Structure-function studies of mammalian purple acid phosphatases
Funhoff, E.G.
Substrate positioning by His92 is an important factor in purple acid phosphatase catalysis

Enrico G. Funhoff, Yunling Wang, Goran Andersson, and Bruce A. Averill.
Abstract

Proteolysis of single polypeptide mammalian PAPs results in the loss of an interaction between the loop residue Asp146 and the active site residues Asn91 and/or His92. Although His92 is known to contribute to the catalytic mechanism, its specific role is unknown. Site directed mutagenesis of Asn91 and His92 was performed to examine the roles of these residues. Conversion of His92 into either Ala, which eliminates the interaction of this residue with the active site, or Asn, which cannot function as a proton donor but can provide hydrogen bonding interactions, resulted in enzymes with a more acidic pH optimum and a pKₐ,1 that can only be attributed to an Fe³⁺-hydroxide unit. The EPR spectrum of the His92Ala mutant resembled that of wild-type mammalian PAPs above pKₐ,2. The Kₘ for the His92Asn mutant increased 15-30-fold and kₐₐₚ increased 3-fold. Together these observations support a role of His92 in either nucleophile or substrate positioning rather than in acid or base catalysis. The effect of increasing the negative charge on the divalent site was explored by mutating Asn91 into Asp; the observed kinetics parameters of the mutant indicate that the ferrous ion contributes to the value of pKₐ,1. The presence of a hydrogen bonding network between both metal ions, Asn91, His92, the bridging hydroxide, and a solvent molecule coordinated terminally to Fe³⁺ could account for these observations.
Introduction

The binuclear metalloenzyme purple acid phosphatase (PAP), which may be involved in disorders such as osteoporosis (1, 2), Gaucher disease (3), hairy cell leukemia (4), AIDS (5), and Alzheimer's disease (6), is widely distributed in mammalian tissues (7). In these disorders the expression level of PAP (also referred to as tartrate resistant acid phosphatase (TRAP) or type 5 acid phosphatase (AcP5; EC 3.1.3.2)) is elevated, suggesting a relationship between the increased levels of the enzyme and the clinical picture. The presumed role of PAP makes it important to develop drugs devoted to the inhibition of PAP activity. In order to facilitate this process, the function of each residue involved in catalysis must be known in order to elucidate the precise catalytic mechanism.

Although the available crystal structures of PAPs (8-11) provide structural information about the residues involved, to date only structures of inactive redox and protonation states of the enzymes have been determined. All structures show an active site composed of two metal ions bridged by a solvent molecule and an aspartate residue. In addition, the ferric ion is coordinated by a tyrosinate (giving a LMCT that is responsible for the purple color), a histidine, and an aspartate residue. The site that is ferrous in the active enzyme is coordinated by two histidine residues and an asparagine. The ligating residues are conserved in several other phosphatases (12), including the closely related protein phosphatases (PPs). As shown by several crystal structures (13-17), however, the PPs lack the tyrosinate coordinated to the ferric ion. Because of the similarity of their active sites, the catalytic mechanisms of PAPs and PPs are believed to be similar (18). No systematic studies that involve the metal coordinating residues have yet been performed in PAPs, making it difficult to interpret the functions of the individual residues. Several site-directed mutagenesis studies have been performed with PPs, however, which makes them potentially valuable for the understanding of the PAP mechanism.

Both the PAPs and the PPs contain a highly conserved histidine residue, His92 (numbering of the residues according to the human PAP sequence (19) will be used throughout the paper, although the numbering differs in PPs). His92 is one of three residues proposed to be responsible for substrate binding, and it also appears to be involved in the catalytic cycle. The other two possible substrate-coordinating residues (based on X-ray structures) are Asn91, which is coordinated to the divalent metal site, and His195. Mutagenesis of His195 to alanine in KBPAP resulted in a sharp decrease in activity (20), while in PPs an Arg residue is found in place of His195. In PPs and KBPAP, His92 is part of a histidine/aspartate pair, which with a solvent molecule could be analogous to the catalytic aspartate/histidine/serine triad of serine proteases (21). A number of mutagenesis studies on this residue in PPs have been published. Initially, the loss of catalytic activity of the His92Ala and His92Ser mutants was interpreted as due to either improper folding and decreased stability of the protein or complete loss of catalytic activity (22). Subsequent studies by Rusnak and others revealed that at pH 7.8 the catalytic activity of mutants of His92 was 150-1000 fold reduced, without a significant change in $K_M$ (21, 23, 24). These findings suggested that His92 was not involved in substrate binding, but only in catalysis. His92 could therefore function as: (1) an active site nucleophile; (2) a general acid catalyst involved in leaving group protonation; or (3) a general base that deprotonates a solvent molecule coordinated to iron (21).
direct transfer of the substrate to water is observed and $k_{\text{cat}}$ is independent of the $pK_a$ of the leaving group, options (1) and (2) are highly unlikely. Isotope effect studies, however, showed no clear evidence for option (3). Moreover, the Asn mutant showed a basic limb in the pH optimum that should not have been present (23). Thus, the precise role of His92 is still unclear. In other zinc and manganese enzymes (e.g. arginase and carbonic anhydrase), a similar histidine is present, which has been proposed to act as a shuttle that switches between an “in” and “out” conformation to regenerate the metal-bound hydroxide (25). In principle, a similar role for this residue is possible in PAP.

Examination of the structures of PAP from pig uterine fluids (Uf) and recombinant rat bone PAP (recRPAP) reveals that sufficient space is available to rotate the His92 side chain. Another possible role is the positioning of the nucleophilic hydroxide or substrate for optimal in-line attack (18), a phenomenon known as orbital steering (26).

Studies on single polypeptide and proteolytically cleaved recHPAP suggested that His92 could interact with a residue, Asp146, present in a loop in the vicinity of the active site. Asp146 could also hydrogen bond to Asn91, a ligand to the ferrous ion (27).

Mutagenesis (28) and studies of the pH dependence (29) showed that the interaction between Asp146 and Asn91 is primarily responsible for the observed differences between single polypeptide and proteolytically cleaved mammalian PAPs. As mentioned earlier, Asn91 could also be involved in substrate binding. In the crystal structures of mammalian PAPs with bound phosphate (8, 10), the distance between the asparagine amido hydrogen and the phosphate oxygen is 1.75 Å. Mutation of Asn91 in PPs to aspartate or alanine reduced $k_{\text{cat}}$ significantly, while $K_M$ was hardly affected (22, 30-32). These findings argue against a role in coordinating substrate. The pH optimum of the Asn91Asp mutant was shifted a full pH unit down, while the Asn91Ala mutant lost the basic limb of its pH profile (22). The observed increase in activity of the N91A mutant at higher pH was attributed to increasing solvent hydroxide concentration generated by the solvent, rather than to the action of the metal ions.

To study the interaction between Asp146 and the residues Asn91 and His92, we prepared mutants of the two latter residues and characterized their kinetics and spectroscopic properties. The kinetics parameters of the Asn91Asp mutant are consistent with involvement of the divalent site in catalysis. The characteristics of the His92Asn and His92Ala mutants suggest an important role for this residue in positioning of the substrate or the nucleophile. They do not, however, support the proposal that His92 is involved in the catalytic process either as a proton donor for the leaving group or as a base that regenerates the nucleophilic hydroxide.

**Experimental procedures**

**General procedures**

Enzyme concentrations were determined by measuring the absorbance of the Tyr$^*$ to-Fe$^{3+}$ charge transfer at $A_{\text{max}}$ (510-550 nm; $\varepsilon$=4080 M$^{-1}$cm$^{-1}$) on a Cary 50 or HP8452A photodiode array spectrophotometer.
Generation of mutant proteins

RecRPAP mutant enzymes were prepared as previously described with the QuickChange™ Site-Directed Mutagenesis Kit (Stratgene, USA) (28). Primers used for specific mutations were as follows:

N91A, 5’- GTACGTGCTGGCTGGAGCCCATGATCACCTTGGC-3’;
N91D, 5’- GATCGTGCTGGCTGGAGACCATGATCACCTTGGC-3’;
H92A, 5’- CTGGCTGGAAACGCTGATCACCTTGGC-3’;
H92N, 5’- GGCTGGAAACAATGATCACCTTGGC-3’.

The underlined bases indicate changes compared to the wild-type sequence.

Recombinant baculovirus stocks containing regions coding for the N91D, N91A, H92N, and H92A mutants were used to infect High 5 cells cultured in 500-1000 ml Excel 405™ SFM at 27°C; the cell density was 0.7-0.9x10^6 cells/ml, and a low MOI (0.001-0.01) was used. After 5 days the cells were removed by centrifugation (10,000xg), and the enzyme was purified from the medium as previously described (28).

Kinetics measurements

The pH dependence of H92N and H92A was measured in 100 mM buffer (Na-acetate, MES and HEPES), 300 mM KCl, 10 mM Na-K tartrate, 6.7 mM Na-ascorbate, 0.37 mM Fe(NH₄)₂(SO₄)₂, and substrate concentrations between 1 and 100 mM p-NPP as previously described (28). After each assay, the pH of the reaction mixture was measured to ensure that it had not changed. Values of $K_M$ and $V_{max}$ were obtained by fitting the data to the Michaelis-Menten equation using the program Leonora (Athel-Cornish Bowden, version 1, 1994).

The pH optimum of the N91D mutant was measured at a fixed substrate concentration (50 mM p-NPP) in the presence of 10 mM ascorbic acid and 1 mM Fe(NH₄)₂(SO₄)₂. All other kinetics measurements of this mutant were performed in the presence of this higher amount of reducing agent.

Electron paramagnetic resonance (EPR) spectroscopy

Samples were prepared in a buffer of the appropriate pH (100 mM Na-acetate, MES, HEPES; 0.45 M KCl, 20 % glycerol (v/v)), and frozen in liquid N₂. X-band EPR spectra (9.43 GHz) were obtained on a Bruker ECS106 EPR spectrometer equipped with an Oxford Instruments ESR900 helium-flow cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG Magnetic Field Meter, and the frequency was measured with a HP 5350B Microwave Frequency Counter. After recording the spectrum, the sample was thawed and buffer exchanged into a buffer with the appropriate pH by repetitive concentration/dilution using a Microcon (30 kDa cut-off). The N91D mutant enzyme was prepared under an N₂ atmosphere in a degassed buffer mix. After recording the spectrum, the frozen sample was made anaerobic by repetitive vacuum/argon flushing and thawed in an N₂ tent for buffer exchange. Complete buffer exchange during the complete EPR experiment took several hours.
Chapter 7

Results

Production of single polypeptide mutant recRPAP (N91D, N91A, H92N, and H92A) in 1 l shaking flask cultures resulted in good yields of the N91D, H92N, and H92A mutant enzymes. In contrast, the amount of N91A-recRPAP in the culture broth after 6 days post infection was very low. Purification of the mutant enzymes gave protein samples with $A_{\text{max}}/A_{280}$ values of 17-21, and a single band with small impurities (~5%) was observed.

![Figure 7.1: $V_{\text{cat}}$ versus pH for single polypeptide N91D-recRPAP at 50 mM p-NPP and 22°C. The line represents fits of the data to the rapid equilibrium diprotic model. The kinetics data obtained from fits of $V_{\text{cat}}$ versus pH are presented in the Table.](image)

<table>
<thead>
<tr>
<th></th>
<th>$p_{\text{H,optim}}$</th>
<th>$pK_{a1}$</th>
<th>$pK_{a2}$</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_{M}$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>RecRPAP$^{(1)}$</td>
<td>5.5</td>
<td>4.5</td>
<td>6.6</td>
<td>240</td>
<td>4.5</td>
</tr>
<tr>
<td>N91D$^{(2)}$</td>
<td>5.4 (0.1)</td>
<td>5.1 (0.2)</td>
<td>5.7 (0.2)</td>
<td>7.5 (0.2)</td>
<td>6.4 (0.9)</td>
</tr>
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$^{(1)}$ Obtained from a fit of $V_{\text{cat}}$ (50 mM p-NPP) as a function of pH.  
$^{(2)}$ from (28)

for each mutant in SDS-PAGE gels stained with Coomassie brilliant blue. The amounts of N91A mutant enzyme obtained were insufficient for kinetics or spectroscopic studies, due to further loss of the enzyme during purification. The N91D mutant was difficult to obtain in a reduced form. The enzyme sample was therefore prior to use reduced with 2.5 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 25 mM ascorbic acid in an anaerobic tent and eluted over a Sephadex G-50 size exclusion column and kept under anaerobic conditions. EPR experiments on the N91D mutant were performed under anaerobic conditions, and the amount of reducing agent in the buffer assay solution was increased for kinetics experiments.

Kinetics characteristics

In order to examine the pH dependence of the single polypeptide mutants, the pH optimum was measured at a single substrate concentration (50 mM p-NPP) for all mutants. The pH optima obtained with this procedure differ slightly from the actual pH optima (as determined from a plot of $k_{\text{cat}}$ versus pH), due to non-saturating substrate conditions at higher pH's.
Figure 7.2: Plots of $k_{\text{cat}}$ and $K_m$ versus pH for single polypeptide H92A-recHPAP (upper panel) and H92N-recRPAP (lower panel) at 22°C. The lines represent fits of the data to the rapid equilibrium diprotic model. The kinetics data obtained from fits of $k_{\text{cat,obs}}$ versus pH are presented in the Table.

<table>
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<th></th>
<th>$\text{pH}_{\text{opt}}$</th>
<th>$\text{pK}_{a,1}$</th>
<th>$\text{pK}_{a,2}$</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RecRPAP$^{(a)}$</td>
<td>5.5</td>
<td>4.5</td>
<td>6.6</td>
<td>240</td>
<td>4.5</td>
</tr>
<tr>
<td>H92N$^{(a)}$</td>
<td>4.4 (0.1)</td>
<td>3.2 (0.1)</td>
<td>5.6 (0.1)</td>
<td>760 (51)</td>
<td>23</td>
</tr>
<tr>
<td>H92A$^{(a)}$</td>
<td>3.8 (0.1)</td>
<td>2.6 (0.2)</td>
<td>5.2 (0.2)</td>
<td>21 (1.7)</td>
<td>35</td>
</tr>
</tbody>
</table>

$k_{\text{cat}}$ is defined as the number of substrate molecules hydrolyzed per enzyme molecule per second; $\text{pK}_{a,1}$ and $\text{pK}_{a,2}$ are for deprotonation/protonation events of a group of the enzyme-substrate complex.

$^{(a)}$ Obtained from a fit of $k_{\text{cat}}$ as a function of pH

$^{(1)}$ from (28)

As shown in Figure 7.1, the N91D mutant enzyme has a Gaussian pH optimum near pH 5.4, with $\text{pK}_{a,\text{ap}}$ values of 5.1 and 5.7. The specific activity of the N91D mutant is only 5% that of wild-type recRPAP (12.5 versus 240 s$^{-1}$), although $K_m$ is unchanged (Figure 7.1). The low value of $K_m$ indicates that below the pH optimum saturating substrate concentrations were present. The V versus pH plot was analyzed according to the rapid equilibrium diprotic model (33) to give the values of $\text{pK}_a$ presented in Table 1. Proteolytic cleavage of N91D-recRPAP with trypsin did not change the turnover number, but $\text{pK}_{a,1}$ shifted to lower pH (3.9 versus 5.1). In addition, $\text{pK}_{a,2}$ increased by almost a full pH unit.
upon cleavage, from 5.7 to 6.6. No change in pH optimum or $K_M$ was observed (not shown).

In contrast to the N91D mutant, mutation of His92 into Ala resulted in a sharp increase in the $K_M$ value at the optimal pH, to approximately 30 mM. In order to define the pH optimum more accurately, $k_{cat}$ was measured as a function of pH (Figure 7.2). The $k_{cat}$ versus pH plot showed a broad optimum at pH 3.8 with $pK_a$ values of 2.6 and 5.2. Because of the low activity, incubation times during the assay were increased, but even at the lowest pH the enzyme was stable over the time range measured. Although the $k_{cat}$ values have rather small errors, the plot of $K_M$ versus pH suffers from very large error bars at each pH. Apparently $K_M$ parallels the behavior of $k_{cat}$ as a function of pH, rather than simply increasing with increasing pH. The maximum activity at pH 3.8 is 21 s$^{-1}$, less than 10% that of wild-type enzyme, while $K_M$ is 15-30-fold larger.

The H92N mutant shows an optimum at pH 4.4, with $pK_a$ values of 3.2 and 5.6 (Figure 7.2) and a turnover number of approximately 760 s$^{-1}$, three times that of the wild-type recRPAP. $K_M$ increases with increasing pH, and is about 10-15 times larger than that of the wild-type at its optimal pH. Upon tryptic cleavage, the pH optimum shifts to 5.6 with $pK_a$ values of 3.7 and approximately 7.5 and a fitted $k_{cat}$ of approximately 470 s$^{-1}$ is found. The value of $K_M$ is almost constant at the pH optimum (not shown).

*Spectroscopic characteristics*

The EPR spectrum of single polypeptide N91D-recRPAP at pH 6.5 shows several $g_{av}$=1.74 species (Figure 7.3). Simulation of the spectra shows that the signal could be a superposition of two spectra with features at $g_{xy}$=1.65, 1.74, 1.93 and $g_{xyz}$=1.58, 1.73, 1.86 respectively with the latter being the major species. Upon decreasing the pH to 4.3, the intensity of the signal decreases with a slight relative increase in the intensity of the

Figure 7.3: EPR spectra of N91D-recRPAP at different pH's. EPR conditions: microwave frequency, 9.423 GHz; modulation, 12.7 G at 100 kHz; temperature 4.5-5.5 K. All spectra were normalized for gain, temperature, and power.
Mutational analysis of Asn91 & His92

Figure 7.4: EPR spectra of H92A-recRPAP at different pH's. EPR conditions: microwave power 2 mW; microwave frequency, 9.423 GHz; modulation, 12.7 G at 100 kHz; temperature, 4.5-5.5 K. All spectra were normalized for gain, temperature, and protein concentration.

Figure 7.5: EPR spectra of H92N-recRPAP at different pH's. EPR conditions: microwave frequency, 9.423 GHz; modulation, 12.7 G at 100 kHz; temperature, 4.5-5.77 K. All spectra were normalized for gain, temperature, power, and protein concentration. The spectrum in the lower panel of the Figure is a 5-fold enlargement of the pH 2.7 spectrum.
Chapter 7

g_z=1.58, 1.73, 1.86 species. The observed decrease in intensity could be either an intrinsic pH dependence or the result of some loss of protein at lower pH. At all pH's, the largest contribution originated from species between g=4.3 and 8 (not shown), and the g_z=1.74 signal was due to a small fraction of the protein (~20-30%). Integration of the spectra showed that at pH 5.3 the spin concentration was approximately 40% of that found at pH 6.5. The presence of high spin Fe^{3+} species was not due to oxidation by oxygen, because the enzyme was kept under strictly anaerobic conditions after reduction and during all EPR experiments. Before thawing, the frozen samples were also made anaerobic by flushing with argon.

The EPR spectrum of the H92A mutant shows the presence of a single species at its pH optimum (Figure 7.4), with apparent g-values of g_{xyz}=1.58, 1.73, 1.96. Measuring the spectrum at pH ≤ pK_{as,1} was not possible, due to the instability of the mutant enzyme upon prolonged incubation at this pH during buffer exchange. Increasing the pH to pH ≥ pK_{as,2} gave no change in the EPR spectrum. Thus, a single EPR detectable species is present over the pH range 3.7-6.5. The intensity of this species is reduced at pH 4.1 and 3.7 from ~0.5-0.7 to ~0.1-0.15 spins per molecule, suggesting a correlation with the protonation of a catalytically important residue. In the visible spectra only small shifts in λ\text{max} are observed: at pH 3.7 λ\text{max} is 504 nm vs. 508 nm at pH 6.5.

The EPR spectrum of the H92N mutant at pH 6.5 shows apparent g-values at 1.96, 1.85, 1.71, 1.60, and 1.41 (Figure 7.5), indicating the presence of several species. Simulation of the spectra suggests that two main species are present, with g_{xyz}=1.57, 1.70, 1.85, and g_{xyz}=1.41, 1.60, 1.74, respectively. A weak feature with g_{xyz}=1.93, 1.74, 1.65 could also be present. Decreasing the pH to 2.7 causes the intensity of the g_{xyz}=1.41, 1.60, 1.74 species to increase relative to that of the g_{xyz}=1.57, 1.70, 1.85 species. At pH ≤ pK_{as,1} (2.7), the total intensity of the spectrum decreased from ~0.5 spins per molecule to <0.05 spins. Together with the loss of signal intensity at g_{av}=1.74, an increase in the intensity of the signal due to high-spin Fe^{3+} species was observed, from ~1-2% to ~45% of the total signal intensity, respectively. A shift of λ\text{max} to higher wavelength that parallels the loss of signal intensity in the EPR spectrum was observed: λ\text{max} of the H92N mutant shifted from 530 nm at pH 6.5 and pH 4.4 to 560 nm at pH 2.7. Increasing the pH of the sample to pH 6.5 restored the original λ\text{max} of 530 nm.

Discussion

To date, no site-directed mutagenesis (SDM) studies of active site residues of PAP have been published. However, several studies have appeared on the closely related PPs (12), in which all active site residues have been mutated (21-24, 30-32, 34). In most of these studies, the kinetics parameters were determined under only one set of conditions, which did not result in a clear understanding of the function of these residues. A few studies, mostly performed by the Rusnak group, have given more detailed insight in the role of several active site residues (21-23). Two of these residues, Asn91 and His92, are proposed to be involved in proteolytic activation of mammalian FeFe-PAPs. The interaction of these two residues with Asp146 in the exposed loop reduces the catalytic activity and shifts pK_{a,1}, which is the pK_{a} of the metal coordinated nucleophilic residue
Mutational analysis of Asn91 & His92

(29), to lower values (28). Both the Asp146 coordinating residues have been mutated to elucidate their role in the catalytic process.

Mutation of Asn91 to Asp in APP reduced the catalytic activity significantly without affecting $K_m$. Although a stoichiometry of 1.6 [Mn$^{2+}$]/[protein] was found, the low temperature EPR spectrum of this Mn$^{2+}$Mn$^{2+}$ APP mutant, which probes the high affinity metal site (divalent site in PAP), showed a dramatic change in line shape, indicating large differences in the ligand field. At higher temperature, where the signal due to the spin-coupled [(Mn$^{2+}$)$_2$] cluster dominates, the spectrum of N91D-APP is quite similar to that of the wild-type enzyme, suggesting that the parameters of the [(Mn$^{2+}$)$_2$] unit are quite similar to those in the wild-type system (32). In PP1, mutation of Asn91 to Asp resulted in a 5-fold reduction in $k_{cat}$. No significant change in $K_m$ with phosphorylase a as substrate was observed, but the mutant was devoid of activity towards $p$-NPP. The pH optimum of N91D-PP1 was shifted a full pH unit to lower values (6.5 versus 7.5) (22). For N91D-recRPAP, no shift in pH optimum was observed; both the wild-type and the mutant enzyme have an optimum around pH 5.5. The difference in activity is, however, significant; $k_{cat}$ for the mutant is only 3% that of the wild-type enzyme. The $K_m$ value was not affected by the mutation, which suggests that Asn91 is not involved in substrate binding, as has been proposed due to the short distance (1.75 Å in the Uf structure) between the amido hydrogen of Asn and the phosphate oxygen (10). Mutation to a negatively charged carboxylate would be expected to decrease the affinity for phosphate oxygen and therefore increase $K_m$. Furthermore, mutation of Asn91 to Asp should partly neutralize the positive charge of the ferrous ion, decreasing the Lewis acidity of the ferrous ion, and increase the $pK_a$ of a coordinated solvent molecule to higher pH. The value of $pK_a$ of the N91D mutant has increased from 4.5 for the wild-type enzyme to 5.1 for the mutant protein, and $k_{cat}$ is significantly reduced, in agreement with the effects of mutation discussed above. These results support the proposal that the nucleophilic hydroxide is coordinated to the divalent site (29, 35). However, EPR spectroscopy shows that the spectrum of a sample of the mutant prepared anaerobically consists of more than 70 % of high spin Fe$^{3+}$ species; only a minor fraction can be assigned to the mixed-valent Fe$^{3+}$Fe$^{2+}$ unit. A possible explanation for the decrease in intensity of the EPR signal of the unpaired spin and the increase of the intensity of high spin ferric ion species is the loss of the Fe$^{2+}$ leading to an unstable half-apo N91D-recRPAP. Some loss of protein was observed.

The second residue that could interact with Asp146 is His92. Mertz et al. suggested that His92 in PPs is not required for protonation of the leaving group, because the same relative $k_{cat}$ value was found for two substrates with different leaving group $pK_a$'s. Instead, they suggested that His92 could function in concert with the nucleophilic water molecule to either position a lone pair on the oxygen atom for optimum in-line attack on the phosphorus atom of the substrate or serve as a general base to take up a proton concomitant with solvent nucleophilic attack (21). A subsequent isotope effect study of the wild-type and H92N-APP showed that the increase in $^{15}$N(V/K) and $^{18}$O(V/K)$_{bridge}$ isotope effects for the substrate $p$-NPP were analogous to those observed for protein tyrosine phosphatases observed upon mutation of their general acid, but smaller in magnitude. This study did not clearly answer the question of whether His92 functions as a general base (23).
For recRPAP, the pH optima of both the H92N and H92A mutant are shifted 1-1.5 pH units to lower values due to a 1.5-2 pH unit shift in \( pK_a \); in addition \( pK_a \) decreased one pH unit. The pH optimum of H92N-\( \lambda \)PP shows more than a full pH unit decrease, together with a significant increase in \( K_m \) at low pH (23). The presence of a basic limb in the pH profile of both H92N-recRPAP, H92N-\( \lambda \)PP (23), and H92A-recRPAP mutant shows that \( pK_a \) is not due to the (de)protonation of the imidazole group of this residue. The 15-30-fold increase in \( K_m \) of H92A-recRPAP indicates that this residue is involved in coordination of the substrate in PAP, in contrast to the conclusions of a previous SDM study of \( \lambda \)PP (21). A subsequent study of the pH dependence of \( k_{cat} \) and \( K_m \) showed that below pH 7 a large difference is found between the wild-type and the H92N mutant. At pH above the \( pK_a \) of \( p \)-NPP, \( K_m \) increases significantly, suggesting that the monoanion is the substrate and that the dianion does not bind to the enzyme (23). An SDM study of PP1 could not detect any activity from a His92Ala mutant (22), while mutation of His92 to Asn did not affect the \( K_m \) value very much (21, 23, 24). Mutation to a negatively charged glutamate in calcineurin resulted in a 10-fold reduction in \( K_m \) for \( p \)-NPP, while the \( K_m \) for [P]-R11 peptide was unaffected (21). For all these mutants, \( k_{cat} \) was \( \leq 1\% \) of that of the wild-type enzyme. These values of catalytic activity contrast with the observed \( k_{cat} \) values for H92A and H92N-recRPAP. The almost 3-fold increase in \( k_{cat} \) of the H92N mutant compared to the wild-type enzyme is especially surprising. The shift of \( pK_a \) to lower pH upon mutation of His92 into Ala and the increase in \( k_{cat} \) for the H92N mutant strongly suggest that the interaction of the positively charged His92 imidazole group with the nucleophilic solvent molecule increases the \( pK_a \) of the nucleophile, but has no effect on its nucleophilicity. Thus, His92 has no direct function in the catalytic cycle, such as regeneration of the nucleophile as has been proposed for metal-containing enzymes such as arginase or carbonic anhydrase (25), or as an acid or base catalyst. His92 does, however, play a major role in substrate coordination.

The EPR spectrum of H92A-recRPAP shows a single species that resembles those observed above \( pK_a \) in the EPR spectra of recHPAP (29) and recRPAP (E.G. Funhoff, G. Andersson, B.A. Averill, unpublished results) over the pH range 4 to 8. The intensity of this species is reduced at pH 3.7 and 4.1 compared to the spectrum at pH 6.5, which could suggest a correlation with \( pK_a \). Apparently, His92 is capable of interacting with the metal site in PAP, even though it is positioned 4.5-5 Å away from both metals, as observed in the X-ray structures of PAP (8-10) and PP (13-17). The structures of uncomplexed PP1 (14) and calcineurin (13) show that His92 does, however, interact with the metals via two solvent molecules in the active site. Thus, it could well be that in the wild-type enzyme a change in the position of His92 at pH \( \geq pK_a \) is responsible for the observed shift in the g feature of the EPR spectrum (from 1.94 to 1.97) and the loss of activity (29). Although His92 is apparently not directly involved in the catalytic process, it or another hydrogen bonding residue is apparently crucial for the catalytic process.

The presence of this residue influences the \( pK_a \) of the nucleophilic group as mentioned before. Subtle changes in its position, due to, e.g., proteolytic cleavage (27, 28), leading to loss of an Asp146-His92 interaction via a hydrogen bonding network of water molecules, could affect the orientation of the imidazole group of His92 in such a way that this residue positions either the nucleophilic hydroxide solvent molecule or the substrate better for optimal nucleophilic attack (18). This mechanism is analogous to "orbital steering" (26, 36), a phenomenon that explains differences in catalytic efficiency due to
small structural perturbations in enzymes. For example, in the Mg$^{2+}$-containing enzyme isocitrate dehydrogenase, the activity is significantly decreased by substituting Mg$^{2+}$ for Ca$^{2+}$, but not Cd$^{2+}$, even though both metals are considered to be "hard" Lewis acids and have a larger cation radius (0.65 for Mg$^{2+}$ versus 0.99 for Ca$^{2+}$ and Cd$^{2+}$). The activity of the Cd$^{2+}$ substituted enzyme is equal to that of the wild-type dehydrogenase, even though the radius of the metal has increased significantly. The difference between these metals is that Ca$^{2+}$ prefers to be 7- or 8-coordinate, while Cd$^{2+}$ prefers 6-coordinate octahedral coordination. Consequently, in the crystal structure of the Ca$^{2+}$-substituted enzyme, the metal-coordinated water molecules occupy different positions, resulting in a sharp reduction in activity (37). In arginine kinase, a histidine residue was expected to be involved in acid/base catalysis (38), but this was not supported by mutagenesis studies (39). An X-ray structure of arginine kinase in the presence of transition state analogous, however, showed that the only role of this analogue His was to bring the substrate into close proximity with the other reacting groups (40), in other words orbital steering. An analogous role of His92 in PAP could well be responsible for the observed differences.

The results show that pK$_{a,2}$ is not due to deprotonation of His92, but the identity of the group involved is unknown. The group responsible for pK$_{a,1}$ is a metal-bound nucleophilic solvent molecule (29, 41). However, the identity of the water/hydroxide is unknown: possibilities include a terminally coordinated Fe$^{3+}$ or Fe$^{2+}$ hydroxide, a bridging hydroxide ion, or a second coordination sphere hydroxide (42). It is assumed that PAPs and PPs catalyze the hydrolysis reaction via the same reaction path (18): hence, the groups responsible for pK$_{a,1}$ and pK$_{a,2}$ are identical in the two clusters of enzymes. The observed pK$_{a,1}$ values for PAP are 4.5 for wild-type single polypeptide, 5.5 for proteolytically cleaved (27, 28), and 2.6 for H92A single polypeptide mutants. Based on the pK$_{a}$ values of the hexaaquo complexes of metal ions (43, 44), it is almost impossible to attribute a pK$_{a,1}$ of 2.6 (H92A) or 3.2 (H92N) to a water group coordinated to a divalent metal ion. Thus, the most plausible assumption is that a solvent molecule coordinated terminally to the trivalent ion coordinated is involved. This is supported by the pH optimum of λPPs.

Figure 7.6: Active site structure of calcineurin (2.1 Å resolution) showing the hydrogen bonding network between the divalent metal (II), trivalent metal (III), Asn91, and His92. The distances between the residues and solvent molecules are given in Å. The figure was generated using the program Rasmol with the coordinates of 1AU1.
which is 8.3, with a $pK_{a1}$ of 7.7 (23). However, the trivalent site in these PPs is occupied by a loosely bound divalent Mn$^{2+}$ ion (14, 45), whose hexaaquo complex has a $pK_a$ of 10.7, several pH units higher than the $pK_a$ of the hexaaquo complex of Fe$^{3+}$, which is 2.2. This difference nicely explains the higher pH optimum of these phosphatases vs. the earlier proposal that the substrate coordinating residues, arginines in PPs versus His in PAPs, are responsible for the higher pH optimum (46). Arguments against a terminal trivalent coordinated nucleophile are: (1) metal substitution of the trivalent metal site has no effect on kinetics properties, while substitution of the divalent site results in significant changes (35, 47, 48); and (2) fluoride is capable of replacing the nucleophile if protonated (29), but the NMR spectrum of the enzyme-fluoride complex disappears below $pK_{a1}$, suggesting a significant change in the super-exchange interaction between the two iron ions, which is only possible if the bridging hydroxide/water has been replaced (49). The presence of a hydrogen bonding network (Figure 7.6), observed in calcineurin (13) and partly in uteroferrin (10), between the ferrous ion, the bridging hydroxide, Asn91, His92, and the terminal Fe$^{3+}$ coordinated solvent molecule could account for these contradictions. In general, the results are in favor of a model in which the nucleophilic hydroxide is that coordinated to the ferric ion, which is optimally tuned by positioning of the substrate by His92 and by the contribution of the bridging hydroxide (which is affected by the divalent site).

To obtain insight into the (transition state) structure of PAP, it is of utmost importance to obtain high-resolution crystal structures of a mammalian PAP in a redox and pH active state in the presence and absence of a substrate analogues, such as AMP, in combination with a method to visualize the solvent molecules, such as ENDOR.

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References
