Spectroscopic and Kinetics Studies of a High-Salt-Stabilized Form of the Purple Acid Phosphatase from Bovine Spleen†

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ABSTRACT: Use of a revised purification procedure that maintains the enzyme in a high-salt environment has resulted in the isolation of a new form of the bovine spleen purple acid phosphatase. This enzyme cannot be distinguished from that previously described [Davis, J. C., Lin, S. S., & Averill, B. A. (1981) *Biochemistry 20*, 4062] by electrophoresis, isoelectric focusing, Western blot analysis, or N-terminal amino acid sequence and exhibits identical catalytic properties and EPR spectra in the reduced (pink) form. It does, however, possess a much more highly ordered structure as shown by CD spectra and exhibits markedly different reactivity upon oxidation and different visible spectra upon binding of inhibitory anions or changing pH. The properties of the new high-salt-stabilized form of the enzyme have permitted an extensive examination of the visible absorption spectra of complexes of the oxidized and reduced enzyme with inhibitory anions. It is found that these anions may be grouped into three classes on the basis of their effect on the visible absorption maximum and their sensitivity to pH: phosphate, arsenate, and AMP; tungstate and molybdate; and fluoride. This grouping is reinforced by a detailed examination of the steady-state kinetics of the enzyme in the presence of these inhibitors, which reveals that the first class exhibits mixed-type inhibition due to the presence of competitive and noncompetitive binding sites, while the second class exhibits simple noncompetitive inhibition. Fluoride exhibits complex inhibition behavior characterized by curved Lineweaver–Burk plots; this behavior cannot be attributed to the presence of inhibitory aluminum fluoride complexes. Taken together, the spectral and kinetics data are consistent with a picture in which tetrahedral oxyanions bind in a noncompetitive fashion by bridging the two iron atoms in the dinuclear center, with the smaller anions also being able to bind in a competitive manner at a single iron atom.

The purple acid phosphatases (PAP's)[1] constitute a class of novel enzymes that catalyze the hydrolysis of certain phosphate esters, including nucleoside di- and triphosphates and aryl phosphates (Vincent & Averill, 1990; Doi et al., 1988a; Antanaitis & Aisen, 1983). The mammalian enzymes, the most thoroughly studied members of the class, possess a dinuclear iron active site and highly conserved amino acid sequences, with the human and porcine enzymes displaying 85% absolute homology and the porcine and bovine enzymes >90% homology (Hunt et al., 1987; Ketcham et al., 1989; Lord et al., 1990). The enzymes exist in two forms: a reduced, pink form containing a mixed-valent Fe(III)–Fe(II) center, which is the enzymatically active species, and an oxidized, purple form containing a diferric center, which is catalytically inactive. In both oxidation states, the irons are antiferromagnetically coupled, giving rise to $S = {}^1/_2$ and $S = 0$ ground states, respectively. The mixed-valent Fe(III)–Fe(II) unit exhibits a distinctive EPR signal centered at $g < 2$ (Doi et al., 1988a; Antanaitis & Aisen, 1983; Vincent et al., 1990).

Spectroscopic investigations have shown that the novel purple (or pink) color of the proteins arises from a tyrosine phenoxide to ferric iron charge-transfer band, which is sensitive to the oxidation state of the second iron and to the presence of several inhibitors of the phosphatase activity (Gaber et al., 1979; Antanaitis & Aisen, 1985; Averill et al., 1987). Despite the sequence homology between the enzymes, spectroscopic and kinetics studies on the enzymes from different sources have yielded some significantly different and occasionally contrasting results; this difference is most striking in a comparison of results reported for the bovine spleen and porcine uterine fluid (uteroferrin, Uf) enzymes, which because of their availability are the two most studied proteins. For example, addition of oxidizing agents to reduced Uf has been shown to result in conversion of the enzyme to its oxidized form, concomitant with a red shift of its intense visible absorption band (Debrunner et al., 1983); similar treatment of the bovine spleen enzyme results in bleaching of the purple color (Davis, 1982). The inhibition of PAP's by fluoride has been reported to be uncompetitive (Robinson & Glew, 1980), partially competitive (Lau et al., 1987), complex (curvilinear) (Davis et al., 1981), and noncompetitive (Rezyapkin et al, 1985; Hayman et al., 1989). Previously, some of the differences between the bovine enzyme and Uf have been attributed to the proteolytic cleavage of the bovine enzyme (which is isolated as two "subunits" of apparent moleular weights 24 000 and 15 000), whereas Uf is isolated as a single ~32 000 molecular weight unit (Campbell & Zerner, 1973; Averill et al., 1987; Campbell et al., 1978). Recently, sufficient quantities of the "native" bovine spleen enzyme have been isolated to allow an examination of some of its spectroscopic and reactivity properties, which more closely approximate those of Uf (J. Orlando, T. Zirino, and B. A. Averill, manuscript in preparation).

In this report, a new form of the bovine spleen enzyme is described that allows the visible absorption and kinetics properties of the enzyme to be examined in greater detail. Attempts to elucidate the molecular basis for the different properties of this form of the enzyme are also reported. In addition, the origins of the reported discrepancies between the

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[1] Abbreviations: EPR, electron paramagnetic resonance; *p*-NPP, *p*-nitrophenyl phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; CD, circular dichroism; Uf, uteroferrin; PAP, purple acid phosphatase; TYR(P), phosphotyrosine; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate.

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bovine spleen enzyme and Uf are discussed.

Experimental Procedures

Protein Purification. Method A. The protein was isolated by the procedure of Davis et al. (1981). Briefly, this involves homogenization of ca. 3 kg of bovine spleen in 2 mL/g 0.25 M KCl, acid precipitation and extraction at pH 3.0, centrifugation and adjustment of pH to 5.5, batch absorption with cellulose phosphate (P-11) in the presence of 0.1 M ascorbate and extraction with 2 M KCl, dialysis against deionized H_2O, and chromatography on (carboxymethyl)cellulose, Sephadex G-75, and hydroxyapatite, all at 4 °C. As isolated, the protein has a visible λ_{max} at ~550 nm.

Method B. The procedure is identical with that in method A through extraction of enzyme from cellulose phosphate with 2 M KCl. All subsequent operations are also performed at ca. 4 °C. Ammonium sulfate (144 g/L) is added, and the resulting suspension is centrifuged at 25420*g* for 20 min and filtered through glass wool. The supernatant is applied to a 1.5 × 45 cm phenyl-Sepharose (CL-4B, Pharmacia) column, equilibrated with 0.05 M acetate buffer, pH 5.0, containing 144 g/L $(NH_4)_2SO_4$ (buffer A). An intense pink band forms near the top of the column, which is washed with circa 100 mL of buffer A. The protein is then eluted with a 144–0 g/L $(NH_4)_2SO_4$ gradient in 0.05 M acetate buffer, pH 5.0. Fractions with visible pink color are collected and concentrated to ca. 5 mL by ultrafiltration (Amicon 8200 with PM 10 or PM 30 membrane). The intense pink solution is loaded onto a Sephadex G-75 column (2.5 × 80 cm) and eluted with 0.05 M acetate buffer, pH 5.0, containing 2 M KCl. Pink–purple fractions are collected and concentrated as described above to a volume of approximately 20–30 mL. The resulting enzyme solution normally has a visible λ_{max} of 536 nm and an A_{280}/A_{536} ratio of ca. 12.5.

Spectral Characterization. Spectral data for protein estimation, determination of enzyme activity, and kinetics were obtained on a Beckman DU spectrophotometer equipped with a Gilford Model 252-D accessory. Visible spectra were obtained on a Cary 219 spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer. The EPR spectra were run on a Varian 109 spectrometer equipped with an Air Products liquid helium cryostat. CD spectra were performed by the Protein Sequencing Facility at the University of Virginia Medical School on a Jasco J-600 spectrometer.

Analytical Methods. Phosphatase activity was determined as previously described with 0.1 M MES buffer at pH 6.0 (Campbell et al., 1978). Ascorbate and ferrous iron were omitted for assays under nonreducing conditions. All assays for kinetics were performed in triplicate. Protein concentration of purified enzyme was determined by using an extinction coefficient of 4.08×10^3 for the visible band (Campbell et al., 1978). Western blotting, isoelectric focusing, and discontinuous nondissociating gel electrophoresis (native) were variations on standard procedures that will be described in detail elsewhere (Orlando et al., manuscript in preparation). Predictions of average secondary structure from amino acid sequence were performed by using the algorithms of Chou and Fasman (1974a,b) and of Garnier, Osguthorpe, and Robson (Garnier et al., 1978).

Miscellaneous. Stock solutions of inhibitors for kinetics were made in 0.1 M MES buffer, pH 6.0, with the exception of the MoO_4^{2-} and WO_4^{2-} solutions, for which the pH was adjusted to 8.0 to avoid formation of polyoxoanion species. Aliquots (30 μL/mL) of the stock solutions of inhibitors were added to the protein solution to give a final inhibitor concentration of $>10K_i$ for the visible spectral studies. Enzyme solutions at pH 3 were prepared by dialysis against 0.1 M citric acid buffer, pH 3.0. Other pH adjustments were performed by titrating with microliter aliquots of 6 M HCl, 1.5 M HCl, or 3 M NaOH. After addition of inhibitors or adjustment of pH, protein samples were centrifuged by using a Tomy MTX-150 micro refrigerated centrifuge or Sorvall RC-5 refrigerated centrifuge to remove any denatured enzyme. Removal of the 2 M KCl from protein isolated by method B was accomplished by successive cycles of dilution with 0.05 M acetate buffer, pH 5.0, containing 0.15 M KCl, followed by concentration by ultrafiltration (Amicon 8200 or 8050 with PM 10 or 30 membranes and Centricon 10 or 30). Reduced enzyme from method A was generated by adding a solution of 200 mM ascorbate and 12 mM Fe^{2+} at pH 4.5 to an equal volume of protein. After the absorbance maximum shifted to ~505 nm, the ascorbate and ferrous iron were removed by desalting on a Sephadex G-25 column, equilibrated with either 0.05 M acetate buffer, pH 5.0, containing 0.15 M KCl or 0.1 M citrate buffer, pH 3.0, containing 0.15 M KCl. The method B reduced enzyme was oxidized with H_2O_2 according to the procedure of Debrunner et al. (1983).

Results and Discussion

Purification. Previously this group has described the use of cellulose phosphate in the purification of bovine spleen PAP (Davis et al., 1981); the utilization of this material as a batch absorbent, replacing the more conventional ammonium sulfate precipitation (Singer & Fruton, 1957; Campbell & Zerner, 1973), resulted in a greater purification with higher yield. Unfortunately, both procedures left the protein in a high-salt environment that required dialysis prior to further purification by electrophoresis (Glomset, 1959) or (carboxymethyl)cellulose chromatography. This dialysis resulted in substantial loss of activity [20% in Davis et al. (1981)], and in at least the case of the cellulose phosphate extract (of method A), the dialysis is accompanied by oxidation of the reduced form of the enzyme to the oxidized form containing one tightly bound molecule of phosphate. In an attempt to prevent the problems associated with the dialysis and concomitant oxidation (vide infra), a phenyl-Sepharose column (combined with an ammonium sulfate precipitation) was utilized (see also Orlando, et al., manuscript in preparation). By maintaining the high salt concentration, PAP eluted from this column as a tight pink band (visible λ_{max} 515 nm), indicative of the reduced form of the enzyme in the presence of high sulfate concentrations (vide infra). Further purification on a Sephadex G-75 column, equilibrated with 0.05 M acetate buffer, pH 5.0, containing 2 M KCl, yields reduced protein with λ_{max} at 536 nm (as a result of the removal of the sulfate) and a ratio of A_{280}/A_{536} of approximately 12.5. The overall yield and purity are comparable to those obtained by method A.

The visible λ_{max} at 536 nm is distinctly different from that reported for protein from the earlier purification scheme, which after being reduced with ascorbate is at 505 nm. Yet, the isolated protein from method B has been shown unequivocally to contain the *reduced*, mixed-valence diiron center by phosphatase activity assays in the absence of reducing agents, which show no activity loss compared with the standard assay containing ascorbate and ferrous iron (data not shown), a lack of a shift in λ_{max} in the presence of 100 mM ascorbate or 100 mM 2-mercaptoethanol, an EPR spectrum with a signal centered at *g* less than 2 (vide infra), the appearance of paramagnetically shifted resonances in its ^1H NMR spectrum (not shown), and a shift of λ_{max} to higher wavelenths upon treatment of the isolated enzyme with H_2O_2 or other oxidants (vide infra).

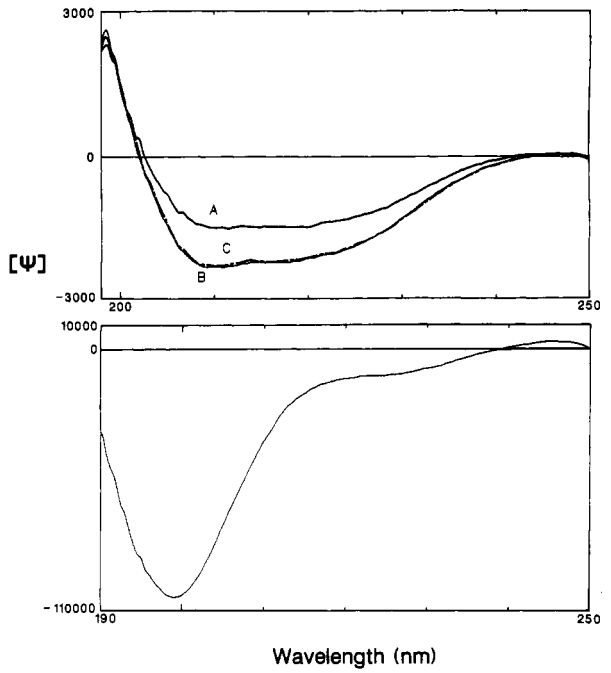


FIGURE 1: Circular dichroism spectra of (top) (A) reduced method B enzyme, 0.101 mg/mL, (B) oxidized method B enzyme, (C) (dashed line) oxidized method B enzyme plus phosphate; (bottom) as-isolated method A enzyme, 0.0995 mg/mL. All spectra obtained in 0.05 M acetate, pH 5.0, with 0.15–0.35 M KCl in 0.1-cm cells.

Circular Dichroism and EPR Spectra. Despite the difference in the visible absorption maximum for the enzyme isolated by methods A and B, the proteins obtained from the two methods cannot be distinguished by native gel electrophoresis, isoelectric focusing, N-terminal amino acid sequencing, or Western blot analysis (data not shown). Thus, the proteins appear not to differ in charge or size, suggesting that they differ only in protein conformation. In addition, neither the activity nor the optical spectrum of the method B enzyme is affected when 2 M KCl is replaced by 2 M KBr or $NaNO_3$, indicating that the spectral differences are not due to formation of a chloride complex. Consequently, circular dichroism spectra in the range 198–250 nm were obtained for oxidized and reduced forms of the PAP's in order to investigate their conformations. As shown in Figure 1, distinct differences exist in the CD spectra of the enzyme from the two preparations.

The spectra of the reduced and oxidized forms of the enzyme from the high-salt preparation (method B) and its oxidized phosphate complex are qualitatively similar, exhibiting strongly negative ellipticities with a shoulder at approximately 210 nm. The spectrum of the reduced enzyme, however, is flatter with the shoulder less distinct, indicating an apparent lack of α-helical structure compared to the oxidized enzyme. Binding of phosphate to the oxidized enzyme results in no change in the spectrum, as indicated by a comparison to the spectrum of the oxidized enzyme prior to the addition of phosphate, suggesting that it is not accompanied by a significant rearrangement of the protein structure. The general shape of the spectra of all three forms of the method B enzyme also resembles that of the spectra reported for the corresponding forms of the uterine fluid enzyme, Uf (Antanaitis et al., 1982, 1983). In contrast, the protein isolated by method A displays very strongly negative ellicipticity with a peak at ca. 198 nm; thus, it is readily apparent that protein from method A contains considerably less (if any) ordered structure. Consequently, the dialysis of the cellulose phosphate extract in method A and concomitant oxidation and binding of phosphate appear to be accompanied by an appreciable decrease in ordered protein secondary structure (although this has no effect on catalytic activity). Conversely, the high concentration of salt in this stage of the method B preparation appears to stabilize a more ordered conformation of bovine PAP.

Table I: Average Secondary Structure of Purple Acid Phosphatases

source/form	α helix (%)	β sheet (%)	turn (%)	random coil (%)
reduced bovine spleen (method B)[c]	22.5	67.5	2.5	7.5
reduced Uf[a]	18	11		71
oxidized bovine spleen (method B)	37.5	40	2.5	15
oxidized Uf[a]	17	11		74
oxidized bovine spleen + PO_4^{3-} (method B)	37.5	40	2.5	15
oxidized Uf + PO_4^{3-}[a]	12	6		82
oxidized bovine spleen + PO_4 (method A)	0	0	0	100
calculated (Chou–Fasman)[b]	58.0	57.7	12.1	
calculated (Garnier)[b]	21.3	32.5	24.9	21.3

[a] Antanaitis et al. (1983). [b] Using human PAP sequence. [c] Protein samples in high salt isolated by method B were diluted with 0.05 M acetate and 150 mM KCl, pH 5.0, buffer just prior to use.

Results of the quantitation of the average secondary structure of the polypeptide backbone are collected in Table I with the previously reported results on Uf (Antanaitis et al., 1982, 1983). The fits of the circular dichroism spectra of the forms of the high-salt preparation of the bovine spleen enzyme indicate that the backbone structure is dominated by β sheet. Thus, the secondary structure of PAP inferred from CD appears to be similar to that of other phosphatases that have been structurally characterized by X-ray diffraction. For example, inorganic pyrophosphatase, which in its active form binds two divalent metal ions at its active site, has about half of its amino acid residues involved in β structure; about one-fifth of the residues occupy α helices (Kuranova, 1989). In contrast, alkaline phosphatase, with two atoms of zinc and one magnesium atom at the active site, contains only 16% β structure and 27% α-helical structure (Sowadski et al., 1985; Kim & Wyckoff, 1989). Both enzymes are of the α/β types. Prediction of the structural type for PAP using the method of Chou (1989) and the sequence of human PAP (Ketcham et al., 1989) (GenBank Accession Number J04430) [the bovine spleen sequence is incompletely known (Hunt et al., 1987)] indicates that the purple acid phosphatases also should belong to the α/β class, which possess alternating α helices and β strands.

The secondary structure quantitations on the CD spectra of the high-salt form of the bovine spleen enzyme and Uf are hard to reconcile. Certainly, the ca. 80% and 100% random structure reported for Uf and the bovine spleen enzyme prepared by method A, respectively, are difficult to envision, given the high degree of ordered structure possessed by most enzymes. Indeed, prediction of the secondary structure of PAP's by the method of Chou and Fasman using the sequence of human PAP is in excellent agreement with the bovine spleen CD results (Table I), indicating a high probability that the PAP's possess considerable nonrandom secondary structure. Prediction of the average secondary structure of human PAP using the method of Garnier (Garnier et al., 1978) again suggests that the predominant backbone structure is based on β chains; however, more detailed analysis of the secondary structure must await X-ray crystallographic studies of one of these enzymes. The high percentage of random structure found for Uf may suggest that this protein also undergoes a change to a less ordered conformation approximating that of the method A bovine enzyme during purification [which like the method A procedure involves the use of (carboxy-

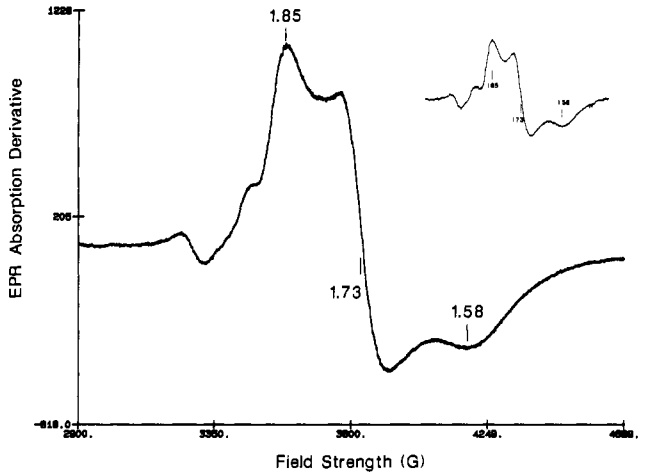


FIGURE 2: X-band electron spin resonance spectrum of reduced method B enzyme. Conditions: ~0.4 mM protein; microwave frequency, 9.30 GHz; microwave power, 10 mW; receiver gain, 4×10^3; modulation amplitude, 10 G; T ~ 5 K; 50 mM acetate and 2 M KCl, pH 5.0. Inset: EPR of reduced enzyme from method A reproduced from Averill et al. (1987).

methyl)cellulose and similar ionic strength buffer]; replacement of the cation-exchange resin with phenyl-Sepharose and higher ionic strength buffers during the Uf purification might prove interesting in this regard.

Although the PAP's appear to lose their α and β structure in low ionic strength media, they do not denature to the point of precipitation, and they retain their activity in phosphate ester hydrolysis. However, removal of the iron atoms gives apoprotein, which is extremely unstable toward denaturation and precipitation (Nuttleman & Roberts, 1990), suggesting that the iron atoms play an important role in maintaining the integrity of the enzyme.

The EPR spectrum of enzyme isolated by method B is presented in Figure 2, along with that of ascorbate-reduced method A enzyme (Averill et al., 1987). The high-field regions of both spectra display the complex $g < 2$ signal, indicative of the mixed-valence diiron active site. Surprisingly, given the apparent major differences in secondary structure, the two signals are identical (within error) both in general features and in g values. As EPR is particularly sensitive to changes in the electronic structure of the iron atoms, this result strongly suggests that the differences in secondary structure do not manifest themselves in any appreciable change in the environment of the diiron active site. As the purple (or pink) color of the enzyme arises from a tyrosine phenoxide to ferric iron charge-transfer band and the diiron centers of the PAP from both purification procedures appear to be essentially identical, the difference in the visible spectra of the reduced enzymes from methods A and B (λ_{max} 505 and 536 nm, respectively) may arise from a difference in the orientation of the tyrosine phenoxide ligand.

Visible Spectra of Oxidized and Reduced Forms of Bovine Spleen Enzyme and Their Inhibitor Complexes. While the bovine spleen enzyme and uteroferrin display a high degree of sequence homology (ca. 90%) and exhibit many similar spectral characteristics, one difference previously reported is the ability of the mixed-valence diiron center to be converted to the diferric form by a variety of oxidants. Oxidizing agents including hydrogen peroxide and ferricyanide cleanly oxidize the reduced diiron center of Uf, with a corresponding shift of λ_{max} from 515 nm to approximately 560 nm (Debrunner et al., 1983; Antanaitis et al., 1983). In the case of the spleen enzyme isolated by method A, treatment with these reagents

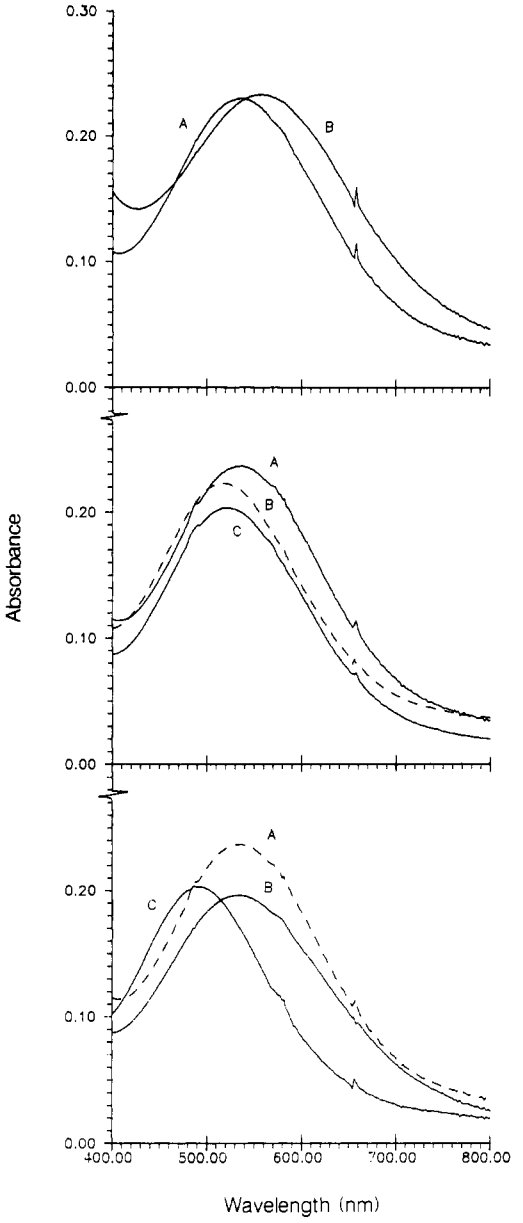


FIGURE 3: (Top) Visible spectra of reduced method B enzyme (A) and enzyme oxidized with H_2O_2 (B) in 0.05 M acetate and 2 M KCl, pH 5.0. (Middle) Visible spectra of reduced method B enzyme (A), reduced enzyme plus 150 μM MoO_4^{2-} (B), and reduced enzyme plus 150 μM WO_4^{2-} (C), in 0.05 M acetate and 2 M KCl, pH 5.0. (Bottom) Visible spectra of reduced method B enzyme (A), enzyme plus 60 mM PO_4^{3-} (B), and enzyme plus 30 mM AsO_4^{3-} (C) in 0.05 M acetate and 2 M KCl, pH 5.0. Intensities not corrected for dilution upon addition of anions or any accompanying protein denaturation.

leads to destruction of the diiron center (Davis, 1982). Consequently, a number of spectroscopically intriguing forms of the spleen enzyme have been inaccessible until this work.

Treatment of the as-isolated PAP from method B in 0.05 M acetate buffer, pH 5, containing 2 M KCl, with a 10-fold excess of hydrogen peroxide [as described in Debrunner et al. (1983) for Uf] or other oxidants such as *m*-chloroperoxybenzoic acid or *tert*-butyl hydroperoxide does not result in bleaching of the pink–purple color, but in a rapid shift in the color of the solution to violet (λ_{max} 560 nm), indicative of the formation of the oxidized diiron center (Figure 3, top). Interestingly, the oxidation is accompanied by essentially no change in the intensity of the absorption band. As suggested previously for the bovine enzyme (Averill et al., 1987) and uteroferrin (Pyrz et al., 1986), this indicates that the tyrosine

Table II: Visible Absorbance Maxima (nm) for Various Forms of Purple Acid Phosphatases

	bovine spleen PAP				Uf	
	method A		method B			
	pH 3.0[j]	pH 5.0[k]	pH ≤ 4.0[l]	pH 5.0[l]	pH 3.0	pH 4.9
reduced	525[a]	510[a]	[b]	536	524,[a,e] 536[d,f]	510[a,d-h]
reduced + WO_4^{2-}	524	518	516	522		530[f]
reduced + MoO_4^{2-}	524	518	516	518		515,[d] 510,[f] 525[e]
reduced + AsO_4^{3-}	520[a]	486[a]	556	492		510,[a,e] 505[a,h]
reduced + PO_4^{3-}	560[a]	530[a]	560	536	561,[a,e] 564[a,f]	542,[a,e] 536[a,h]
reduced + AMP	554	554	560	536		
reduced + F^-	510	510	524	518		
oxidized[c]			556	560		550,[g] 570[i]
oxidized + WO_4^{2-}			552	552		
oxidized + MoO_4^{2-}			552	552		550[d]
oxidized + AsO_4^{3-}			548	542		550[e,f]
oxidized + PO_4^{3-}	545	545	548	544	550[e]	545,[d] 550[e,f]
oxidized + AMP			550	538		
oxidized + F^-			554	530		550[d]

[a] Unstable toward oxidation in air. [b] Unstable, λ_{max} 536 nm over range pH 5–9. [c] H_2O_2 as oxidant. [d] Antanaitis and Aisen (1985). [e] Pyrz et al. (1986). [f] Pyrz (1986). [g] Debrunner et al. (1983). [h] David and Que (1990). [i] Antanaitis et al. (1983). [j] 0.1 M citrate. [k] 0.05 M acetate and 0.15 M KCl. [l] 0.05 M acetate and 2 M KCl.

phenoxide ligand binds to the iron that remains trivalent. Addition of tetrahedral oxyanions (i.e., PO_4^{3-}, AsO_4^{3-}, MoO_4^{2-}, and WO_4^{2-},[2] the nonhydrolyzable substrate analogue AMP, and F^- to this species results in the generation of the corresponding oxidized enzyme–anion complexes, which possess visible maxima shifted to higher energy.

Previously, phosphate has been shown to be a competitive inhibitor of the bovine enzyme by using *p*-nitrophenyl phosphate (*p*-NPP), while *p*-nitrophenol, the other product of *p*-NPP hydrolysis, was not [Davis et al. (1981) and vide infra], suggesting that the reaction follows a pseudo Uni Bi hydrolytic two-step transfer mechanism (in which nitrophenol is released prior to phosphate). Thus, phosphate must bind at the active site of the enzyme. Examination of oxyanion binding should therefore serve as a technique to probe the active site structure; however, as will be discussed below, some of the oxyanions are found to interact at a separate site. The use of AMP is suggested by its similarity to ADP and ATP, substrates that are turned over especially efficiently by PAP's; AMP is not, however, hydrolyzed at any appreciable rate. Thus, AMP is expected to bind tightly to the enzyme active site without being dephosphorylated. As will be shown, the same classification of anions also arises from an analysis of their effects on the visible spectrum of the reduced enzyme and on the kinetics of substrate hydrolysis, suggesting a clear difference in their mode of interaction with the dinuclear iron center.

Oxidized PAP. The anions can be divided into three classes according to their effect on the visible spectrum of the oxidized enzyme: molybdate and tungstate; phosphate, arsenate, and AMP; and fluoride. The molybdate and tungstate complexes exhibit λ_{max} of 552 nm, very close to that of the uncomplexed enzyme; the position of their absorption maxima shows no dependence on pH over the range 4–5 (Table II). In contrast, the spectra of the complexes of AMP, phosphate, and arsenate have λ_{max} shifted to slightly higher energies (538–544 nm) and are sensitive to the lowering of the pH to 4. The fluoride complex exhibits anomalous behavior, as its λ_{max} is blue-shifted 30 nm from that of the uncomplexed oxidized diiron center (530 nm). The visible spectra of the oxidized phosphate and arsenate species agree well with those reported for similar Uf species (Table II).

[2] Use of PO_4^{3-} and AsO_4^{3-} as abbreviations for phosphate and arsenate, respectively, is not intended to reflect the degree of protonation of these species.

Reduced PAP. Addition of the above inhibitors to the reduced method B enzyme in 0.05 M acetate buffer, pH 5.0, containing 2 M KCl results in either a blue shift or no apparent shift of the visible λ_{max} (Table II). Tungstate and molybdate shift the absorbance maximum of the reduced diiron center from 536 to ca. 520 nm (Figure 3, middle), and their spectra exhibit little if any pH dependence. Treatment of the enzyme with arsenate produces a dramatic blue shift (to 492 nm), while phosphate and AMP result in no apparent shift in λ_{max} (Figure 3, bottom). However, adjusting the pH from 5 to below 4 results in a distinct red shift of the λ_{max} of the arsenate-, phosphate-, and AMP-treated enzyme to ca. 560 nm. As the reduced enzyme does not exhibit this behavior upon lowering of pH (indeed, the enzyme denatures rapidly at pH <4), this result suggests that the reduced enzyme–phosphate and –AMP complexes are formed, despite the lack of change in the visible spectra. [Addition of phosphate, arsenate, or AMP to the reduced enzyme results in loss of the characteristic EPR signal, also indicating complex formation (J. B. Vincent, M. W. Crowder, and B. Averill, unpublished results).] It is apparently coincidental that the λ_{max} values for the phosphate and AMP complexes and the reduced method B enzyme are identical at pH 5. This conclusion is supported by results reported for the reduced Uf–phosphate complex (Pyrz et al., 1986) and by the results of the addition of anions to the ascorbate-reduced enzyme from method A (λ_{max} ~510 nm) (Table II). The reduced oxyanion complexes of method A enzyme at pH 5.0 are indistinguishable for practical purposes from their method B enzyme counterparts. The existence of a reduced PAP–phosphate complex until recently has been a point of substantial controversy (Pyrz et al., 1986; Doi et al., 1987; Burman et al., 1986; Day et al., 1988); the results presented here are in agreement with the conclusions of Que and co-workers that such a species does in fact exist (Pyrz et al., 1986). This represents the first report of a reduced phosphate complex of the bovine spleen enzyme.

Addition of fluoride to reduced method B enzyme results in a blue shift of λ_{max} of 18 nm; in the case of the method A enzyme, fluoride treatment has no apparent effect on the enzyme. In neither case is instability of the fluoride–enzyme adduct to air oxidation observed, as is the case for Uf (Antanaitis et al., 1983).

As previously reported for the bovine spleen enzyme (Davis et al., 1981) and Uf (Antanaitis et al., 1983), the reduced method A enzyme in the presence of phosphate or arsenate

is unstable toward air oxidation. In the case of phosphate, the oxidation is accompanied by a red shift of λ_{max} to 545 nm; the resulting spectrum is identical with that of the oxidized phosphate complex of method B enzyme. (Consequently, the visible spectra of the reduced phosphate and arsenate complexes of the method A enzyme were obtained in the presence of 30 mM ascorbate.) Uniquely, while complete air oxidation of the phosphate and arsenate adducts of the method A complexes requires only a few hours, no shift in λ_{max} is observed for the method B reduced phosphate species after 24 h. Only a tiny shift is observed for the corresponding arsenate species (because of the difference in λ_{max} of nearly 70 nm between the reduced and oxidized arsenate complexes, oxidation of only a small percentage of the enzyme is required to give a detectable shift in λ_{max}). Surprisingly, given the generally similar behavior of the AMP–enzyme complexes to their phosphate and arsenate counterparts, reduced method A enzyme in the presence of AMP is not rapidly oxidized by air. Also, this reduced AMP species does not exhibit any pH dependence of the optical spectrum over the pH range 3–5; it remains in a form with λ_{max} ca. 560 nm, similar to those of the arsenate and phosphate complexes at pH 3. The substrate analogue, with its increased bulk, may thus make the diiron center inaccessible to dioxygen.

The behavior of the method B enzyme resembles in some ways that of the recently reported high molecular weight form of Uf, which possesses a second subunit (Baumbach et al., 1986). This protein is isolated in its reduced form and is not susceptible to air oxidation. Likewise, the enzyme remains reduced in the presence of phosphate and air, although phosphate still serves as a competitive inhibitor. The function of the second subunit was reported to be to stabilize Uf in a special conformation (Baumbach et al., 1986). However, determination of the secondary structure (for example, by CD) was not reported; it would be most interesting to see if the second subunit maintains Uf in a conformation with appreciably more α and β structure, similar to that of the method B bovine spleen enzyme.

Like Uf (Pyrz et al., 1986), the reduced method A enzyme exhibits pH-dependent visible spectra. On reduction of pH from 5 to 3, the λ_{max} of the protein shifts from 510 to 525 nm. In the case of Uf, the visible absorption maximum has been reported to red-shift from 510 nm to as far as 536 nm. This shift is the same as the difference in the position of λ_{max} of the method A and method B bovine spleen enzyme, suggesting that the conformation of the phenoxide diiron center of Uf at pH 5 and 3 are similar to the difference in conformation between the method A and B bovine spleen enzymes, respectively. Antanaitis and Aisen (1982) reported that addition of ferrous iron to as-isolated bovine spleen PAP (primarily the oxidized phosphate complex) resulted in a shift in λ_{max} from 550 to 525 nm, a value intermediate between method B enzyme and reduced method A enzyme. Their enzyme was purified according to method A except that the dialysis against deionized water was replaced with a dialysis against 0.1 M acetate, pH 4.9 buffer. Thus, at this higher salt concentration, it is conceivable that some of their protein remained in the conformation stabilized by high salt or that the enzyme adopted a conformation intermediate between that of the method A and B bovine spleen enzymes. Indeed, a strong correlation exists between the percent random coil structure in the protein backbone and the position of the visible maximum of the reduced protein (Figure 4). This strongly suggests that disruption of the backbone structure (by exposure to low-salt conditions, etc.) results in a change in the environment or orientation of the tyrosine phenoxide ligand to one iron of the diiron center.

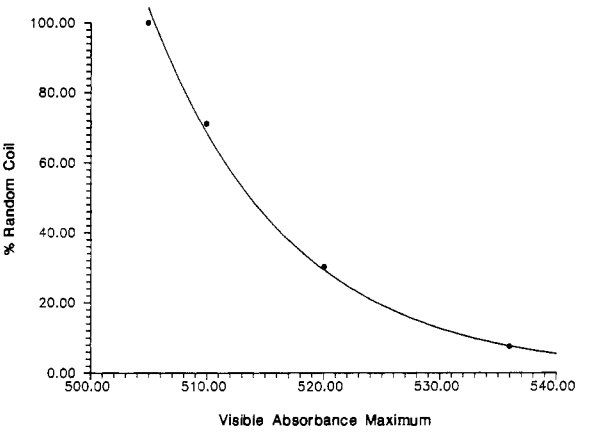


FIGURE 4: Correlation between percent random coil structure and visible maxima of reduced PAP's. Includes data from Orlando, et al., manuscript in preparation; Antanaitis et al. (1983); and Davis et al. (1981).

Table III: Visible Maxima (nm) for Method B Enzyme in Low-Salt Buffer[a]

anion	reduced	oxidized[b]
	536	554
WO_4^{2-} (150 μM)	522	552
MoO_4^{2-} (150 μM)	520	550
AsO_4^{3-} (30 mM)	496	544
PO_4^{3-} (60 mM)	534	548
AMP (60 mM)	532	552

[a] 0.2 M KCl and 0.05 M acetate, pH 5.0. [b] H_2O_2 used as oxidant.

While the method B form of the reduced bovine spleen enzyme is stabilized by high salt concentrations during purification, once purified it is reasonably stable under low-salt conditions (0.15 M KCl). The visible spectra of the desalted enzyme after addition of inhibiting anions or of hydrogen peroxide followed by the anions are essentially identical with the spectra in 2 M KCl (Table III). However, if the reduced method B enzyme in low-salt buffer is stored at room temperature, a shift in λ_{max} to ca. 515 nm occurs over a period of days, accompanied by some protein precipitation; this effect is not reversed by addition of 2 M KCl. The protein appears thus to convert to the conformation of reduced method A enzyme under these conditions. It is notable that reduced method A enzyme is quite susceptible to denaturation, especially at low protein concentrations.

As previously mentioned under Experimental Procedures, the enzyme that elutes from the phenyl-Sepharose column in method B possesses a visible absorption maximum at 515 nm. To test whether this was the result of the high concentration of sulfate in the buffer, the reduced enzyme from method B was treated with $(NH_4)_2SO_4$. As shown in Figure 5, high concentrations of sulfate result in a shift of λ_{max} to lower wavelengths, with an apparent isosbestic point at 530 nm and a value of λ_{max} = 515 nm at 25% saturated $(NH_4)_2SO_4$. Determining whether this shift is due to binding of sulfate to the diiron active site or to a conformational change must await more detailed spectroscopic studies.

Published data exhibit a discrepancy in the visible absorption maxima reported for some reduced forms of Uf and bovine spleen PAP (Davis, 1982; Davis et al., 1981). Our results suggest that this discrepancy is due to the presence or absence of reducing agents in the buffers. While low concentrations of ascorbate ($\leq$30 mM) have no effect on the λ_{max} of the

Table IV: Effect of 100 mM Ascorbate on Visible Maxima (nm)

anion	reduced method A[a]	reduced method B[b]
	505–510	536
WO_4^{2-}	490	490
MoO_4^{2-}	490	490
AsO_4^{3-}	480	480
PO_4^{3-}	490	490
F^-	505–510	

[a] 0.05 M acetate and 0.15 M KCl, pH 5.0. [b] 0.05 M acetate and 2.0 M KCl, pH 5.0.

reduced PAP complexes, high concentrations of ascorbate (≥100 mM) have drastic effects (Table IV). Addition of oxyanions to method A or B enzyme in the presence of 100 mM ascorbate results in all cases in blue-shifting of the spectra to λ_{max} less than 500 nm, together with significant increases (>33%) in intensity. The visible band actually becomes a shoulder on an enhanced ultraviolet absorption band that tails into the visible region; presumably an ascorbate–enzyme–anion complex is formed.

Because of the degree of dependence of the position of λ_{max} on the protein conformation, it is difficult to draw any firm conclusions about anion binding or pH dependence solely from visible absorption data. For example, the difference between the reduced method A enzyme at pH 5 and 3 (~15 nm) or between the reduced method A and B enzymes (~25 nm) is greater than the shift observed upon addition of some of the inhibitory anions. Thus, a shift of the visible λ_{max} does not guarantee that an anion is binding to the diiron active site; likewise, the absence of a shift does not necessarily imply the absence of anion binding.

Kinetics Studies. As discussed in the previous section, the reported visible spectra of PAP's from different sources can now be readily explained by carefully examining the histories of the samples and the conditions under which the spectra were obtained. The question arises as to whether this is the case for the reported kinetics of the purple acid phosphatases. For example, molybdate has been reported to inhibit the hydrolysis of *p*-NPP by the hairy cell spleen enzyme in both a competitive fashion (Lam et al., 1977) and a noncompetitive fashion (Ketcham et al., 1985). While some of these discrepancies may arise from plots of kinetics data based on a limited number of data points (because of available quantity of enzyme, etc.), it is possible that some of these conflicting results may also be the product of using different conformations of the enzymes, due to previously unrecognized differences in sample preparation, etc. Consequently, herein are reported the most detailed kinetics studies to date on the inhibition of a PAP by tetrahedral oxyanions and the substrate analogues TYR(P) and AMP. The results are summarized in Table V and compared with previously reported data on bovine spleen PAP and Uf.

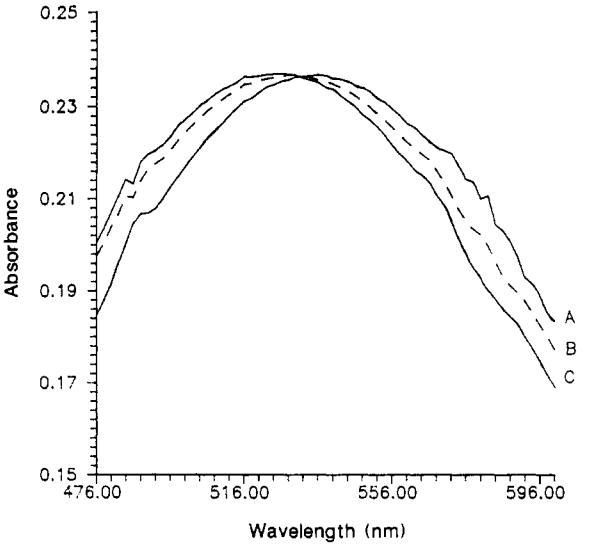


FIGURE 5: Effect of addition of sulfate on visible spectra. (a) Reduced method B enzyme in 0.05 M acetate and 2 M KCl, pH 5.0, (B) enzyme plus 150 mM $(NH_4)_2SO_4$, and (C) enzyme plus 300 mM $(NH_4)_2SO_4$. Spectra corrected for dilution upon addition of $(NH_4)_2SO_4$.

Unfortunately, however, method A and method B enzyme cannot be differentiated by the kinetics data obtained by using the normal assay. (Assays in the presence of 2 M KCl, KBr, or $NaNO_3$ reveal that neither form of the enzyme is inhibited, and in fact both exhibit significantly higher specific activities in the presence of high salt concentrations than in the standard assay. The origin of this effect is currently under investigation.) Although the method B enzyme retains its conformation for at least 1 week in acetate buffer containing 0.15 M KCl, as evidenced by CD spectra, it is conceivable that the enzyme might convert to the less ordered method B form under the assay conditions (10^3 times more dilute than CD conditions). Within experimental error, the K_m and specific activity for the enzymes toward the hydrolysis of *p*-NPP are indistinguishable (K_m = 3 mM, specific activity = 2100 units/mg). Likewise, no statistically significant differences were seen in the results of the inhibition studies discussed below.

On the basis of their mode of inhibition, the inhibitory anions can be divided into three categories that are identical with those based on their behavior in the visible studies. The first group contains the tetrahedral oxyanions, phosphate and arsenate, the substrate analogue TYR(P), and AMP. Lineweaver–Burk plots of the data for each of these inhibitors exhibit lines that intersect at a single point to the left of the $1/V$ axis and above the $1/S$ axis, but in close proximity to the $1/V$ axis.

Table V: Inhibition of Phosphatase Activity of Bovine Spleen PAP and Uf by Selected Anions

anion	source	substrate	mode of action	K_i	pH	ref
PO_4^{3-}	bovine spleen	*p*-NPP	competitive	0.13 mM	6.0	*a*
		p-NPP	intersecting noncompetitive (mixed)	5.4 mM	6.0	this work
	uteroferrin	*p*-NPP	competitive	2.3 mM	4.9	*b*
		p-NPP	competitive	22 mM	5.5	*c*
AsO_4^{3-}	bovine spleen	*p*-NPP	intersecting noncompetitive (mixed)	0.18 mM	6.0	this work
	uteroferrin	*p*-NPP	competitive	2 mM	4.9	*c*
MoO_4^{2-}	bovine spleen	[^{32}P]ATP	competitive	0.37 μM	5.8	*e*
		p-NPP	noncompetitive	1.9 μM	6.0	*d*, this work
	uteroferrin	*p*-NPP	noncompetitive	4 μM	4.9	*f*
		p-NPP	noncompetitive		7.0	*d*
WO_4^{2-}	bovine spleen	*p*-NPP	noncompetitive	10 μM	6.0	this work
	uteroferrin	*p*-NPP	noncompetitive	4 μM	4.9	*f*

[a] Davis et al. (1981). [b] Baumbach et al. (1986). [c] Pyrz et al. (1986). [d] Ketcham et al. (1985). [e] Rezyapkin et al. (1985). [f] Pyrz (1986).

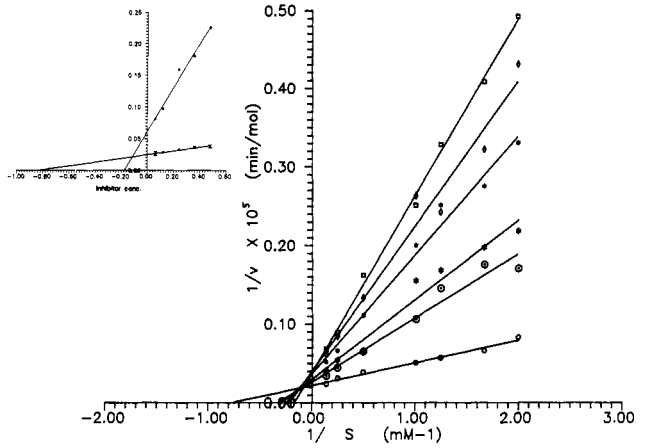
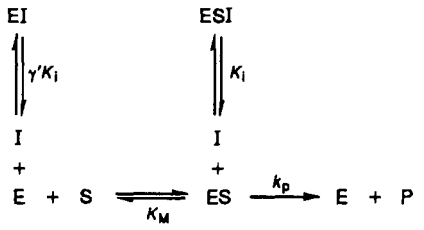


FIGURE 6: Lineweaver–Burk plot of inhibition of *p*-NPP hydrolase activity by arsenate. Inset: Slope (○) and intercept (□) replots. Error bars on the intercept plot show the standard deviation on the intercept values when it is greater than the size of the symbol.

Scheme I: γ' Mechanism



The results for arsenate are shown in Figure 6, and plots for the other inhibitors are available in the supplementary material. On the basis of similar plots or Dixon plots [which can only distinguish between noncompetitive and other forms of inhibition, but not between competitive and mixed inhibition (Segal, 1975)] for PAP's from a variety of sources, these inhibitors have been assumed to act by a competitive mode of action (Table V). If such were the case, replots of the kinetics data (slope or *y*-intercept versus inhibitor concentration) should be linear, and only the slope replot should intercept the inhibitor axis at a point corresponding to K_i. As can be readily seen in the inset to Figure 6, this is not the case; the two lines intercept at a point well removed from the [I] axis, and the intercept replot does not parallel the inhibitor axis. This behavior is characteristic of mixed-type or intersecting noncompetitive inhibition (Segal, 1975).

At least three scenarios can give rise to this type of inhibition: (i) two inhibitor sites, one competitive and the other uncompetitive (Scheme I); (ii) two inhibitor sites, one competitive and the other noncompetitive (Scheme II); or (iii) separate noncompetitive inhibitor and substrate binding sites, where binding of either inhibitor or substrate to its respective site affects the binding of the other. Given the structural similarity between the substrate *p*-NPP and the inhibitor TYR(P) and the fact that the inhibitor phosphate is also the last product of the hydrolysis reaction to dissociate from the active site, the presence of mutually exclusive binding sites (scenario iii) is unlikely. Consequently, only the first two scenarios are plausible. These imply that two binding sites exist on the enzyme for phosphate, arsenate, TYR(P), and AMP: one at the enzyme active site, and a second at a site that could be considerably removed from the active site. As shown in Schemes I and II, the K_i values for the competitive and non- or uncompetitive inhibition are related by a factor γ' or γ, respectively. Phosphate, TYR(P), and AMP have

Scheme II: γ Mechanism

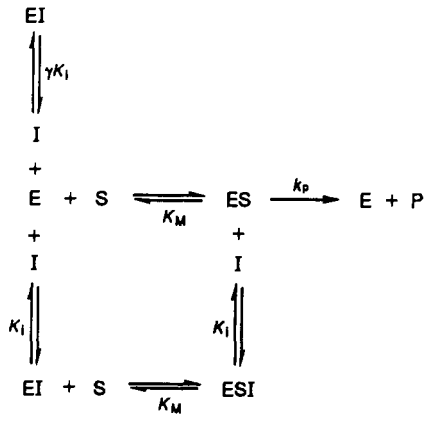


Table VI: Inhibition Constants and γ and γ' Values

inhibitor	K_i (mM)	γ'	γ
PO_4^{3-}	5.4	0.14	0.16
AsO_4^{3-}	0.18	0.20	0.24
MoO_4^{2-}	0.0019		
WO_4^{2-}	0.010		
AMP	4.5	0.14	0.16
TYR(P)	2.2	0.13	0.15
F^-	22		

essentially identical values for γ and γ', while that for arsenate is distinctly larger (Table VI); thus, the larger size of arsenate appears to direct it more to the non- or uncompetitive site. The charge of the anions does not appear to be reflected in the γ and γ' values; despite their differing pK_a values, phosphate, TYR(P), and AMP possess similar γ and γ' values.

Molybdate and tungstate are noncompetitive inhibitors of *p*-NPP hydrolase activity. Lineweaver–Burk plots for these inhibitors are comprised of lines that intercept at a point in close proximity to the 1/[S] axis. Unlike the previous case, replots of the data result in lines that intersect on the [I] axis, indicative of purely noncompetitive inhibition (supplementary material, Figures S-4 and S-5). Thus, *molybdate and tungstate*, presumably because of their size, *bind at a site different from the active site.* This result is most surprisingly, as ENDOR studies of the reduced molybdate complex of Uf have shown that the anion binds to the diiron center (Doi et al., 1988b), while EPR experiments indicate that phosphate also binds to the diiron center (David & Que, 1990). An appreciable pocket(s) must be present at the dinuclear iron active center to allow formation of a ternary substrate–inhibitor–enzyme complex (i.e., $O_3POR{\cdot}MoO_4{\cdot}PAP$). The results presented for molybdate and tungstate are in excellent agreement with earlier studies on Uf, but conflict with those reported for the enzymes from rat spleen, human osteoclast, and bovine bone (Lam et al., 1977; Hayman et al., 1989; Hara et al., 1984). This is most disturbing, because along with tartrate insensitivity, the sensitivity to molybdate inhibition has been used as a diagnostic for this class of acid phosphatases (i.e., PAP's). Another factor suggesting that tungstate and molybdate bind to the PAP's in a different manner than phosphate, etc., is that the former are significantly better inhibitors, with K_i's 2–3 orders of magnitude lower (Table VI).

As the arsenate is directed more toward the noncompetitive or uncompetitive site than the smaller phosphate, and the even larger molybdate and tungstate ions are noncompetitive inhibitors, this suggests that Scheme II (one competitive and one noncompetitive site) is in operation. Two inhibitor binding sites are thus present on the enzyme at or near the active site,

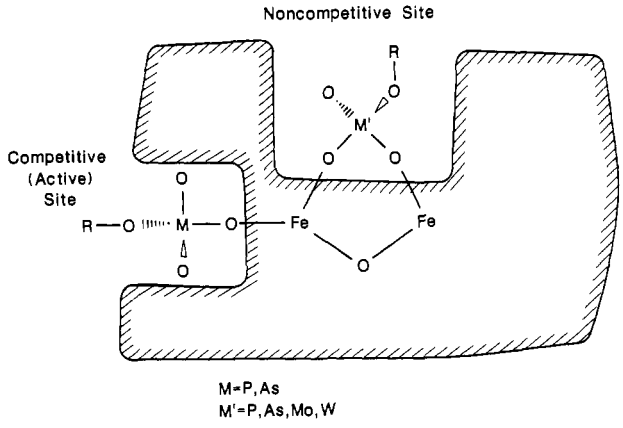


FIGURE 7: Proposed model for tetrahedral oxyanion binding to PAP's.

and their respective occupancy apparently is a function of the size of the MO_4 (M = P, As, Mo, W) moiety.

Phosphate, arsenate, and the substrate analogues AMP and phosphotyrosine bind at a site that results in competitive inhibition of *p*-NPP hydrolysis (the active site). These anions and molybdate and tungstate also bind at a second site removed from the active site (noncompetitive site). As molybdate apparently binds to the iron(s), the noncompetitive binding site must be at the diiron center. Likewise, recent EPR studies with ^{17}O-labeled phosphate (David & Que, 1990) indicate that phosphate also interacts directly with the diiron center. This observation does not, however, necessarily mean that the active site involves the diiron center, because the phosphate ligand observed by EPR could well be bound at the noncompetitive site. Consequently, to date no unequivocal evidence exists that substrate hydrolysis occurs at the diiron center. Removal of iron from the enzyme is accompanied by a corresponding loss of activity (Nuttleman & Roberts, 1990), but other effects due to iron loss such as protein denaturation (vide supra) may be the cause.

On the basis of a series of spectroscopic studies of Uf, Que and co-workers (Pyrz et al., 1986) have previously suggested that two binding sites for phosphate might exist: one that gives rise to the color of the reduced enzyme–phosphate complex (λ_{max} 536 nm, pH 5.5; 561 nm, pH 3.0) and an oxidation effector site that does not affect the active site spectral properties. This fits well with the anion binding scheme described above. Molybdate and tungstate bind in the noncompetitive site, which would correspond to the spectral effector site; consequently, the reduced molybdate and tungstate complexes would not be expected to exhibit enhanced susceptibility toward air oxidation. Doi et al. (1988b) have reported that addition of phosphate to the reduced molybdate complex of Uf (λ_{max} 520 nm) results in no shift in λ_{max} and suggested that this indicates that molybdate "blocks" the effect of phosphate. This, however, was inconsistent with prior kinetics studies of Uf, which indicated that molybdate was a noncompetitive inhibitor and phosphate a competitive inhibitor (Pyrz, 1986; Ketcham et al., 1985). In agreement with the earlier results on Uf, addition of phosphate to the reduced molybdate complex of the high-salt form of the bovine enzyme produced no shift in λ_{max}. It seems more likely that, instead of blocking the effect of phosphate, molybdate prevents only the binding of phosphate to the noncompetitive (spectral effector) site.

Lastly, the unique behavior of fluoride reported previously has been confirmed (Davis et al., 1981). Lineweaver–Burk plots of the kinetics data are curvilinear, suggesting that two forms of enzyme differing greatly in the degree to which they are inhibited by fluoride may be present. While this behavior has also been noted for Uf (Ketchum et al., 1985), fluoride inhibition for enzymes from other sources has been reported to be noncompetitive, (Rezyapkin et al., 1985; Hayman et al., 1989), uncompetitive (Robinson & Glew, 1980), and partially competitive (Lau et al., 1987). In this case, the discrepancies are easily explained, as the curvature of the lines cannot be readily detected unless a wide range of inhibitor and substrate concentrations are examined. [The inhibiting species in this system has been conclusively shown *not* to be AlF_4^-, as has been proposed for other phosphatases (Lunardi et al., 1988) (M. W. Crowder, and B. A. Averill, unpublished results).]

Conclusions. Use of high salt concentrations during the purification of the purple acid phosphatase from bovine spleen results in isolation of a new form of the enzyme that appears to differ from that previously studied in having a much more highly ordered conformation, which is accompanied by significant differences in the optical spectra of various forms of the enzyme. The increased stability of this high-salt form has permitted the spectroscopic properties of anion complexes of the previous inaccessible oxidized (diferric) state of the enzyme to be examined.

Proposed interactions of the oxyanions with the enzyme consistent with the spectral and kinetics data are shown in Figure 7. The anions binding to the noncompetitive site are depicted as bridging the two irons, as previously proposed for Uf (David & Que, 1990); this mode of binding could account for the tighter binding (lower K_i values) of molybdate and tungstate versus arsenate, phosphate, and the substrate analogues. [This may be too simple an explanation, as phosphate, arsenate, and the substrate analogues appear to bind more tightly to the competitive site compared to the second site (i.e., $\gamma K_i < K_i$); however, hydrogen-bonding effects may be important in these cases (David & Que, 1990).] Despite the lack of unequivocal proof, the competitive site has also been placed at the diiron center.

EPR, NMR, and Mössbauer experiments are now in progress to test this binding scheme. [Removal of iron and reconstitution of enzyme activity upon addition of iron can now be accomplished in high yield by using the method B enzyme (Vincent, Crowder, and Averill, unpublished results), allowing [^{57}Fe]iron-enriched samples for Mössbauer spectra to be obtained as has been done for Uf.] While molybdate and tungstate serve as excellent probes of the noncompetitive site, a selective probe of the active site has not yet been identified; efforts to identify one are currently underway.

ACKNOWLEDGMENTS

We acknowledge J. L. Orlando for developing the purification procedure for the native enzyme, which in slightly modified form was used to produce the method B enzyme described herein.

SUPPLEMENTARY MATERIAL AVAILABLE

Figures S-1 to S-5 depicting Lineweaver–Burk plots and slope and intercept replots for inhibition of bovine spleen PAP by phosphate, phosphotyrosine, AMP, molybdate, and tungstate, respectively (6 pages). Ordering information is given on any current masthead page.

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