1$ mM) and potentiates the air oxidation of Uf_r as well (Pyrz et al., 1986; David & Que, 1990). Electrochemical studies on uteroferrin–anion complexes support these chemical observations; the binding of phosphate and arsenate decreases the midpoint reduction potential of Uf_r at pH 6 by 193 and 89 mV, respectively (Wang et al., 1991). In contrast, tungstate and molybdate are more potent inhibitors of uteroferrin with noncompetitive inhibition patterns. Furthermore, both these anions form air-stable complexes with Uf_r, with molybdate increasing the midpoint potential at pH 6 by 192 mM (David & Que, 1990; Wang et al., 1991).

Vanadate is both electronically and structurally a phosphate analog (Chasteen, 1983). Aqueous solutions of vanadate undergo a series of rapid reactions to generate vanadate oligomers and millimolar concentrations of vanadate at neutral pH contain monomer, dimer, tetramer, and pentamer (Crans et al., 1990a). Specifically, vanadate monomer and oligomers have been reported to interact with various biological systems. Vanadate is a known enzyme inhibitor for phosphatases, ribonucleases, and myosin. Vanadate monomer is a potent inhibitor for phosphatases and ATPases (VanEtten et al., 1974; Chasteen, 1983; Crans et al., 1989a,b), vanadate dimer for human seminal fluid acid phosphatase and dehydrogenases (Crans et al., 1989a,b; Crans & Schelble, 1990; Crans & Simone, 1991), and tetramer for dehydrogenases (Crans & Schelble, 1990; Crans et al., 1990b; Crans & Simone, 1991; Crans et al., 1992a). Vanadate decamer also inhibits or binds to several enzymes including Ca^{2+}-ATPase (Csermely et al., 1985), muscle phosphorylase (Soman et al., 1983), adenylate cyclase (DeMaster & Mitchell, 1973), and hexokinase and phosphofructokinase (Boyd et al., 1985). Vanadate is reduced in the presence of thiols in aqueous solution (Cohen et al., 1987), and redox chemistry between thiol-containing enzymes, such as aldolase, and vanadate has indeed been observed (Crans et al., 1992a). The presence of thiols in an enzyme does not necessarily ensure redox chemistry with vanadate, because glycerol-3-phosphate dehydrogenase, which contains an essential thiol group in the cofactor binding site, regains all enzyme activity upon removal of vanadium (Crans & Simone, 1991). Reaction of vanadate with biomolecules, such as glycerol or NAD, leads to formation of analogs of substrate (Nour-Eldeen et al., 1985; Drueckhammer et al., 1989) and cofactor (Crans et al., 1992b) and these derivatives are converted by respective enzymes to products.

The interaction of vanadate with uteroferrin is of particular interest since Uf_r contains a redox active diiron center. Most phosphatases are inhibited by monomeric vanadate for one of three reasons. First, vanadate is structurally and electronically similar to phosphate and may inhibit the enzyme by occupying the phosphate binding site. Second, vanadate may form a stable covalent enzyme–vanadate complex. Third, vanadate can form a stable pentacoordinated transition-state analog of phosphate ester hydrolysis. Vanadate may inhibit the enzyme

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activity of uteroferrin for the above reasons, but the presence of a diiron center in uteroferrin raises the possibility that redox chemistry may be involved as a novel mechanism for inhibition, since only the Fe(II)–Fe(III) form of the enzyme is catalytically active. Indeed, an earlier EPR study suggested the involvement of redox chemistry, but a thorough investigation was not carried out (Antanaitis & Aisen, 1985). In light of the recent studies of oxoanion interactions with uteroferrin, a detailed kinetic study of vanadate inhibition combined with spectroscopic studies should assist in further characterizing the oxoanion binding sites and the diiron center of uteroferrin.

EXPERIMENTAL PROCEDURES

Materials

Water was distilled and further deionized by passage through an ion-exchange column. Oxometallates used as inhibitors were obtained from Aldrich Chemical Co. Stock solutions of molybdate (100 mM) and tungstate (100 mM) were prepared at pH 8.0 to avoid formation of polyoxoanions. A vanadate stock solution (25 mM) was prepared at pH 7.0 from ammonium metavanadate. At no time was acid added to this solution; acid addition generates decamer, and once formed at neutral pH, it only slowly converts to an equilibrium mixture of rapidly exchanging vanadate oligomers. Stock solutions (1 mM) of vanadyl cation [V(IV)] were prepared in 0.1 M HCl to prevent oxidation to V(V). All other chemicals were obtained from Sigma and were used without further purification.

A solution of reduced uteroferrin (Uf_r) in 100 mM acetate buffer, pH 4.9, and 200 mM NaCl was isolated as described previously and kept frozen at all times (Pyrz et al., 1986). The activity of this sample was determined to be ~410 units/mg of protein, and the A_{280}/A_{510} ratio was 15. Small quantitites of this solution were removed and diluted in 400 mM acetate, pH 5.5, when needed, and these solutions were stable for several hours at 0 °C. A solution of FeZn uteroferrin in 100 mM acetate buffer, pH 4.9, and 200 mM NaCl was prepared as described previously and kept frozen at all times (David & Que, 1990; Keough et al., 1980). The activity of this sample was ~460 units/mg of protein, and the A_{280}/A_{510} ratio was 20. Small quantities of this solution were removed and diluted in 400 mM acetate, pH 5.5, when needed.

Methods: General

UV Spectroscopy. Spectrophotometric measurements of assay absorbances were conducted on a λ 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. All enzymatic reactions were conducted in the presence of oxygen unless specified otherwise and were terminated by the addition of 2.000 mL of 0.1 M NaOH. The *p*-nitrophenol formed as product in the reactions was quantified by the absorbance of *p*-nitrophenolate at 405 nm. Control assays containing no enzyme were measured in a similar manner, and the enzyme reactions were adjusted accordingly.

When vanadyl cation was used, the assay solutions were purged for approximately 4 h with nitrogen before addition of enzyme and inhibitor (Chasteen, 1981). In order to maintain proper pH in the vanadyl cation assay solutions, 0.1 M NaOH was added in equal volumes as vanadyl cation (which was prepared in 0.1 M HCl). The pH of these solutions was measured before and after these additions to ensure no changes had occurred.

For anaerobic studies, solutions were purged with nitrogen for approximately 4 h before use. All work for the studies was carried out in a nitrogen-purged, desktop glovebox under constant nitrogen flow.

Vanadate Anion Speciation (Crans et al., 1990a). Aqueous solutions containing vanadium can undergo a variety of reactions (eqs 1–4) to form vanadate oligomers, where vanadate monomer (V_1), dimer (V_2), tetramer (V_4), and pentamer (V_5) are the predominant species. The distribution of the oligomers is dependent on concentration, temperature, ionic strength, and pH (Pope, 1983). The concentrations of the vanadate species in solution can be readily quantified by ^{51}V NMR. The constants used in this work were $K_{12} = 110$ M^{-1}, $K_{14} = 6.5 \times 10^8$ M^{-3}, and $K_{24} = 5.5 \times 10^4$ M^{-1}.

$$2\,V_1 \overset{K_{12}}{\rightleftharpoons} V_2 \quad (1)$$

$$4\,V_1 \overset{K_{14}}{\rightleftharpoons} V_4 \quad (2)$$

$$2\,V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (3)$$

$$5\,V_1 \overset{K_{15}}{\rightleftharpoons} V_5 \quad (4)$$

^{51}V NMR Spectroscopy (Heath & Howarth, 1981; Gresser & Tracey, 1985). ^{51}V NMR spectra were recorded at 132 MHz on a Bruker AM-500 (11.7 T) spectrometer at ambient temperature. Typical conditions include a spectrum width of 8064 Hz, a 90° pulse angle, an accumulation time of 0.2 s, and no relaxation delay. No change in integration was observed if the relaxation delay was increased. The chemical shifts are reported relative to the external reference standard $VOCl_3$ (0 ppm), although in practice a solution of vanadate–diethanolamine complex was used as a reference (–490 ppm) (Crans & Shin, 1988).

Samples containing 200 mM NaOAc, pH 5.5, and 0.0–1.5 mM vanadate, corresponding to assay conditions, were prepared and analyzed by ^{51}V NMR. The concentrations of the various vanadate species were determined by integration of the spectra, assuming the total vanadium concentration equals the sum of all vanadate species.

EPR Spectroscopy. EPR spectra were obtained at the X-band frequency with a Varian E-109 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat. The integrations of the VO^{2+} signals were carried out at 40 K.

Oxoanion Inhibition Studies of Purple Acid Phosphatase

Inhibition by Oxoanions. Assay solutions for inhibition studies contained 200 mM acetate, pH 5.5, 2.0–6.0 mM *p*-nitrophenyl phosphate [PNPP bis(cyclohexylammonium) salt], and approximately 0.05 mg/L (0.02 unit/L) uteroferrin. Various inhibitors were added to the solutions in the following concentration ranges: phosphate (0.0–4.0 mM), arsenate (0.0–2.0 mM), vanadate (0.0–1.5 mM), molybdate (0.0–0.015 mM), tungstate (0.0–0.0015 mM), and vanadyl cation (0.0–0.080 mM). All assays were incubated 5.00 min following addition of enzyme, with the exception of those for vanadyl cation, which were incubated 1.00 min. The extent of the reactions was determined at 405 nm and 25 °C as described above.

Time-Dependent Inhibition Studies: Varying Time. Assay solutions containing 200 mM acetate, pH 5.5, 2.0 mM PNPP, approximately 0.05 mg/L (0.02 unit/L) uteroferrin, and (a) 0.08 mM vanadate, (b) 1.7 mM phosphate, or (c) no inhibitor were prepared. Aliquots (1.000 mL) were removed at 5-min

intervals for 90 min after the assay was begun and were immediately added to 2.000 mL of 0.1 M NaOH. Reaction progress was monitored at 405 nm and 25 °C as described above.

Time-Dependent Inhibition Studies: Varying Vanadate Concentration. Assay solutions containing 200 mM acetate, pH 5.5, 2.0 mM PNPP, and 0.0–0.040 mM vanadate were prepared. Aliquots (1.000 mL) were removed at several time intervals after the addition of approximately 0.038 mg/L (0.016 unit/L) uteroferrin to each assay solution. The aliquots were immediately added to 2.000 mL of 0.1 M NaOH. Reaction progress was monitored at 405 nm and 25 °C as described above. An analogous study was carried out under anaerobic conditions using 0.027 mg/L (0.011 unit) uteroferrin.

Reaction of Uteroferrin or Vanadate-Treated Uteroferrin with Mercaptoethanol. Approximately 0.05 mg/L (0.02 unit/L) uteroferrin was allowed to preincubate in 200 mM acetate, pH 5.5, for 15 min at room temperature with (a) no inhibitor, (b) 0.03 mM vanadate, (c) no inhibitor, or (d) 0.03 mM vanadate. This was followed by the addition of (a) 0.03 mM vanadate and 0.3 mM EDTA, (b) 0.3 mM EDTA, (c) 0.03 mM vanadate, 0.3 mM EDTA, 20 mM mercaptoethanol, and 0.4 mM ferrous ammonium sulfate, or (d) 0.3 mM EDTA, 20 mM mercaptoethanol, and 0.4 mM ferrous ammonium sulfate. The assays were all started by adding 4.0 mM PNPP and were incubated 5.00 min before the addition of 2.000 mL of 0.1 M NaOH. The extent of the enzymatic reactions was determined at 405 nm and 25 °C as described above.

Uteroferrin and Vanadyl Cation. Approximately 0.05 mg/L (0.02 unit/L) uteroferrin was allowed to preincubate in 200 mM acetate, pH 5.5, for 15 min at room temperature with 0.2 mM vanadate [V(IV)]. This was followed by the addition of (a) 0.2 mM EDTA and (b) 0.2 mM EDTA, 0.3 mM vanadyl cation [V(V)] in 3 mM HCl, and 3 mM NaOH. The assay solutions were incubated 5 min before the addition of 0.3 mM EDTA. The assays were started by the addition of 4.0 mM PNPP and were incubated for 5.00 min before the addition of 2.000 mL of 0.1 M NaOH. The extent of the enzymatic reactions was determined at 405 nm and 25 °C as described above.

Approximately 0.05 mg/L (0.02 unit/L) uteroferrin was allowed to oxidize in 400 mM acetate, pH 5.5, for 2 days at room temperature. The enzyme activity in assays which contained 200 mM acetate, pH 5.5, and (a) 0.3 mM EDTA, (b) 0.3 mM vanadyl cation [V(IV)], or (c) 0.3 mM vanadyl cation [V(IV)] and 0.3 mM EDTA was measured. The assay solutions were incubated 5 min before the addition of 0.3 mM EDTA. The assays were started by the addition of 4.0 mM PNPP and were incubated for 5.00 min before the addition of 2.000 mL of 0.1 M NaOH. The extent of the enzymatic reactions was determined at 405 nm and 25 °C as described above.

Simultaneous Inhibition of Uteroferrin by Two Oxoanions (Yonetani & Theorell, 1964; Vincent et al., 1991b). Assay solutions contained 200 mM acetate, pH 5.5, 2.0 mM PNPP, and approximately 0.05 mg/L (0.02 unit/L) uteroferrin. Multiple inhibitors were added to the solutions in the following combinations and concentration ranges: vanadate (0.0–0.080 mM) and phosphate (0.0–4.0 mM), vanadate (0.0–0.080 mM) and molybdate (0.0–0.0040 mM), vanadate (0.0–0.080 mM) and tungstate (0.0–0.00040 mM), and vanadate (0.0–0.080 mM) and vanadyl cation (0.0–0.10 mM). All assays were incubated 5.00 min following addition of enzyme, with the exception of those containing vanadyl cation, which were incubated 1.00 min. The extent of the reactions was determined at 405 nm and 25 °C as described above.

Table I: Summary of Inhibition Constants of Various Oxoanions for Uteroferrin

inhibitor	K_i (mM)	inhibition pattern	reference
phosphate	3.4 ± 0.4[a]	competitive	this work
	2.3[b]	competitive	Baumbach et al. (1986)
arsenate	0.64 ± 0.06[a]	competitive	this work
	2.0[b]	competitive	Pyrz et al. (1986)
molybdate	0.0050 ± 0.001[a]	noncompetitive	this work
	0.004[b]	noncompetitive	Pyrz (1986)
tungstate	0.00070 ± 0.0003[a]	noncompetitive	this work
	0.004[b]	noncompetitive	Pyrz (1986)
vanadate	0.040 ± 0.005[a]	noncompetitive	this work
vanadyl cation	0.082 ± 0.008[a]	noncompetitive	this work
perrhenate	~30	nd[c]	this work

[a] 200 mM acetate, pH 5.5, in the presence of O_2. [b] 100 mM acetate, pH 4.9, in the presence of O_2. [c] nd, not determined.

Oxoanion Inhibition Studies with FeZn Uteroferrin

Determination of Inhibition Constants. Assay solutions containing 200 mM acetate, pH 5.5, 1.5–6.0 mM PNPP, and 0.0–0.2 mM vanadate were prepared. Samples were incubated 5.00 min at room temperature after the addition of FeZn uteroferrin. The extent of the reactions was determined at 405 nm and 25 °C as described above.

Assay solutions containing 200 mM acetate, pH 5.5, 2.0–6.0 mM PNPP, and 0.0–0.4 mM vanadyl cation were prepared. Samples were incubated 1.00 min at room temperature after the addition of FeZn uteroferrin. The extent of the reactions was determined at 405 nm and 25 °C as described above.

Time Dependence of Inhibition Studies. Assay solutions containing 200 mM acetate, pH 5.5, 2.0 mM PNPP, and 0.0–1.0 mM vanadate were prepared. Aliquots (1.000 mL) were removed at several time intervals after the addition of FeZn uteroferrin from 0.0 to 300 min. These aliquots were immediately added to 2.000 mL of 0.1 M NaOH. The extent of the reactions was determined at 405 nm and 25 °C as described above.

RESULTS AND DISCUSSION

Inhibition of Purple Acid Phosphatase by Oxoanions. The inhibition of uteroferrin by a series of oxoanions was measured for comparison with the inhibition by vanadate and vanadyl cation. Inhibition constants for phosphate, arsenate, molybdate, tungstate, vanadate, and vanadyl cation were determined in assays containing 200 mM acetate at pH 5.5, 1.5–6.0 mM PNPP, and inhibitor concentration ranges described under Experimental Procedures. Under these conditions, $K_{m,PNPP}$ was determined to be 6.4 mM, in agreement with that previously reported under different conditions (Schlosnagle et al., 1974). The low solubility of this substrate in water limited analysis at concentrations higher than 6.0 mM. Competitive inhibition patterns were found for phosphate and arsenate using Lineweaver–Burk plots; noncompetitive patterns were found for molybdate, tungstate, vanadate, and vanadyl cation. Slope replots gave K_i values of 3.4, 0.64, 0.0050, 0.00070, 0.040, and 0.082 mM, respectively. The inhibition constants are in general agreement with those reported previously for uteroferrin (Table I) (Schlosnagle et al., 1974; Baumbach et al., 1986; Pyrz et al., 1986; Ketcham et al., 1985; Pyrz, 1986).

Inhibition of Purple Acid Phosphatase by Vanadate Monomer and Vanadyl Cation. Inhibition studies were carried out using vanadate concentrations up to 1.5 mM in order to

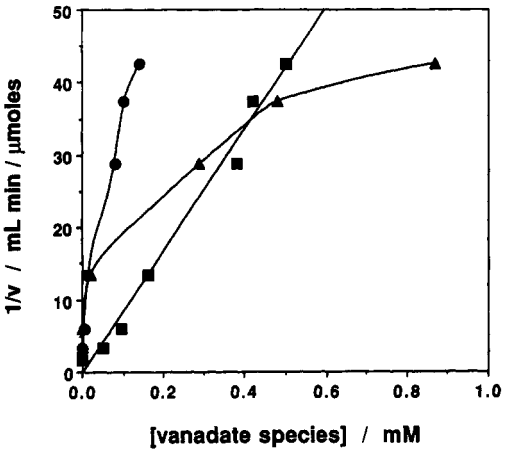


FIGURE 1: Correlation of enzyme inhibition with concentration of vanadate oligomers: vanadate monomer (■), dimer (●), or tetramer (▲). Assay solutions contained 200 mM acetate at pH 5.5, 1.5–6.0 mM PNPP, and 0.0–1.5 mM vanadate.

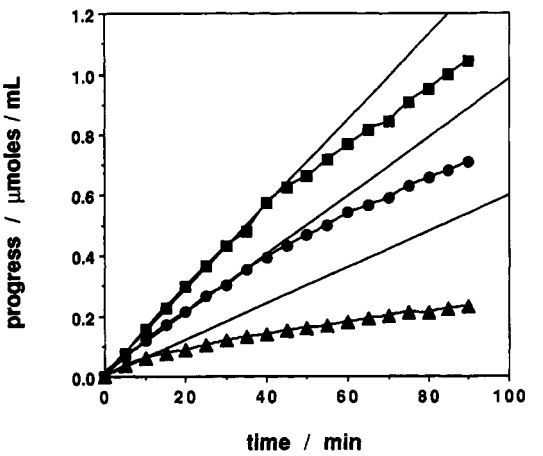


FIGURE 2: Time-dependent deactivation of uteroferrin by vanadate. Assay solutions contained 200 mM acetate at pH 5.5 and 2.0 mM PNPP. This experiment consists of a control (■), a phosphate deactivation assay (●), and a vanadate assay (▲).

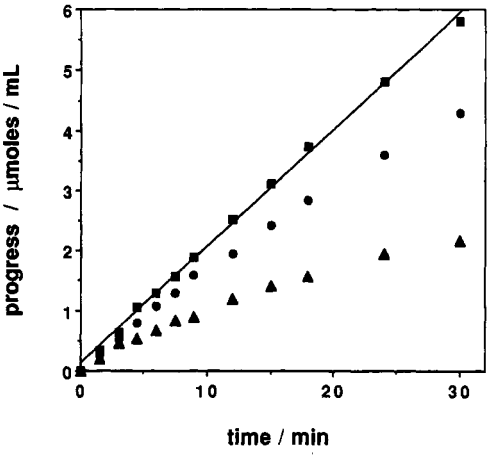


FIGURE 3: Reaction progress curves in the presence of various concentrations of vanadate. Assay solutions contained 200 mM acetate at pH 5.5, 2.0 mM PNPP, approximately 0.038 mg/mL (0.016 unit/mL) uteroferrin, and 0.000 mM (■), 0.010 mM (●), or 0.040 mM (▲) vanadate. Aliquots of the solutions were removed at several time intervals after the addition of enzyme and were added to 2.000 mL of 0.1 M NaOH.

determine which vanadate anion exhibits the observed inhibitory activity (Crans et al., 1989a, 1990a,b; Crans & Schelble, 1990; Crans & Simone, 1991). ^{51}V NMR was used to measure the concentrations of the various vanadate species present in the assay solutions. Dixon plots of the reciprocal rates of the enzyme reactions versus the concentration of each vanadate species were made to explore which species is responsible for the inhibition. The concentration of the inhibitory species will correlate linearly with the observed reciprocal rates. Vanadate monomer showed the only linear correlation and is therefore the only species that can account for the observed inhibition. Both dimer and tetramer gave nonlinear fits, with significant inhibition in a concentration range where none of these anions are present. This suggests that these species are not responsible for the observed inhibition (Figure 1) (Crans et al., 1989a,b). To obtain an accurate inhibition constant, rates of hydrolysis of PNPP in the presence of five different concentrations of vanadate ranging from 0.0 to 0.040 mM were measured, leading to the observed K_i of 0.040 mM for monomeric vanadate.

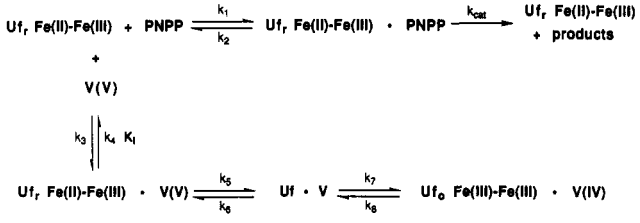
The K_i for vanadyl cation was 0.082 mM, compared to a K_i of 0.040 mM for vanadate monomer. These two oxovanadium derivatives, despite the fact that one is a cation and the other is an anion, have similar affinities for PAP. Vanadate, however, may be capable of undergoing redox chemistry with the diiron center of the protein, which would lead to loss of enzyme activity. If such a reaction occurred in the vanadate–PAP complex, it may be somewhat slow and may only be observed in experiments with incubation times beyond 5 min. The possibility that redox chemistry occurs in the uteroferrin–vanadate complex upon prolonged incubation will be discussed below.

Time-Dependent Inhibition by Vanadate. In monitoring the rates of product formation in the presence of 0.080 mM vanadate, a linear increase in absorbance corresponding to product formation was obtained for 7–8 min, after which point the absorbance increased nonlinearly, reflecting a decrease in enzyme activity (Figure 2). For comparison, nonlinear absorbance curves were observed in the purple acid phosphatase control sample and in the presence of 1.7 mM phosphate only after 45 min. We conclude that vanadate promotes the nonlinear response and inhibition of the purple acid phosphatase in short periods of time.

The time-dependent inhibition was examined in more detail at three concentrations of vanadate (0.020, 0.040, and 0.080 mM). Low vanadate concentrations (0.020 mM) led to a 30% loss of enzyme activity in 15 min. In the same time frame, 60% of the activity was lost with 0.080 mM vanadate (Figure 3). This indicates that the time-dependent inhibition of uteroferrin changes with varying vanadate concentration. Higher concentrations of vanadate lead to more loss of activity. The percentages of activity losses at 0.040 and 0.080 mM vanadate were independent of variations in the enzyme concentration (data not shown). These findings are consistent with the formation of a complex between vanadate and purple acid phosphatase ($Uf_r \cdot V_1$), which is transformed into a second complex proposed to be $Uf_o \cdot V(IV)$ as shown in (5). The independence of the enzyme concentration can be rationalized by recalling that the vanadate concentration is much greater than the enzyme concentration and that the enzyme giving rise to activity is enzyme that does not follow the pathway indicated in (5). The fraction of the enzyme that reacts with the substrate to form product remains constant as the enzyme concentration varies. The ratio of the activity of the inhibited uteroferrin and the original activity should therefore be independent of the enzyme concentration, assuming no significant concentration changes are observed with other essential components in the assay.

$$Uf_r + V_1 \rightleftharpoons Uf_r \cdot V_1 \rightleftharpoons Uf_o \cdot V(IV) \qquad (5)$$

Scheme I



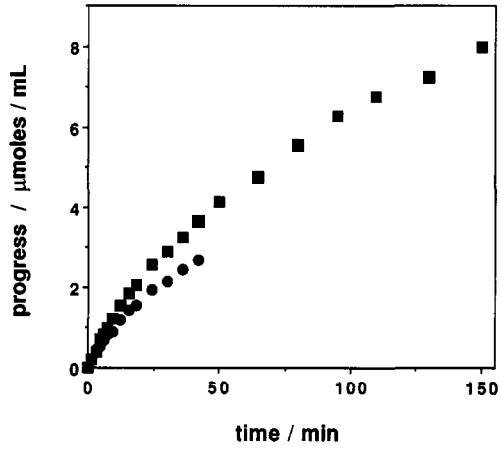


FIGURE 4: Aerobic (■) and anaerobic (●) reaction progress curves in the presence of vanadate. Assay solutions contained 200 mM acetate at pH 5.5, 2.0 mM PNPP, and 0.040 mM vanadate. Aerobic studies contained approximately 0.038 mg/mL (0.016 unit/mL) uteroferrin, while anaerobic studies were conducted with approximately 0.027 mg/mL (0.011 unit/mL) uteroferrin. Aliquots of the solutions were removed at several time intervals after the addition of enzyme and were added to 2.000 mL of 0.1 M NaOH.

If formation of the second complex is rate limiting, a kinetic scheme shown in Scheme I can be suggested and tested using a kinetic model for slow binding inhibitors. The model was tested experimentally using assay solutions which contained 200 mM acetate, pH 5.5, 2.0 mM PNPP, and 0.0–0.040 mM vanadate. Aliquots were removed at appropriate time intervals after the addition of enzyme to monitor reaction progress. Since the enzyme loses activity after 40 min, the time frame of these experiments was limited. The time studies yielded progress curves similar to those observed with slow binding inhibition (Figure 4) (Morrison & Walsh, 1988).

The mechanism shown in Scheme I proposes the establishment of an initial, rapid equilibrium, which is followed by a slow, rate-limiting step. The progress curve can be described by (6), where P is the concentration of p-nitrophenolate formed, v_s is the final steady-state velocity, v_0 is the initial velocity, t is the time after enzyme is added, and k is the apparent first-order rate constant. The displacement term, d, is zero in our series of measurements because control experiments carried out during the assays found that all initial readings are identical. The rate constants k_5 and k_6 are described in (7) and (8). K_i and K_i^* are determined from noncompetitive inhibition kinetics using v_0 and v_s, respectively. In Scheme I, K_i is identified as the initial noncompetitive inhibition constant for the rapid preequilibrium and K_i^* represents the overall inhibition constant and is defined in (8).

$$P = v_S t + (v_0 - v_S)(1 - e^{-kt})/k + d \quad (6)$$

$$k_6 = (v_s)(k)/v_0 \quad (7)$$

$$k_5/k_6 = (K_i/K_i^*) - 1 \quad (8)$$

Table II: Summary of Inhibition Constants and Rate Constants for Vanadate Interaction with Uteroferrin under Aerobic and Anaerobic Conditions

	aerobic[a] (40-min study)	anaerobic[a] (40-min study)	anaerobic[b] (150-min study)
K_i (mM)	0.042 ± 0.005	0.065 ± 0.005	0.066 ± 0.006
K_i^* (mM)	0.0064 ± 0.0005	0.0089 ± 0.0004	0.0034 ± 0.0004
k_5	0.12 ± 0.01	0.12 ± 0.01	0.088 ± 0.017
k_6	0.023 ± 0.001	0.019 ± 0.002	0.0046 ± 0.0002
k_5/k_6	~5/1	~6/1	~19/1

[a] Product formation is linear for 40 min and then control assays become nonlinear. [b] Product formation is linear for 150 min and then control assays become nonlinear.

Using nonlinear least-squares analysis, K_i and K_i^* were calculated to be 0.042 ± 0.005 mM and 0.0064 ± 0.0005 mM, respectively (Table II). This K_i is in agreement with the K_i determined using initial rate kinetics. Determination of k_5 and k_6 yielded rate constants of 0.12 ± 0.01 min^{-1} and 0.023 ± 0.001 min^{-1}, respectively. This gives a k_5/k_6 ratio of about 5/1. A large k_5/k_6 ratio is indicative of a mechanism where k_6 is rate limiting and is an essential requirement for slow binding inhibition. The kinetic findings are therefore consistent with the formation of two purple acid phosphatase·vanadate complexes, one which is rapidly formed and a second complex which forms much more slowly.

Phosphate promotes the oxidation of the Fe(II) in the diiron center of the purple acid phosphatase in the presence of O_2. Upon this oxidation of the metal center, the phosphate becomes tightly bound to PAP. Phosphate is known to inhibit the reduced enzyme reversibly without the presence of oxygen, and this phosphate can be removed reversibly from PAP. Since vanadate is a phosphate analog, similar modes of interaction may take place between the purple acid phosphatase and vanadate (Antanaitis & Aisen, 1985; Doi et al., 1987; Pyrz et al., 1986; Day et al., 1988; David & Que, 1990; Wang et al., 1991), and the nature of the interaction is explored in further detail below.

If the second complex forms reversibly as suggested by the experiments above, it should be possible to reach the complex from both Uf$_o$ and Uf$_r$. The experiment described above suggests the inhibition is indeed reversible; however, this finding may be an artifact of the limited duration of the experiments. The reaction progress curves may not have been followed to a plateau due to the loss of enzyme activity in the control samples, which imposes a 40-min upper time limit on the enzyme assays. Accurate analysis of slow binding inhibitors is only carried out if the final equilibrium (k_5 and k_6) is reached. Since the autooxidation of the enzyme in the control and the consequent loss of activity hampered the accurate analysis of the slow binding inhibition, it was important to explore whether the final equilibrium condition could be established under anaerobic conditions.

Time-Dependent Anaerobic Inhibition by Vanadate. A series of time-dependent vanadate inhibition studies of purple acid phosphatase were carried out under anaerobic conditions otherwise identical to those described above for the studies in the presence of O_2. The loss of enzyme activity in control samples was not observed for 150 min, extending the usable time period in these experiments. By use of nonlinear least-squares analysis, K_i and K_i^* were determined to be 0.066 ± 0.006 mM and 0.0034 ± 0.0004 mM, respectively (Table II). Determination of k_5 and k_6 yielded results of 0.088 ± 0.017 min^{-1} and 0.0046 ± 0.0002 min^{-1}, respectively. This gives a k_5/k_6 ratio of about 19/1, a significantly larger ratio than that obtained under aerobic conditions. Nevertheless, this

study supports the nonzero k_6 obtained in the previous results and the reversible formation of the second vanadium–uteroferrin complex.

Conducting the vanadate experiments with or without oxygen may affect the mechanism by which vanadate interacts with purple acid phosphatase. The results of experiments conducted aerobically for 40 min differ from the results of experiments conducted anaerobically for 150 min. These differences may be caused by the O_2 or by the limited time period in the former experiment, or by a combination of these factors. Anaerobic experiments were performed for a time period of 40 min for comparison with the aerobic experiments described above. These experiments, when analyzed using the nonlinear least-squares method as above, yielded K_i and K_i* values of 0.065 ± 0.005 mM and 0.0089 ± 0.0004 mM, respectively (Table II). Determination of k_5 and k_6 gave results of 0.12 ± 0.01 min^{-1} and 0.019 ± 0.002 min^{-1}, respectively. This gives a k_5/k_6 ratio of about 6/1. Considering experimental uncertainties, these results are indistinguishable from those obtained aerobically, suggesting that oxygen does not significantly influence the kinetics of formation of the second vanadium–uteroferrin complex.

Inhibition of FeZn Uteroferrin by Vanadate Monomer and Vanadyl Cation. (The zinc as a prefix implies that zinc has substituted iron, as in FeZn Uf.) Experiments with the FeZn uteroferrin were carried out to show that the Fe(II) of uteroferrin is crucial to the time-dependent vanadate inhibition. Replacement of the Fe(II) in uteroferrin by Zn(II) leads to a form of uteroferrin which retains nearly all catalytic activity and binds inhibitors such as phosphate, arsenate, and molybdate with affinities similar to the native enzyme (David & Que, 1990). Since FeZn uteroferrin has no possibility of undergoing redox chemistry at the metal center, different behavior upon treatment with vanadate should be observed if the redox property of vanadium is important for enzyme inhibition.

The vanadate inhibition was examined as a function of time in order to determine whether the FeZn phosphatase is inhibited by vanadate in a manner similar to that of the native enzyme. Assay solutions containing 200 mM acetate, pH 5.5, 2.0 mM PNPP, and 0.0–1.0 mM vanadate were used for this determination, and time intervals up to 5 h, as described in detail under Experimental Procedures, were examined. FeZn purple acid phosphatase showed linear product formation over a period of 5 h, suggesting the enzyme is not losing enzyme activity. The inhibition of the FeZn acid phosphatase by vanadate correlates with vanadate monomer and showed no signs of formation of a tight second complex.

Inhibition studies were carried out using assay solutions which contained 200 mM acetate, pH 5.5, and 1.5–6.0 mM PNPP. Phosphate is a competitive inhibitor for FeZn uteroferrin, with a K_i of 1.6 mM compared to 3.4 mM for the natural enzyme under identical conditions (David & Que, 1990). Vanadate inhibition for FeZn uteroferrin is noncompetitive, and a K_i of 0.36 mM was measured. Inhibition by vanadyl cation yields a K_i of 0.46 mM and a noncompetitive inhibition pattern. For the Fe(II)–Fe(III) enzyme under the same conditions, the vanadate K_i is 0.040 mM and the vanadyl K_i is 0.080 mM. These results suggest that both vanadate and vanadyl cation are capable of binding to the purple acid phosphatase after replacement of the Fe(II) with Zn(II) and that the affinity is somewhat reduced. However the absence of the time-dependent inactivation when Zn(II) is substituted for Fe(II) suggests that the inactivation reaction is related to the redox properties of Fe(II). These combined studies suggest

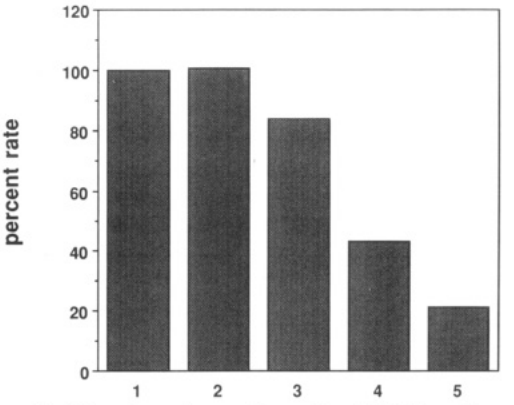


FIGURE 5: Time dependence of vanadate inhibition illustrated by recovery of activity upon EDTA additions. Assay solutions contained 200 mM acetate, pH 5.5, 2.0 mM PNPP, approximately 0.05 mg/mL (0.021 unit/L) uteroferrin, and 0.040 mM vanadate, with the exception of (1), which contained no vanadate. The solutions containing vanadate were preincubated for (2) 0 min, (3) 1 min, (4) 5 min, and (5) 15 min before the addition of 0.2 mM EDTA.

that the substitution of Fe(II) with Zn(II) does not significantly alter the coordination to the dimetal core but affects the binding of vanadate and vanadyl cation and completely prevents the electron transfer reaction.

Inhibition of Purple Acid Phosphatase by Perrhenate. Studies using perrhenate (ReO_4^-) and chromate as inhibitors were carried out to explore the effects of other redox active anions. Our attempts to carry out analogous studies with chromate failed, because chromate reacted with the substrate in the assay system. Perrhenate is a weaker oxidant than chromate but should have sufficient redox potential to oxidize Fe(II). However, perrhenate was found to be a weak inhibitor ($K_i \sim 30$ mM) for uteroferrin. It is possible that its single negative charge modulates its interactions with uteroferrin. More importantly, the inhibition of uteroferrin by perrhenate showed no time-dependent loss of enzyme activity over the course of 30 min. We conclude that an appropriate redox potential is not sufficient to effect loss of enzyme activity.

Nature of the Second Complex. The nature of the second complex is of particular interest. One possibility is a reduced uteroferrin complex containing V(V) which is distinct from Uf_r·V_1. The second possibility is a complex in which the Fe(II) has been oxidized to Fe(III) upon reduction of vanadate to vanadyl cation, i.e., a Uf_o·V(IV) complex. Spectroscopic and reversibility studies will probe the above possibilities.

When monitored by EPR, the addition of vanadate to Uf_r elicited the appearance of signals typical of VO^{2+} that quantitate to be about 85% ± 5% of the Uf_r concentration; in contrast, incubation with FeZnUf did not elicit this signal. The appearance of the vanadyl EPR signal in the Uf_r case alone suggests that vanadate may have been reduced by the Fe(II)–Fe(III) center, affording Uf_r·V(IV). Detailed spectroscopic studies characterizing these interactions are in progress.

Reversibility experiments support the mechanism proposed in Scheme I. Preincubation of the enzyme with vanadate for 15 min caused a loss in enzyme activity which could not be retained by simply removing the vanadate (or vanadyl) by, for example, complexation with EDTA (Figure 5) (Crans & Shin, 1988; Crans et al., 1989b; Scheidt et al., 1971; Crans & Simone, 1991). It has previously been demonstrated that excess EDTA will remove all inhibiting vanadium species from enzyme assay solutions to regenerate enzyme activity (Crans et al., 1989b; Crans & Simone, 1991). Analogous experiments were conducted where vanadate was removed by size-exclusion

chromatography. No increase in phosphatase activity was observed following the vanadium removal by the column after quantification of the enzyme and measurement of enzyme activity. When the phosphatase was incubated with vanadate for only 1 min, EDTA was able to recover some of the enzyme activity (Figure 5). These experiments show that vanadate forms two types of complexes with purple acid phosphatase, one (Uf_r·V_1) which is conveniently reversed by the addition of EDTA and a second complex which is not reversed upon removal of vanadate by chromatography or by EDTA addition.

Additional kinetic experiments were used to examine the possibility that the enzyme activity from a second complex could be regained. These experiments took advantage of the reduction of oxidized diiron center in PAP using mercaptoethanol and ferrous ion (Antanaitis & Aisen, 1985). Purple acid phosphatase was incubated for 15 min with vanadate in order to ensure the second complex was generated. EDTA was then added to this solution to complex the free vanadate, removing it from solution. Ferrous ion and mercaptoethanol were then added to the solution. The EDTA concentration was carefully controlled such that nearly all of the vanadate (or vanadyl cation) and iron were complexed, since only catalytic Fe^{2+} is needed to reactivate the enzyme (Day et al., 1988). Enzyme treated in such a manner regained some phosphatase activity. This experiment suggests that vanadate could oxidize the diiron center of the enzyme, which is then reduced by mercaptoethanol. Alternatively, it is possible the second complex may be a tight Uf_r·V(V) complex which, in the presence of Fe^{2+}, produces a Uf_o·V(IV) complex and free Fe^{3+}. This complex may or may not release vanadium, but if it does PAP activity can be generated. The latter complex, however, would not be consistent with the EPR spectrum showing formation of 85% ± 5% vanadyl cation.

Oxidation of the diiron center of uteroferrin by vanadate should produce some V(IV), which may be either bound to the protein or free in solution. The formation of a Uf_o·V(IV) complex may take place as shown is Scheme I. An additional set of experiments was conducted to explore how V(IV) interacts with Uf_o. If the reactions are reversible, V(IV) should regenerate some activity of vanadate-inhibited enzyme (the second vanadium–uteroferrin complex). The second complex was generated by preincubation of purple acid phosphatase (0.05 mg/L, 0.02 unit/L) with 0.2 mM vanadate [V(V)] for 15 min followed by addition of equimolar amounts of EDTA to remove the vanadate from the system. This enzyme retained only 17% of the original activity, presumably because vanadate had oxidized a large fraction of the enzyme. As shown earlier, vanadyl cation inhibits the reduced form of uteroferrin (Uf_r). Incubation with 0.3 mM vanadyl cation [V(IV)] for 5 min with Uf_o (0.05 mg/L, 0.02 unit/L) resulted in an enzyme with 1% of the original enzyme activity. This was expected since vanadyl cation is a potent phosphatase inhibitor. EDTA (0.3 mM) was added to this solution to remove the vanadyl cation, and the resulting enzyme had 42% of the activity of the original enzyme. The combined treatments of adding vanadyl cation to allow some reduction of the enzyme followed by addition of EDTA led to significant recovery of enzyme activity. Similar experiments were carried out with uteroferrin which had been allowed to air oxidize for several days. The oxidized enzyme (Uf_o) (0.05 mg/L, 0.02 unit/L) retained about 30% of the original activity of the reduced enzyme (Uf_r). Addition of V(IV) (0.3 mM) to the oxidized enzyme, followed by complexation with EDTA (0.3 mM), yielded an enzyme with nearly 80% of the activity of the reduced enzyme (Figure 6). These observations show that V(IV) is able to reverse

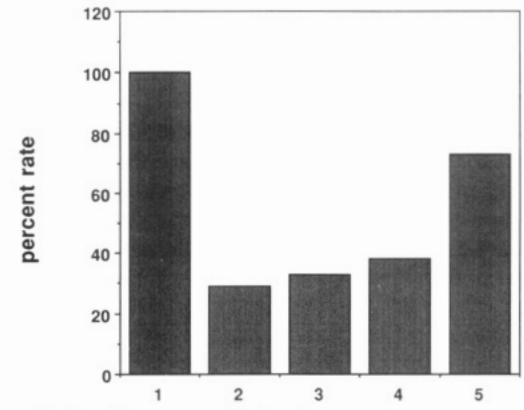


FIGURE 6: Partial recovery of phosphatase activity upon incubation of air-oxidized enzyme with V(IV). Assay solutions contained 200 mM acetate, pH 5.5, 4.0 mM PNPP, and (1) reduced enzyme, (2) air-oxidized enzyme, (3) air-oxidized enzyme and 0.3 mM EDTA, (4) air-oxidized enzyme and 0.3 mM V(IV), or (5) air-oxidized enzyme incubated for 5 min with 0.3 mM V(IV), followed by addition of 0.3 mM EDTA. The enzyme concentration was approximately 0.05 mg/L (0.02 unit/L).

some of the presumed redox reaction which initially formed the inactive phosphatase. The results are in agreement with the previous findings suggesting that redox chemistry between V and the diiron center in PAP are truly reversible. This result can be explained by the reaction of a Uf_o·V(IV) complex to generate a Uf_r·V(V) complex. That is, the second complex can be accessed through Uf_o or Uf_r. To further explore the binding sites of the various oxoanions, inhibition studies with two simultaneous oxoanions were done.

Simultaneous Inhibition of Uteroferrin by Two Oxoanions. The interaction of phosphate, molybdate, tungstate, vanadate, and vanadyl cation with purple acid phosphatase may involve similar or identical binding sites, and kinetic studies with two simultaneous inhibitors can explore these possibilities. Assay solutions for these inhibition studies contained 200 mM acetate, pH 5.5, 2.0 mM PNPP, and multiple inhibitors in combinations and concentration ranges described in detail under Experimental Procedures. The experiments using vanadate and phosphate as inhibitors generated data which led to intersecting lines when plotted as reciprocal rates versus vanadate concentration at various phosphate concentrations (data included in supplementary material). Since phosphate is a competitive inhibitor and vanadate is a noncompetitive inhibitor, these two anions are not mutually exclusive. Experiments with molybdate and phosphate gave intersecting lines, suggesting thay also bind at different sites on the enzyme (data included in supplementary material). The parallel lines observed with vanadate and molybdate, vanadate and tungstate, and vanadate and vanadyl cation for uteroferrin suggest the inhibitors are mutually exclusive. This supports the possibility that these inhibitors bind to the same noncompetitive site of the enzyme (Figure 7) (Yonetani & Theorell, 1964; Vincent et al., 1992). Molybdate, which is also a noncompetitive inhibitor for uteroferrin, is believed to bind at the Fe(III) center of the enzyme (Wang et al., 1991). Vanadate may be binding near the same site as molybdate. It is therefore possible that vanadate, tungstate, and vanadyl cation also binds at the diiron center of the enzyme. The shift in the UV absorption maximum upon binding of vanadate supports such a conclusion.

These results are different from those observed for the bovine spleen purple acid phosphatase (Vincent et al., 1992). Early data reported phosphate to be a competitive inhibitor for the bovine enzyme (Davis et al., 1981); however, recent studies

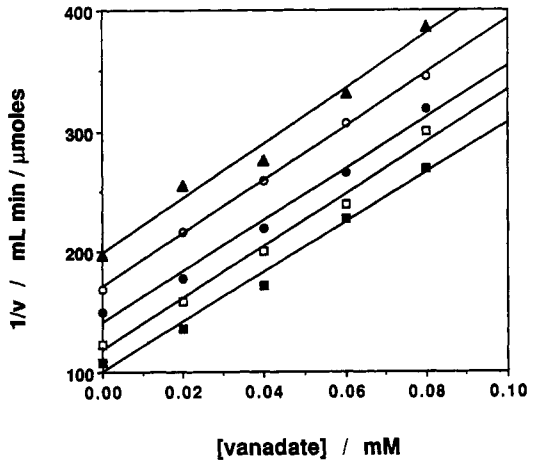


FIGURE 7: Yonetani–Theorell plot study using vanadate and molybdate as inhibitors. Assay solutions contained 200 mM acetate at pH 5.5, 2.0 mM PNPP, approximately 0.05 mg/mL (0.02 unit/mL) uteroferrin, 0.00–0.08 mM vanadate, and 0.0000 mM (■), 0.0010 mM (□), 0.0020 mM (●), 0.0030 mM (○), or 0.0040 mM (▲) molybdate.

show a more complex mixed-type inhibition pattern for the bovine enzyme (Vincent et al., 1992). The inhibition data for phosphate with uteroferrin is most consistent with a competitive inhibition pattern (data included in supplementary material), although it is possible to interpret the data as having a mixed-type inhibition pattern (data included in supplementary material). Nevertheless, the differences between these interpretations are not as large as those exhibited by the bovine enzyme (Vincent et al., 1992), suggesting there are some differences in the phosphate interactions between the bovine and the porcine enzyme. The differences between the two enzymes when responding to simultaneous interactions of phosphate and molybdate reported in this manuscript are, however, striking. The observed parallel lines for the bovine enzyme are significantly different from the intersecting lines observed for uteroferrin.

In summary, vanadate inhibits uteroferrin in a time-dependent manner which probably involves redox chemistry. The reaction is reversible. A minimum of two different vanadium–uteroferrin complexes form and the expected differences are observed in studies with the non-redox-active Fe(III)–Zn(II) enzyme. The interaction of phosphate and molybdate with uteroferrin is significantly different from that observed for the bovine purple acid phosphatase. It is possible that further characterization of vanadate and multiple inhibitor interactions will continue to uncover differences between the porcine and bovine enzymes.

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SUPPLEMENTARY MATERIAL AVAILABLE

Five figures showing Yonetani–Theorell plots for vanadate/phosphate and molybdate/phosphate inhibition of uteroferrin and Lineweaver–Burk plots for phosphate inhibition of bovine spleen PAP (5 pages). Ordering information is given on any current masthead page.

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