Direct observation of multiple protonation states in recombinant human purple acid phosphatase

Enrico G. Funhoff, Thyra E. de Jongh, and Bruce A. Averill.
Abstract

Most spectroscopic studies on mammalian purple acid phosphatases (PAPs) have been performed at a single pH, in almost all cases pH 5. Detailed analysis of the $k_{cat}$ versus pH profiles of several mammalian PAPs, however, revealed that their pH optimum is approximately 0.5 to 1 pH unit higher than pH 5 (depending on the form). Moreover, the pH optimum of PAPs isolated as single polypeptides is more than half a pH unit lower than that of proteolytically cleaved PAPs, while the catalytic activity of single polypeptide PAPs is also 4-5-fold lower than that of the cleaved enzymes. Both forms of recombinant human PAP (recHPAP) have been studied over the pH range 4 to 8 using several spectroscopic methods.

The EPR spectra of single polypeptide recHPAP are pH dependent, and show the presence of three species: a low pH form (pH<$pK_{a,1}$), an active form ($pK_{a,1}<$pH<$pK_{a,2}$), and a high pH form (pH>$pK_{a,2}$). The previously observed shift in $pK_{a,1}$ upon proteolysis is also observed in the EPR spectra. The enzyme-phosphate complex (which should mimic the coordination of substrate), the enzyme-fluoride complex, and the enzyme-fluoride-phosphate complex (which should mimic the ternary complex) were also examined. Spectroscopic results showed no evidence for binding of phosphate to the diiron center of the single polypeptide form of the enzyme. Fluoride was unable to substitute for hydroxide in the active protein state but appeared to replace a coordinated water in the low pH form.
Introduction

Purple acid phosphatase (PAP), also known as tartrate-resistant acid phosphatase (EC 3.1.3.2.) or type 5 acid phosphatase, is a member of the αβ-hydrolase family (1-4). It is also related to the family of non-heme diiron enzymes (1, 2), such as methane monoxygenase (3), ribonucleotide reductase (4), and hemerythrin (5).

To date, research has centered on the elucidation of the active site structure of PAP. With the published structures of kidney bean PAP (KBPAP) (6, 7), pig uterine fluids, also called uteroferrin (Uf) (8, 9), and rat bone PAP (recRPAP) (10, 11) the focus of attention has turned to ascertain the physiological function of these enzymes (12). Mammalian PAPs show a broad phosphatase activity towards phosphoproteins (13-15) and are able to perform Fenton type chemistry (15-17). PAPs have been proposed to be involved in the transport of iron in fetal pigs (18, 19), in osteoporosis (20, 21), in the immune response (22-24), and possibly in pathological processes such as Alzheimer's disease (25). Although the cDNA sequence indicates that the mammalian enzymes are translated as single polypeptide proteins (26, 27), purification often yields an enzyme that consists of two fragments with masses of 20 and 16 kDa respectively (13, 28, 29). This form differs from the single polypeptide form in catalytic activity, pH optimum, and characteristic EPR spectrum at pH 5.0 (13, 28), due to differences in the interaction between a loop residue, Asp146, and the active site residues (30).

Despite the detailed knowledge of the structure, the catalytic mechanism of PAPs remains unresolved. Experiments with bovine spleen PAP (BSPAP) and the chiral substrate S₇p-2',3'-methoxymethylidene-ATP-γSr¹⁸O₂¹⁷O showed that hydrolysis resulted in net inversion of configuration at phosphorus (31), ruling out a mechanism with a phosphoenzyme intermediate that has been proposed earlier (32) and supporting a mechanism in which the substrate is directly attacked by water. The mode of coordination of the substrate in the active enzyme is not known, nor is it known which metal bound water/hydroxide acts as the nucleophile. Possibilities include: (1) a terminally bound Fe³⁺ hydroxide; (2) a hydroxide bridging the two Fe³⁺Fe²⁺ ions; (3) a terminally bound Fe²⁺ hydroxide; and (4) a water/hydroxide molecule in the second coordination sphere (33). The absence of burst kinetics for BSPAP at pH 7 has been interpreted in terms of a model in which the hydrolysis of the phosphate ester is the rate limiting step, rather than the release of phosphate (33).

Because it is assumed to mimic the binding mode of the substrate, phosphate has been used extensively as a substrate analogue. Several kinetics (34, 35) and spectroscopic studies at pH 5 (e.g. Mössbauer (34, 36, 37), NMR (38-40), EPR (35), EXAFS (41-43), and CD/MCD (44)) have shown that phosphate is a competitive inhibitor of the enzyme and that it binds in a bidentate fashion to the two metal ions. Merkx et al., however, showed that these studies were performed at a pH that is well below the optimal pH, and proposed that at the pH optimum phosphate binds in a monodentate fashion to the Fe²⁺ site (33). Recent X-ray structures of recRPAP crystallized at pH 7 (11), and λ phage protein phosphatase (APP) (45), in which the active site residues are almost identical to those of PAP (46), with sulfate bound to the binuclear site support this proposal. It should be noted, however, that in both protein structures an inhibiting cation is present (Zn in RPAP and Hg in APP) which may distort the active site structure.
EPR and kinetics studies, using fluoride as a hydroxide analogue and phosphate as a substrate analogue, have shown that FeZn-BSPAP forms a ternary enzyme-phosphate-fluoride complex in which fluoride presumably replaces a water/hydroxide bound to the ferric ion (47). Based on the shift in the Fe\(^{3+}\)-\(\mu\)-OH vibration of FeZn-Uf and FeZn-Uf-AsO\(_4\) in a resonance raman study, however, Que and coworkers proposed that the bridging water/hydroxide acts as the nucleophile. Because this shift was not observed when phosphate was used as oxyanion, the mode of orientation of phosphate, and thus of substrate, remains an open question (48).

In the present study we examined the pH dependence of the kinetics and spectroscopic properties of the single polypeptide and the proteolytically cleaved form of Fe\(^{3+}\)Fe\(^{2+}\)-recHPAP in the absence and presence of the substrate analogue phosphate and the nucleophile analogue fluoride. The results provide new insights into the mode of coordination of these anions to the diiron site of mammalian PAPs under various conditions.

**Experimental procedures**

**General**

Single polypeptide recHPAP was expressed by a baculovirus expression system and the reduced form was purified, as described (28). Reduced proteolytically cleaved protein was obtained by trypsin digestion (28). Enzyme concentrations were determined from the maximal absorbance in the UV-vis spectrum (\(\lambda_{\text{max}}=505-550\); \(\varepsilon=4080\ \text{M}^{-1}\ \text{cm}^{-1}\)) on a Carey 50 spectrophotometer.

**Kinetics**

All assays were performed using the fixed-point assay at 22°C (28). The assay buffer contained 100 mM Na-acetate, 100 mM MES, and 100 mM HEPES. Enzyme dilutions were made in 50 mM MES pH 6.5, containing 2M KCl and 0.5 mg/ml BSA. Values of \(K_i\) were determined by measuring the rate of hydrolysis of \(p\)-NPP, using at least 6 different \(p\)-NPP concentrations with several fixed inhibitor concentrations. The results were fitted to the mixed competitive inhibition equation using the program Leonora (Athel Cornish-Bowden, version 1.0, 1994).

**pH dependence of the EPR spectrum of reduced recHPAP**

Electron paramagnetic resonance spectra were obtained at 4-5 K on a X-band Bruker ECS106 EPR spectrometer equipped with an Oxford Instruments ESR900 helium-flow cryostat with an IT6C temperature controller and a AEG magnetic field calibrator.

To follow a pH titration by EPR, 150 \(\mu\)l of recHPAP was taken for each pH from an enzyme stock solution, and buffer exchanged into a buffer mix (150 mM Na-acetate, 150 mM MES, and 150 mM HEPES, 180 mM KCl, and 20% glycerol) at the appropriate pH by repetitive dilution/concentration. The pH of each sample was measured to ensure correct pH. The enzyme concentration and its \(\lambda_{\text{max}}\) at this pH were determined by UV-vis spectroscopy before the sample was transferred to the EPR tube. The protein was frozen in liquid \(N_2\).
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**pH dependence of the EPR spectrum of the enzyme-phosphate complex**

EPR samples containing reduced recHPAP were thawed and made anaerobic by repetitive vacuum/flushing with argon. From anaerobic stock solutions of phosphate, prepared at the correct pH to avoid changes in sample pH, phosphate was added under anaerobic conditions to a concentration of 50 mM and the samples were immediately frozen in liquid N2 after mixing. After recording the EPR spectrum, the sample was thawed and λ\text{max} and its concentration were determined within a minute by measuring its UV-vis spectrum.

**pH dependence of the EPR spectrum of the enzyme-fluoride and enzyme-phosphate-fluoride complex**

Fresh samples of recHPAP were buffer exchanged to the appropriate pH in a buffer containing 150 mM Na-acetate, 150 mM MES, 150 mM HEPES, 180 mM KCl, and 20% glycerol, and EPR spectra were recorded. After thawing, the samples were made 10 mM in fluoride using stock solutions and the samples were frozen in liquid N2. After recording the EPR spectra, the samples were thawed and made anaerobic, and phosphate was added from an anaerobic stock solution that had been adjusted to the destined pH, to give concentrations of 50 mM. After again recording the EPR spectra, the samples were thawed and UV-vis spectra were recorded within a minute at thawing.

**Results**

**pH dependence of single polypeptide and proteolytically cleaved recHPAP**

Earlier studies on the single polypeptide and proteolytically cleaved forms of PAP showed that upon proteolysis the characteristic EPR spectrum at pH 5.0 changes from a signal with features at g\text{xyz}=1.58, 1.73, 1.94 into a spectrum with features at g\text{xyz}=1.58, 1.73, 1.86. Together with this change, a shift in pK\text{a,1} was observed in the k\text{cat} versus pH optimum. It suggested that at pH 5.0 two different species are present. To gain more insight into the pH dependence we measured the EPR spectrum of the single polypeptide recHPAP over the pH range 4.0 to 8.0. Figure 4.1A shows that as the pH is increased from 4.0 to 8.0, three different species are observed with g\text{xyz}=1.86, 1.73, 1.58; g\text{xyz}=1.94, 1.73, 1.58; and g\text{xyz}=1.97, 1.73, 1.60, respectively. Simulation of the EPR spectra of the three species and summation in varying ratios confirmed the observed EPR spectra (not shown). Plotting the relative intensities of the three species versus pH (Figure 4.7) shows that the intensity of the signal of the low pH species (g\text{z}=1.86) decreases as the pH increases. At pH 5.5, (the optimal pH) one species is present (g\text{z}=1.94), the relative intensity of this species increases as the pH increases from 4.0 to 5.5 and the intensity decreases as the pH increases further. As the signal due to the g\text{z}=1.94 species decreases in intensity, a concomitant increase in the intensity of a species with g\text{z}=1.97 is observed. The g\text{z}=1.97 feature is observable as a shoulder on the g\text{z}=1.94 signal at pH below pK\text{a,2}. A similar pH dependency is observed for other mammalian PAPs, such as recombinant rat PAP (E.G. Funhoff, G. Andersson, B.A. Averill, unpublished results). Even although in kinetics the enzyme-substrate complex is studied and in EPR the native enzyme, the similarity of the pH dependence of the g\text{z}=1.94 species and k\text{cat} (28) suggests that pK\text{a,1} and pK\text{a,2} correlate with the conversion from the
Figure 4.1: EPR spectra of native (—) single polypeptide (A) and proteolytically cleaved (B) recHPAP (B) over the pH range 4-8. Addition of 50 mM phosphate (⋯⋯) to the native form was performed under anaerobic conditions. All spectra were corrected for instrument gain and temperature, and the phosphate spectra were normalized using the spin concentrations of the native samples. EPR conditions: microwave power, 2 mW; microwave frequency, 9.42 GHz; modulation, 12.7 G at 100 KHz; T, 4.5-5 K.

$g_z=1.86$ species into the $g_z=1.94$ species and with the conversion from the $g_z=1.94$ species into the $g_z=1.97$, respectively.

If that is the case, then a similar correlation should be observed for the pH dependence of the EPR spectrum of the proteolytically cleaved protein, which has a $pK_a$ of 5.5 versus 4.6 for the single polypeptide form (28). The EPR spectra of recHPAP that has been cleaved with trypsin are shown over the pH range 4.0-8.0 in Figure 4.1B. Again, three different species are observed with the same $g_{xyz}$ values as for single polypeptide recHPAP. The conversion from the $g_z=1.86$ species to the $g_z=1.94$ species, however, occurs at higher pH. A plot of the relative intensities of all three forms versus pH (Figure 4.7) shows that there is indeed a correlation between the $pK_a$ values observed in kinetics and the conversion from one species into another in the EPR.

The UV-vis spectra of single polypeptide and proteolytically cleaved PAP in the presence and absence of various inhibitors show a broad absorption band around 500-560 nm ($\lambda_{max}$). For single polypeptide PAPs as well as for proteolytically cleaved “low salt” BSPAP, $\lambda_{max}$ is observed around 510-515 nm, while for proteolytically cleaved “high salt” BSPAP a maximum around 536 nm is reported. The pH dependence of $\lambda_{max}$ of both single polypeptide and proteolytically cleaved recHPAP are depicted in Figure 4.2A, which shows that for the proteolytically cleaved enzyme $\lambda_{max}$ shifts to higher wavelengths with decreasing pH (from 513 nm at pH 6.0 to almost 535 nm at pH 4.1). The single polypeptide form, however, does not display such a strong pH dependency with a $\lambda_{max}$
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Figure 4.2: pH dependence of $\lambda_{\text{max}}$ of single polypeptide (o) and proteolytically cleaved (*) recHPAP in their native state (A), in the presence of 50 mM phosphate (B), 10 mM fluoride (C), and 50 mM phosphate and 10 mM fluoride (D).

shift from 514 nm to 518 nm. This observation suggests that in the proteolytically cleaved enzyme a change in nuclear charge of the phenolate bound ferric center occurs, while in single polypeptide PAP this hardly takes place.

Phosphate coordination

As a substrate analogue, phosphate either coordinates to the active site in a bridging mode (as observed by EXAFS (49) and in X-ray crystal structures (8)), or in a monodentate fashion to the ferrous ion (33). To follow the pH dependence of phosphate coordination, 50 mM phosphate was added to the EPR samples at various pH's under anaerobic conditions, and the EPR spectra were recorded. The dotted lines in Figure 4.1A represent the spectra of the enzyme-phosphate complex, and show that the $g_z=1.86$ signal is not present in the phosphate complex of single polypeptide PAP.

Together with the loss of the $g_z=1.86$ signal a decrease in the intensity of the total signal is observed; fitting of the relative spin concentrations of free enzyme and enzyme-phosphate complex to the Henderson-Hasselbalch equation gives an apparent $pK_a$ of 4.5. The relative intensities of the remaining signals do not change (Figure 4.7). This result suggests that phosphate is able to bind to the $g_z=1.86$ species in single polypeptide recHPAP, but not to the active $g_z=1.94$ species. In addition, the signal of $g_z=1.97$ species does not change upon addition of phosphate.

Proteolytically cleaved recHPAP behaves differently as shown in Figure 4.1B. In this case, addition of phosphate results in a loss of intensity
of the other two EPR features in addition to the $g_z = 1.86$ signal. Sixty percent of the intensity of the signal remains at pH 7, and fitting the remaining intensities to the Henderson-Hasselbalch equation results in an apparent $pK_a$ of 6.0. The distribution of the remaining features remains the same as for the proteolytically cleaved enzyme in the absence of phosphate throughout the titration (Figure 4.7). In addition the pH at which the $g_z = 1.94$ signal is converted into the $g_z = 1.97$ species shifts from approximately 6.6 for the native form to 7.3 for the enzyme-phosphate complex.

Addition of phosphate to the reduced single polypeptide recHPAP has little effect on $\lambda_{\text{max}}$ (Figure 4.2B). At pH's below 5, a slight increase in $\lambda_{\text{max}}$ is observed, from 518 to 520 nm. For the proteolytically cleaved enzyme $\lambda_{\text{max}}$ increases from 534 to approximately 550 nm at pH 4. No changes are observed for either the single polypeptide or proteolytically cleaved enzyme as the pH increases above 5-6.

The pH dependence of the inhibition constants of phosphate for both the single polypeptide and proteolytically cleaved forms of recHPAP are depicted in Figure 4.3. For the single polypeptide protein, $K_i$ increases at higher pH, while for the proteolytically cleaved form no change in $K_i$ is observed as a function of pH. At pH below 5, a $K_i$ of 3 mM is found, and phosphate is a competitive inhibitor, no uncompetitive element could be found for either single polypeptide or proteolytically cleaved recHPAP when fitting the data to a mixed competitive inhibition equation. At higher pH, $K_i$ increases to approximately 12 mM for the single polypeptide protein at pH 6.5. Plotting $\log K_i$ versus pH shows no evidence that deprotonation of an active site residue is coupled to phosphate binding in this pH range.

**Fluoride coordination**

In principle, fluoride is able to function as an analogue of the hydroxide ion. Fluoride could, therefore, replace three different groups at the diiron site in PAP; a putative terminal ferric hydroxide, a bridging hydroxide, and/or a water/hydroxide bound to the ferrous site. To study the pH dependence of binding of fluoride in EPR, 10 mM fluoride was added to single polypeptide and proteolytically cleaved recHPAP at five different pH values. As shown in Figure 4.4, a very broad EPR spectrum is observed at pH < $pK_{a,1}$. Above pH 5.1 for single polypeptide and pH 6.1 for proteolytically cleaved recHPAP,
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Figure 4.4: EPR spectra of native (—) single polypeptide (A) and proteolytically cleaved recHPAP (B) over the pH range 4-8. 10 mM fluoride (⋯⋯) was added to the native forms. All spectra were corrected for instrument gain and temperature, and the fluoride spectra were normalized using the spin concentrations of the native samples. EPR conditions: microwave power, 2 mW; microwave frequency, 9.42 GHz; modulation, 12.7 G at 100 KHz; T, 4.5-5 K.

Figure 4.5: EPR spectra of single polypeptide (A) and proteolytically cleaved recHPAP (B) over the pH range 4-8 in the absence (—) and presence (⋯⋯) of 10 mM fluoride and 50 mM phosphate. All spectra were corrected for instrument gain and temperature, and the enzyme-fluoride-phosphate complex spectra were normalized using the spin concentrations of the native samples. EPR conditions: microwave power, 2 mW; microwave frequency, 9.42 GHz; modulation, 12.7 G at 100 KHz; T, 4.5-5 K.
addition of 10 mM fluoride causes no significant changes in the EPR spectra, suggesting that fluoride does not bind to the binuclear center of either form. As with phosphate, the pH at which the added ligand perturbs the EPR spectrum of both forms is comparable to $\text{pK}_{a,1}$. This result strongly suggests that fluoride can replace a coordinated water molecule, but not a bound hydroxide ion, and it supports the assignment of $\text{pK}_{a,1}$ to the deprotonation of a coordinated water molecule. Thus, proton transfer events at the active site can be monitored by EPR spectroscopy. In contrast, the pH at which the $g_z=1.94$ feature is converted into the $g_z=1.97$ signal, which correlates with $\text{pK}_{a,2}$ in the native enzyme, is apparently affected by the presence of fluoride, and shifts from approximately 7 to 7.5.

With visible spectroscopy, a shift of $\lambda_{\text{max}}$ to higher wavelength is observed upon addition of fluoride at pH $< 5$ for single polypeptide PAP and at pH $< 6.5$ for proteolytically cleaved. The data for the $\lambda_{\text{max}}$ versus pH graphs of single polypeptide and proteolytically cleaved enzyme-fluoride complex, shown in Figure 4.2C, were obtained by titration of a pH 7.4 sample with HCl, rather than by measuring the visible spectra of the EPR samples. The shift to higher wavelength at low pH is further supported by fluoride titrations of single polypeptide recHPAP at pH 7.0 and proteolytically cleaved recHPAP at pH 4.0. At pH 7.0 no shift in $\lambda_{\text{max}}$ is observed (i.e. $K_d \approx \infty$), while at pH 4.0 a $K_d$ of approximately 0.4 mM was found (not shown).

Kinetic experiments with fluoride as inhibitor show a pH dependent uncompetitive inhibition pattern (Figure 4.6). Values of $K$ are $\sim 0.2$ mM at pH 4-4.5 for both forms, comparable to the $K_d$ found in the previously mentioned fluoride titrations in EPR at this pH, and 7 mM for single polypeptide and 2 mM for proteolytically cleaved recHPAP at pH 6.5. Plots of the logarithm of $K$ versus pH show that fluoride binding to the proteolytically cleaved enzyme is independent of pH between pH 4.0 and 5.4, while a slope of approximately 1 is found above pH 5.4. Although the break in the plot is not as obvious for the single polypeptide enzyme, the data are consistent with a $\text{pK}_a$ of $\sim 4-4.5$. At higher pH the maximum inhibition of fluoride decreases, resulting in only 40 % inhibition at pH 7.3 for single polypeptide and 20 % inhibition for proteolytically cleaved recHPAP, as observed in a plot of fluoride concentration versus relative activity at 50 mM $p$-NPP (not shown). When residual activity was plotted versus pH and fitted to the Henderson-Hasselbalch equation, a $\text{pK}_a$ of 6.4 was fitted for single polypeptide recHPAP and 6.9 for proteolytically cleaved recHPAP respectively.

The enzyme-phosphate-fluoride complex

Figure 4.5 shows the EPR spectra of the enzyme-phosphate-fluoride complex of single polypeptide and proteolytically cleaved FeFe-recHPAP. Anaerobic addition of either phosphate to the enzyme-fluoride complex or fluoride to the enzyme-phosphate complex results in disappearance of the EPR signal due to the formation of an EPR silent species (or a species with a very broad signal under the conditions examined). For the single polypeptide enzyme, the spectrum above pH 6 is almost identical to that of uncomplexed enzyme, i.e., a mixture of the $g_z=1.94$ and 1.97 species. With proteolytically cleaved PAP, the signal due to the uncomplexed enzyme is observed above pH 7. The pH at which the 1.94 species is converted into the 1.97 species is higher, as in the enzyme-fluoride complex. The loss of intensity is not due to oxidation of the sample, as shown by
separate addition of fluoride or phosphate. The change in the EPR spectrum is thus due to the combined effects of phosphate and fluoride.

In the visible spectra a shift in \( \lambda_{\text{max}} \) to higher energy is observed for the enzyme-phosphate-fluoride complex at pH < 5; this shift is most pronounced for proteolytically cleaved recHPAP as depicted in Figure 4.2D.

### Discussion

Previous studies of the pH dependence of the EPR spectrum of PAP were performed on proteolytically cleaved “low salt” FeFe-BSPA P over the pH range 3.1 - 6.1. A change in the EPR spectrum from \( g_{xyz} = 1.65, 1.77, 1.95 \) at pH 3.6 to a species with \( g_{xyz} = 1.59, 1.74, 1.86 \) at pH 6.1, was observed with an apparent pK\(_a\) of 4.5, which was attributed to the deprotonation of a metal bound water (50, 51). Addition of phosphate gave a signal with \( g_{xyz} = 1.49, 1.74, 1.91 \) at pH 3.6 and \( g_{xyz} = 1.45, 1.74, 1.85 \) at pH 6.1, whose intensity increased with increasing pH (50). With the “high salt” BSPAP-phosphate complex, a single species was observed in EPR between pH 4.5 and 7. The intensity of the signal increased with increasing pH with an apparent pK\(_a\) of 6 (33), substantially higher than the pK\(_a\) of 4.5 reported for the “low salt” form. This pK\(_a\) was, however, identical to that observed in subsequent kinetics studies (33). In recHPAP the pK\(_a\) observed by kinetics was shown to be affected by proteolysis, suggesting that it is due to deprotonation of a residue bound to the ferrous ion (28, 30). To gain more insight into the differences in kinetics and spectroscopic characteristics of single polypeptide and proteolytically cleaved PAP, we have examined the pH dependence of both forms using kinetics, EPR spectroscopy, and visible spectroscopy over a pH range that includes both kinetics pK\(_a\) values.

The three different species observed previously by EPR for PAPs purified from different sources (52-54) have been observed in a single system and shown to be due to three different protonation states of the enzyme (E, EH\(^+\), and EH\(_2\)^{2+}\). The pH dependence of the EPR spectra correlates well with the pK\(_a\) values obtained by kinetics studies (28) (Figure 4.7 and the table therein), even though the EPR spectra are due to the native enzyme while kinetics studies give pK\(_a\) values for the enzyme-substrate complex.
Figure 4.7: relative intensities of the EPR signals of single polypeptide (left panel) and proteolytically cleaved (right panel) recHPAP. A: $k_{cat}$ versus pH of recHPAP, from (28); B: relative intensity of the $g_z$=1.86 signal of the native enzyme; C: relative intensity of the $g_z$=1.94 signal in the absence (closed marks) and presence (open marks) of 50 mM PO$_4$; D: relative intensity of the $g_z$=1.97 signal in the absence (closed marks) and presence (open marks) of 50 mM PO$_4$. All relative intensities were normalized between 0 and 1. In the table the apparent $pK_a$ values, derived from kinetics and spectroscopic measurements, for single polypeptide and proteolytically cleaved recHPAP are given.

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Addition of substrate, therefore, appears to result in only minor changes in the pKₐ values of catalytically relevant residues. The proton transfer event that is associated with pKₐ,1 also produces a shift in ₐmax, which is more pronounced for proteolytically cleaved PAP. pKₐ,2 is also observed in the EPR spectrum, as shown by the change in gₓ from 1.94 to 1.97. No change in ₐmax is observed, however, which suggests that the residue responsible does not affect the ferric ion. The gₓ=1.97 signal is present even at lower pH and increases in intensity above pKₐ,2, suggesting that protonation/deprotonation of the residue that is responsible for pKₐ,2 results in a conformational change affecting the diron site. Studies on PPs suggest that deprotonation of a conserved histidine, His92 in recHPAP, is responsible for pKₐ,2 (55), and mutagenesis experiments indicated that this residue might be involved in base catalysis (56). However, neither isotope effect studies nor the expected loss of the basic limb of the pH profile of mutants have been reported to support this proposal (57-59). Another option is that His92 is involved in regeneration of the metal-bound nucleophile (55, 60).

Coordination of the substrate analogue, phosphate, to PAP has been intensively studied. EXAFS (43, 61) and EPR (48, 50) studies have suggested a bridging coordination mode for phosphate, while the second class of oxyanions represented by tungstate and molybdate are bound to the ferric ion in a primarily monodentate fashion (61). In the crystal structures of KBPAP, utofermin, and rat bone PAP complexed with phosphate (8, 10, 62), the phosphate bridges the two metal ions in a 1,3-mode. With tungstate a distorted bridging coordination is observed in KBPAP (62), with a slightly stronger binding to the ferric site, as has been observed in the closely related protein phosphatases (63). Merkx et al. have argued that these studies were performed at non-optimal pH values, and that at the optimal pH of these enzymes phosphate binds to the ferrous site in a monodentate fashion, as shown by EPR spectra of the FeZn-form of “high salt” BSPAP (33, 47). Addition of phosphate to recHPAP at different pH’s showed that the gₓ=1.86 species binds phosphate to give a species that is effectively EPR silent (35, 64). In principle, phosphate can coordinate either in a bridging mode, replacing the presumed water/hydroxide molecules at the ferric and ferrous ions, or to the ferrous ion via replacement of the water by one phosphate oxygen atom. Because ENDOR experiments on FeZn-Uf have shown no evidence for the presence of a water molecule at the ferric site (65), monodentate coordination of phosphate to the ferrous ion seems more likely. In the 2.2 Å resolution recRPAP structure (10) and in the native PP1 (63) and PP2B structures (66), however, water molecules are found at the ferric site. Moreover, the absence of the tyrosine ligand in the PP structures could provide the space necessary for the coordination of water, and in the 2.2 Å resolution recRPAP structure, the presence of an inhibitory Zn ion could alter the active site structure. Thus, the presence and location of solvent molecules in and near the active site of PAPs remains unclear.

Phosphate does not appear to bind to the binuclear metal center in the active (gₓ=1.94) species of single polypeptide recHPAP, as shown by the absence of any change in the EPR and optical spectrum upon addition of phosphate. The pH dependence of Kᵣ did not reveal a pKₐ for a residue involved in the binding of phosphate, as found previously for Uf (34), the log Kᵣ versus pH plot has an apparent pKₐ < 4. Aquino et al. (67) have studied the kinetics of phosphate binding to Uf. The reported rate constant varied from 6.8 s⁻¹ at pH 2.7 to 0.42 s⁻¹ at pH 6, indicative of slow exchange of phosphate with the active site. Thus, the present results suggest that phosphate does not bind to the diron
Chapte rr  4

site of single polypeptide recHPAP at the optimal pH (pH 5.5). Rapid freeze quench experiments with proteolytically cleaved BSPAP at pH 6.5 indicated much higher phosphate exchange values ($k_{obs}>200$ s$^{-1}$). At the pH optimum, the EPR spectrum of proteolytically cleaved recHPAP loses intensity due to partial formation of an enzyme-phosphate complex, but the absence of changes in $\lambda_{max}$ suggests that phosphate does not coordinate to the ferric ion. At pH < $pK_{a,1}$, however, visible spectra clearly show that phosphate binds to the ferric site of proteolytically cleaved recHPAP, as found for the single polypeptide form. Thus, at the optimal pH for activity, phosphate apparently binds to the ferrous site of proteolytically cleaved recHPAP. Phosphate could replace a water coordinated to the ferrous ion and mimic a non-bridging substrate molecule, as proposed by Merkx et al. (33). The significant difference in reactivity with phosphate observed for the single polypeptide and proteolytically cleaved forms of the enzyme might be related to the difference in turnover numbers for the two forms of mammalian PAPs.

On the other hand, "high salt" cleaved BSPAP exhibits very different behavior (33), in that both the EPR and the visible spectra are independent of pH. We observe the same $\lambda_{max}$ (536 nm) at the optimal pH for recHPAP, but only if the enzyme is purified as a proteolytically cleaved protein under "high salt" conditions (E.G. Funhoff, B.A. Averill, unpublished results). In rapid freeze quench experiments using EPR spectroscopy to examine the interaction with phosphate or p-NPP at pH 6.5, spectra exhibiting a mixture of features of "high salt" BSPAP and the $g_z=1.94/1.96$ species were observed (33). These results imply that several species can be formed during the catalytic cycle: the $g_z=1.94$ species, to which phosphate does not bind, and the uncomplexed high salt form, which could be an activated or "resting" state of PAP.

To further explore the difference between single polypeptide and proteolytically cleaved recHPAP, kinetics and spectroscopic studies were performed in the presence of fluoride. Due to its ability to replace a nucleophilic water/hydroxide, fluoride inhibits a number of binuclear metalloenzymes, including urease (68, 69), pyrophosphatase (70-73), and aminopeptidase (74, 75). In contrast to previous results on human PAP (14), inhibition of both forms of recHPAP by fluoride was found to be uncompetitive over the pH range studied. Fluoride has been reported to be either an uncompetitive (76) or a non-competitive inhibitor (77) of other PAPs. In the FeZn form of "high salt" BSPAP, only uncompetitive inhibition was observed below $pK_{a,1}$ (47, 48) and pH independent competitive inhibition was observed above $pK_{a,1}$. The uncompetitive inhibition observed for fluoride in this work below $pK_{a,1}$, together with the loss of the EPR signal for both single polypeptide and proteolytically cleaved recHPAP and the small effects on the absorbance maximum, suggest that fluoride replaces the coordinated water that is responsible for $pK_{a,1}$, which $pK_a$ is apparently affected by changes at the divalent site (30), and whose deprotonation gives the nucleophilic hydroxide. Earlier fluoride titrations of Uf at pH 4.9 monitored by EPR showed that fluoride does not bind at concentrations below $10\times K_a$ (as is found for recHPAP). At higher fluoride concentrations ($>50\times K_a$), a broad isotropic EPR spectrum was observed (65), which resembles the broad spectrum observed at low pH for proteolytically cleaved recHPAP. Thus, fluoride can replace a coordinated water molecule, but not a coordinated hydroxide ion. The same study suggested the presence of a second fluoride binding site, which might explain the loss of fluoride inhibition above pH $\approx 6.5-7$ observed in this study. Because no differences are observed between the visible and EPR spectra of the native enzyme and its fluoride
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complex, the second fluoride does not appear to bind to the binuclear site. Thus, fluoride behaves similarly with both the single polypeptide and proteolytically cleaved forms of recHPAP. Below pKₐ₁, it binds to the metal site, but at a pH where the enzyme is catalytically active it does not.

Although neither phosphate nor fluoride appear to bind to the single polypeptide enzyme form at its optimal pH, addition of both phosphate and fluoride produces a ternary enzyme-fluoride-phosphate complex, which should mimic the ternary enzyme-hydroxide-substrate complex. At its optimal pH, the EPR signal of proteolytically cleaved recHPAP is abolished by addition of both fluoride and phosphate, consistent with formation of a ternary complex even though λₘₐₓ does not change. In "high salt" FeZn-BSPAP and FeZn-Uf, formation of a ternary enzyme-fluoride-phosphate complex has been observed by EPR (47, 48). UV-visible spectroscopy, however, argued against this, because the λₘₐₓ of FeZn-BSPAP-F and FeZn-BSPAP-F-PO₄ are identical at pH 5 and 6.5, even though phosphate is proposed to bind to the ferric site at pH 5 but not at pH 6.5.

Distinguishing between the various mechanistic possibilities based on these results is difficult. Candidates for the nucleophile that attacks the phosphate ester substrate include the following: (1) a hydroxide terminally bound to the ferric ion; (2) a hydroxide terminally bound to the ferrous ion; (3) a hydroxide bridging the two metal ions; and (4) a water in the second coordination sphere of the ferric ion. Arguments for a ferric hydroxide as nucleophile include the fact that fluoride is a 50-100-fold better inhibitor of the Al₂Zn form of BSPAP than of the FeZn or GaZn forms (78, 79), suggesting that it (and, by inference, hydroxide) interacts with the trivalent metal. Similarly, visible and EPR spectra (47) show that fluoride binding results in changes in the spectrum of the high spin ferric ion. The following evidence, however, argues against a terminal ferric hydroxide as the nucleophile: (i) kₐₙ and pKₐ do not change upon replacing the ferric ion with other metal ions (79); (ii) the EPR spectrum of FeZn-recRPAP at pH 5 does not change upon proteolysis, in contrast to the results observed for the FeFe form (28, 30); and (iii) ENDOR experiments give no evidence for coordination of a solvent molecule to the ferric ion (65).

The correlation between fluoride inhibition and the loss of the EPR signal of the enzyme-fluoride complex below pKₐ₁, together with the sensitivity of pKₐ₁ to perturbations at the divalent site (30), suggest that the nucleophile is the bridging hydroxide. Protonation of a bridging hydroxide, however, should decrease the exchange coupling constant J substantially, resulting in much greater changes in the EPR spectrum than are observed. For example, binuclear complexes containing a Ni(II)-OH-Ni(II) or Mn(II)-OH-Mn(II) unit have J values of -4.5 cm⁻¹ and -9 cm⁻¹, respectively, which are decreased to < -2 cm⁻¹ and -1.7 cm⁻¹, respectively, upon protonation (80). The lack of major changes in the value of J (80) and in the EPR spectrum (50) of cleaved BSPAP over the pH range 3.7 to 5.6 argue against the bridging hydroxide as nucleophile.

This leaves the terminally ferrous ion bound hydroxide and a water molecule in the second coordination sphere as the remaining candidates for the identity of the nucleophilic hydroxide. Unfortunately, several pieces of data also argue against these options. For example, a shift in pKₐ of almost 6 pH units (from 10.2 in the hexaaquiferrous ion to 4.5 in the enzyme) is hard to reconcile with the pKₐ of a water bound to a divalent metal ion. The large red shift in λₘₐₓ observed for proteolytically cleaved recHPAP at pH values below pKₐ₁ suggests that protonation results in an
increased positive charge on the ferric ion, which is also difficult to correlate with the protonation of a terminal hydroxide bound to the ferrous ion. However, the large red shift in $\lambda_{\text{max}}$ upon oxidation and the increase in $\lambda_{\text{max}}$ to 525-530 nm for FeZn-BSPAP (81) show that changes at the divalent site do affect the nuclear charge of the trivalent site. Thus, it is clear that further research using a variety of experimental approaches is necessary to resolve the problem of the identity of the nucleophilic hydroxide.

In conclusion, we have shown that three different protonation states of recHPAP can be observed in EPR, and that the pK$_a$ values for interconversion of these species correlate with the pK$_a$ values found from kinetics studies. At the optimal pH for activity, phosphate is apparently unable to bind directly to the diiron site in single polypeptide recHPAP, while it does bind to the ferrous site in proteolytically cleaved PAP. Kinetics studies and visible and EPR spectra show that fluoride, a hydroxide analogue, can replace the coordinated water molecule, but not the nucleophilic hydroxide derived therefrom.

References


References continued