Structure-function studies of mammalian purple acid phosphatases

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Citation for published version (APA):

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The highly exposed loop region in mammalian purple acid phosphatase controls the catalytic activity

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preliminary version published in:

Abstract

Recombinant human purple acid phosphatase (recHPAP) provides a convenient experimental system for assessing the relationship between molecular structure and enzymatic activity in mammalian purple acid phosphatases (PAPs). RecHPAP is a single polypeptide protein with properties similar to those of uteroferrin (Uf) and other PAPs isolated as single polypeptide chains, but its properties differ significantly from those of bovine spleen PAP (BSPAP) and other PAPs isolated as proteolytically “clipped” forms. Incubation of recHPAP with trypsin results in proteolytic cleavage in an exposed region near the active site. The product is a tightly associated two-subunit protein whose collective spectroscopic and kinetics properties resemble those of BSPAP. These results demonstrate that the differences in spectroscopic and kinetics properties previously reported for mammalian PAPs are the result of proteolytic cleavage.

Mass spectrometry shows that a 3-residue segment, D-V-K, within the loop region is excised by trypsin. This finding suggests that interactions between residues in the excised loop and one or more of the groups that participate in catalysis are lost or altered upon proteolytic cleavage. Analysis of available structural data indicates that the most important such interaction is that between Asp146 in the exposed loop and active site residues Asn91 and His92. Loss of this interaction should result in both an increase in the Lewis acidity of the Fe$^{2+}$ ion and an increase in the nucleophilicity of the Fe$^{3+}$-bound hydroxide. Proteolytic cleavage thus could constitute a potential physiological mechanism for regulating the activity of PAP in vivo.
**Introduction**

Purple acid phosphatases (PAPs) are metalloenzymes that catalyze the hydrolysis of phosphate monoesters; they contain a metallophosphoesterase signature sequence motif (1, 2) and have an \( \alpha\beta \)-type structure. PAPs are characterized by their purple color, their ability to efficiently hydrolyze unactivated phosphate esters, their acidic pH optimum, and their insensitivity to tartrate inhibition (3-6).

PAPs, also referred to as tartrate resistant acid phosphatases (TRAPs) or type 5 acid phosphatases (EC 3.1.3.2) (7), have been isolated from mammalian sources such as bovine spleen (BSPAP), porcine uterine fluids (UF), rat spleen and bone, and human tissues. Similar enzymes are present in plants (e.g., red kidney beans (KBPAP), sweet potatoes and *Arabidopsis thaliana*) and in microorganisms (6). The subunit molecular mass of PAP from various sources ranges from approximately 55 kDa for the plant enzymes to 35 kDa for the mammalian enzymes. The primary structures of mammalian PAPs are highly homologous; for example, BSPAP and UF exhibit 84-90% sequence identity to the human enzyme (8-10).

Of the mammalian PAPs, those from bovine spleen and porcine uterine fluids are the best characterized. The enzymatically active form contains a mixed-valence binuclear cluster at the active site, in which a high-spin Fe\(^{2+}\) ion and a high-spin Fe\(^{3+}\) ion are antiferromagnetically coupled to give an \( S = \frac{1}{2} \) ground state (11-13). The one-electron oxidized (Fe\(^{3+}\)Fe\(^{3+}\)) form is also antiferromagnetically coupled (\( S = 0 \) ground state); it is essentially inactive, with \( \leq 10\% \) of the activity of the mixed-valence form. This residual activity has been shown to be due to the presence of small amounts of an Fe\(^{3+}\)Zn\(^{2+}\) form (14). Both the divalent and trivalent metal ions in UF and BSPAP can be replaced by e.g., Zn\(^{2+}\) or Co\(^{2+}\) and Ga\(^{3+}\) or Al\(^{3+}\), respectively, resulting in active enzymes that exhibit relatively minor differences in kinetics parameters and inhibition constants (13-18).

The first X-ray structure of a PAP, the 110 kDa dimer from kidney bean, showed the presence of an FeZn center coordinated by three histidines, two aspartates, a tyrosinate, and an asparagin (19). In addition, three histidines were proposed to act as acidic or basic groups that interact with the substrate during catalysis. The X-ray structures of two Ser/Thr-specific protein phosphatases, protein phosphatase 1 (PP1) (20, 21) and 2B (calcineurin) (22, 23), show the presence of very similar binuclear metal centers in which the same amino acids are coordinated to the metal ions with virtually identical geometries. The only exception is the replacement of the tyrosinate ligand present in the PAPs by a coordinated water molecule in the PPs, accounting for the absence of the purple color in the latter. Very recently, three additional structures of mammalian PAPs have been published (24, 25); those from rat bone (at 2.7 Å and 2.2 Å resolution) and from porcine uterine fluids (at 1.5 Å resolution). These structures show that the amino acids coordinated to the binuclear center and the secondary structure of the mammalian PAPs are identical to those of the plant PAPs and the PPs. A disulfide bond is present in both the UF structure and the 2.7 Å rat bone PAP structure, but is apparently absent in the 2.2 Å rat bone PAP structure. In all three structures the proteins are glycosylated on Asn97 (24, 25).

The mammalian PAPs studied to date are quite similar in their spectroscopic and catalytic properties. All exhibit a rhombic \( g_{\text{av}} = 1.74 \) EPR spectrum, a broad visible absorbance band at ca. 550 nm, and a pH optimum in the acidic region (4.9 - 6.3).
Surprisingly, however, PAPs from different sources and preparations exhibit rather large differences in catalytic activity, with reported turnover numbers ranging from 200 - 3000 s⁻¹. The origin of these differences has long been unclear, although suggestive evidence has been presented linking higher enzymatic activity with proteolysis of the protein. For example, the activity of both bovine spleen PAP and Uf has been shown to increase upon incubation with proteases (26), presumably due to cleavage of the polypeptide. Similar increases in activity, accompanied by a shift in pH optimum, have been reported upon proteolysis of recombinant rat PAP (recRPAP) (27, 28), while recombinant human PAP (recHPAP) is also suspected to be sensitive to proteolysis (29). The loop that is apparently subject to proteolysis is a highly antigenic portion of the polypeptide that is present in the 3-D structure of Uf (24) and in one of the rat bone PAP structures (30), but which could not be resolved in the other rat bone PAP structure (25). Why proteolysis of this loop affects the catalytic activity of mammalian PAPs is, however, not known.

Because published studies have utilized different enzyme preparations and sources, the extent and location of proteolysis have been difficult to ascertain and/or control. In addition, the system for which the proteolysis process is best characterized (recRPAP) is among the least well characterized in terms of spectroscopic and kinetics studies. Conversely, the two benchmarks for spectroscopic and kinetics studies have been BSPAP, which is normally isolated in a proteolytically cleaved form, and Uf, which is isolated as a single polypeptide of 36 kDa. Unfortunately no site-directed mutagenesis studies and very limited proteolysis studies have been reported for these enzymes.

We have utilized recHPAP as a model system with which to study the correlation between proteolysis and the catalytic and spectroscopic properties of PAP. RecHPAP has been isolated, and both the intact enzyme and its proteolytically cleaved form have been characterized. Our results indicate that the differences in kinetics and spectroscopic properties reported for mammalian PAPs are due to variations in the extent of proteolytic cleavage in an exposed loop near the active site. A molecular basis for the effect of proteolytic cleavage upon the catalytic and spectroscopic properties of the enzyme is proposed. These findings are consistent with the possible use of proteolytic activation as a physiological mechanism for regulation of PAP activity.

**Experimental procedure**

**Materials and instruments**

Insect Xpress SFM medium (Bio Whittaker), phosphocellulose (Whatman), CL-4B phenyl-sepharose, SP-Sepharose, p-NPP (Fluka), trypsin (Sigma), and all other chemicals were of highest purified grade.

Recombinant protein was produced in a 10 l Applikon bioreactor, controlled by an ADI 1030 Biocontroller, and purified on a Pharmacia FPLC. Electron paramagnetic resonance spectra were obtained on a X-band Bruker ECS EPR spectrometer equipped with an Oxford Instruments ESR900 helium-flow cryostat with an ITC4 temperature controller and a AEG magnetic field calibrator. An AVIV® model 62ADS circular dichroism spectrometer operating at 25°C and flushed with nitrogen gas was used to measure CD spectra. RP-HPLC was performed on a Pharmacia Biotech SMART system. The alkylated thiohydantoinss were identified online by reversed phase analysis on a Perkin Elmer 140 C
Proteolytic activation

microgradient system. A Perkin Elmer, model 476A protein sequencer operating in the pulsed liquid mode with online PTH analysis was used for N-terminal sequence analysis. C-terminal sequence analysis was performed on a Perkin Elmer Procise 494C sequencer. A Micromass Q-TOF mass spectrometer with a Protana borosilicate Au/Pd coated capillary was used for mass spectrometry measurements.

General procedures

Protein concentrations during purification were determined according to Bradford (31). The concentration of purified enzyme was determined by measuring the absorption of the tyrosinate-to-Fe$^{3+}$ charge transfer band at 520 nm ($\varepsilon=4080 \text{ M}^{-1} \text{ cm}^{-1}$). SDS-PAGE under reducing conditions was performed with 15% gels according to Laemmli (32). All samples for EPR spectroscopy contained 20% glycerol.

Large scale production of recombinant protein

Sf9 cells (cell density of 0.33x10^6 cells/ml) were seeded in a 10 l bioreactor; pO2 (50% air saturation), temperature (27°C), and stirring (80 rpm) were controlled. The culture was infected (1.1x10^6 cells/ml) with recombinant baculovirus containing a coding region for human purple acid phosphatase (MOI = 0.001) (29, 33). After 6 days the suspension was centrifuged (10 min., 5000 x g) to remove the cells, and the supernatant was used for purification.

Purification of recHPAP

P11 was added to the supernatant (1 g per 200 U activity), and the suspension was stirred at room temperature. If negligible activity remained in the supernatant, the P11 was allowed to settle, filtered, washed with buffer A (50 mM MES pH 6.5, 0.1 M KCl), resuspended in 250 ml buffer B (50 mM MES pH 6.5, 2 M KCl), and stirred at 4°C overnight. The P11 was removed by filtration. (NH$_4$)$_2$SO$_4$ was added to the P11 filtrate (25% saturation), and the suspension was stirred for 2 hours at 4°C. The suspension was centrifuged (15 min., 10000 x g) and the supernatant was loaded overnight onto a CL-4B phenyl-Sepharose column (2.5 x 15 cm) preequilibrated with buffer C (50 mM MES pH 6.5, 25% (NH$_4$)$_2$SO$_4$, 0.25 M KCl). After washing (50 ml), a 100 ml linear gradient (25% to 0% (NH$_4$)$_2$SO$_4$) was applied to the column to elute the protein. Fractions containing recHPAP with R values ($A_{280}/A_{320}$) lower than 30 were pooled and concentrated on an Amicon ultrafiltration unit (YM 30). The concentrated sample was diluted with 50 mM MES buffer and concentrated several times to a KCl concentration of 0.1 M, and applied to a SP-Sepharose column (1.6 x 4 cm) preequilibrated with buffer A. A 20 ml gradient to buffer B was applied to elute the protein. Fractions with R < 18 were pooled and concentrated, reduced (5 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 25 mM ascorbic acid, 3 min.), and applied to a HR10/30 Superose 12 column. After elution with buffer B, the reduced enzyme had R = 16 and was stored at -20°C.

Enzyme kinetics

Purification: Enzyme assays were performed by monitoring the formation of the p-nitrophenolate anion at 410 nm. The activity during purification was measured at pH 6.0 (100 mM MES, 300 mM KCl, 10 mM Na-K tartrate, 17.5 mM Na-ascorbate, 0.37 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$) using 20-40 mM p-NPP at 22°C. At several times after enzyme addition,
Chapte rr  2

Aliquote s (250 μl) were removed and quenched (1.0 ml, 0.5 M NaOH) to convert all product to the phenolate form ($E_{410 \text{nm}}=16.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

pH dependence: The pH dependence of $k_{\text{cat}}$ for recHPAP was measured over the range 4 to 8 (100 mM buffer Na-acetate, MES or HEPES, 10 mM Na-K tartrate, 300 mM KCl, 6.7 mM Na-ascorbate, 0.37 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$) with different $p$-NPP concentrations (0.5 to 50 mM). For each determination of $V_{\text{max}}$ and $K_M$, the hydrolysis rate was measured at 8 different $p$-NPP concentrations. After each assay, the pH of the reaction mixture was measured to ensure that the pH of the solution had not changed. Values of $K_M$ and $V_{\text{max}}$ were obtained by non-linear regression fits to the Michaelis-Menten equation, using the program EnzymeKinetics (Trinity Software).

Substrates: For the determination of $V_{\text{max}}$ and $K_M$ of the substrates ATP, ADP, phosphotyrosine, and phenylphosphate a Malachite green procedure was used. Assays were performed as described (34). In time aliquots were removed and to 4 volumes of assay solution, 1 volume of dye solution (2.5 ml 7.5% ammoniummolybdate (freshly every day) added to 10 ml of 0.44 g malachite green in 20% concentrated sulfuric acid with 0.2 ml 11% tween20) was added to stop the reaction. After 10 minutes the absorbance was read at 630 nm. A phosphate standard was used as reference.

Trypsin digestion

Trypsin (0.035 mol/mol PAP) was added to a sample of recHPAP (50 mM MES pH 6.5). The sample was incubated at room temperature for 20 hours, and the specific activity was followed in time. The sample was then diluted/concentrated several times using a Centricon (30 kDa cut off) to remove the trypsin. After reduction (0.5 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 17 mM ascorbic acid, 3 min.), the sample was applied to a HR10/30 Superose 12 column and eluted with buffer B, resulting in proteolytically cleaved protein with $\lambda_{\text{max}} = 520$ nm. The proteolytically cleaved protein obtained via this procedure was used for kinetics and spectroscopic measurements.

To follow cleavage by EPR, trypsin (0.035 mol/mol PAP) was added to recHPAP (300 μl, 70 μM) in buffer D (50 mM Na-acetate pH 5.0, 2 M KCl), and the sample was immediately frozen in liquid $N_2$. Its EPR spectrum was measured at 4.3 K, and then the sample was thawed and buffer exchanged to buffer B in two concentration/dilution steps using a Centricon (10 kDa cut off) to initiate the proteolysis reaction. After incubating for the desired time, the buffer was again exchanged to buffer D to stop the proteolysis reaction. Before freezing in liquid $N_2$ for measurement of a second EPR spectrum, the enzymatic activity was measured, and the PAP concentration was determined by visible absorption spectroscopy. This process was repeated with the same sample in order to obtain data points at longer incubation periods. Control experiments showed no change in activity or EPR spectrum in the absence of trypsin.

Circular dichroism (CD) spectroscopy

Samples were prepared by buffer exchanging concentrated recHPAP samples (50 mM TRIS pH 6.3, 150 mM KCl buffer). The concentrated samples were diluted to 0.100 mg/ml in the same buffer and immediately frozen (-80°C). The sample was thawed and centrifuged immediately before measuring its CD spectrum. The pH and recHPAP concentration were checked after the spectrum was obtained. Data were analyzed with the program CONTIN (part of the package called STRUCTURE, which can be obtained
from K.S. Vassilenko, Group of Protein Spectroscopy, Institute of Protein Research, Pushchino, Russia), and the results of three different analysis methods (Provencher & Glöckner, Venyaminov, Sreearasa & Woody (35-39)) were averaged to give the final amounts of α-helix, β-sheet and turn, and random coil.

Mass spectrometry and C- and N-terminal sequence analysis

Proteolytically cleaved recHPAP was denatured (7 M GuHCl/0.3 M TRIS pH 9) and the disulfide bond was reduced with freshly prepared DTT solution (20 mM, 1:100 molar ratio, 1 h, 60°C). The reduced fragments were separated on a reverse phase HPLC column (C8 2.1×100 mm, 5 μm) installed on the SMART system, with a predefined gradient from solvent A (0.1% TFA/milliQ water) to solvent B (0.08 % TFA/90 % ACN/milliQ water): 0-60 min, 15-70% B; 60-65 min, 70-100 % B; 65-72 min, 100 % B; 72-77 min, 100-15 % B. Fractions were collected in Eppendorf vials (500 μl).

C-terminal sequence analysis was performed using a slight modification of the protocol of Boyd (40). Prior to C-terminal sequence analysis, the protein was absorbed on a ProSorb sample preparation cartridge and, after subsequent washes with milliQ water, treated with phenylisocyanate under basic conditions to modify the lysine side chains (41). The alkylated thiohydantoin s were identified online by reversed phase analysis with a linear gradient using solvent C (3.5 % THF/milliQ water/35 mM Na-acetate pH 3.8) and solvent D (100 % ACN).

For nano-electrospray mass spectrometry, a fraction of the RPLC eluate was dissolved (50 % ACN/49.9 % milliQ water/0.1 % formic acid (v:v:v)) and loaded into a coated capillary, which was then placed into the nanospray source of the Q-TOF mass spectrometer. The needle was held at 1.3 kV while spray formation was initially stimulated by putting a low N2 pressure at the back of the capillary. Spectra were taken from 600-2000 Da using 2s scans and accumulated during 5 minutes. The obtained data were further processed using the MaxEnt software delivered with the instrument.

Results

Production, purification, and proteolytic activation of recHPAP

Production of recHPAP with Sf9 cells from Spodoptera frugiperda resulted in recHPAP expression levels of recHPAP of 1-2 U/ml at 6 days post infection. Purification of the protein was accomplished by a combination of the procedures of Hayman & Cox (33) and Vincent et al. (42). Phosphocellulose (P11) absorption was used to reduce the volume, followed by hydrophobic interaction chromatography, cation exchange chromatography and size exclusion chromatography. This procedure yielded ca. 15 mg pure protein from a 10 l batch; Western blot analysis showed a band at ca. 36 kDa with some heterogeneity due to variable glycosylation (see below). The protein was stored at -20°C for several months without any evidence of cleavage.

Both BSPAP and recRPAP are known to be susceptible to proteolytic activation by trypsin (26, 27). To examine proteolytic activation of recHPAP, trypsin was used to cleave the 36 kDa protein. As shown by SDS-PAGE, the resulting protein consisted of two fragments with masses of 20 kDa and 16 kDa (Figure 2.1).
Figure 2.1: Trypsin digestion of single polypeptide recHPAP followed in time by SDS-PAGE.

**Mass spectrometry and sequencing**

Reverse-phase (RP)-HPLC, mass spectrometry (MS), and N- and C-terminal sequencing were used to discriminate between single cleavage within the loop and partial or complete removal of the loop as well as to determine the site(s) of cleavage. RP-HPLC analysis of both single polypeptide and proteolytically cleaved recHPAP resulted in chromatograms with a single peak with the same retention time. This indicated that after cleavage the 2 fragments were still connected through a cysteine bridge (residues 142 and 200 in the human sequence (10)). After denaturation of the trypsin-cleaved protein and reduction of the SS-bridge with DTT, two peaks were observed, consistent with cleavage to give two fragments.

A molecular mass of 36264.8 (1.3) Da was found for single polypeptide recHPAP, which does not correspond to the calculated mass of 34328.9 Da (Table 2.1). Previous deglycosylation experiments with recombinant human and rat PAP indicate that the difference (1935.9 Da) is almost certainly due to glycosylation (33, 43). Other minor peaks in the MS spectrum could be explained by heterogeneity of glycosylation of recHPAP (e.g., the presence of various extra monosaccharides such as deoxyhexose and hexuronic acid), as suggested by SDS-PAGE. After cleavage with trypsin, electrospray ionization mass analysis (ESI-MS) showed that the mass of the two-subunit protein was 35947.2 (8.1) Da. A fragment with a mass of 335.6 (8.1) Da is thus removed during proteolysis. Quadrupole time-of-flight (Q-TOF) MS analysis of the reduced fractions yielded a large fragment with a mass of 19486.1 (1.0) and a smaller fragment with a mass of 16454.5 (0.5) Da. These results were confirmed by ESI-MS analysis.

<table>
<thead>
<tr>
<th>Mass (Da)</th>
<th>Theoretical mass (Da)</th>
<th>C-terminus</th>
<th>N-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native recHPAP</td>
<td>36264.8 (1.3)</td>
<td>34328.9</td>
<td>A-T-P-A</td>
</tr>
<tr>
<td>Cleaved recHPAP</td>
<td>35947.2 (8.1)</td>
<td>34346.9</td>
<td>L(?)-V-K</td>
</tr>
<tr>
<td>Large fragment</td>
<td>19486.1 (1.0)</td>
<td>17896.2</td>
<td>A-T-P-A</td>
</tr>
<tr>
<td>Small fragment</td>
<td>16454.5 (0.5)</td>
<td>16452.8</td>
<td>L-A-R-T</td>
</tr>
</tbody>
</table>

[a]: Numbers in parentheses are standard deviation values.
Figure 2.2.: Dependencies of $k_{\text{cat}}$, $k_{\text{cat}}/K_m$ and $K_m$ on pH for single polypeptide (A) and proteolytically cleaved (B) recHPAP with p-NPP as substrate at 22°C. The lines represent fits of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ to the rapid equilibrium diprotic model. The derived kinetics constants are depicted in the table.

$k_{\text{cat}}$ is defined as the number of substrate molecules hydrolysed per enzyme molecule per second, $pK_{\text{aa}}$ and $pK_{\text{es,2}}$ as a deprotonation/protonation event of a group of the enzyme-substrate complex and $pK_{\text{es,2}}$ as a deprotonation/protonation event of a group of the enzyme, and $K_m$ as the dissociation constant of the ES complex.

[a] Obtained from a fit of $k_{\text{cat}}$ as a function of pH.
[b] Obtained from a fit of $k_{\text{cat}}/K_m$ as a function of pH with $pK_{\text{aa}}<2$.

c | d

Table 2.2: Kinetics constants$^{[a]}$ for hydrolysis of different substrates by recHPAP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pK$_{\text{aa}}$</th>
<th>Single polypeptide (pH 5.5)</th>
<th>Proteolytically cleaved (pH 6.2)</th>
<th>Act$^{[b]}$</th>
<th>Act$^{[c]}$</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
<td>$k_{\text{cat}}/K_m$</td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
<td>(s$^{-1}$mM$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>3.8</td>
<td>58.7</td>
<td>0.27</td>
<td>217.8</td>
<td>1058</td>
</tr>
<tr>
<td></td>
<td>~6.3</td>
<td>~6.3</td>
<td>1.7</td>
<td>(0.02)</td>
<td>(16)</td>
<td>(0.04)</td>
</tr>
<tr>
<td>ATP</td>
<td>3.9</td>
<td>3.9</td>
<td>47.9</td>
<td>0.64</td>
<td>74.8</td>
<td>971</td>
</tr>
<tr>
<td></td>
<td>~6.5</td>
<td>~6.5</td>
<td>4.2</td>
<td>(0.14)</td>
<td>(98)</td>
<td>(0.25)</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td>10.0</td>
<td>10.0</td>
<td>44.4</td>
<td>0.73</td>
<td>60.8</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>(10.1)</td>
<td>(10.1)</td>
<td>(0.22)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-NPP</td>
<td>7.2</td>
<td>7.2</td>
<td>336.5</td>
<td>5.6</td>
<td>60.1</td>
<td>1268</td>
</tr>
<tr>
<td></td>
<td>(7.3)</td>
<td>(7.3)</td>
<td>(0.3)</td>
<td></td>
<td>(53)</td>
<td>(0.6)</td>
</tr>
<tr>
<td>Phosphotyrosine</td>
<td>10.5</td>
<td>10.5</td>
<td>70.9</td>
<td>2.55</td>
<td>27.8</td>
<td>1648</td>
</tr>
<tr>
<td></td>
<td>(6.7)</td>
<td>(6.7)</td>
<td>(0.38)</td>
<td></td>
<td>(43)</td>
<td>(0.6)</td>
</tr>
</tbody>
</table>

n.d., not determined

[a] Data were obtained from fits of the data with the program Leonora version 1.0, McCormish & Bowden, 1994.
[b] Comparing $k_{\text{cat}}$.
[c] Comparing $k_{\text{cat}}/K_m$.
The calculated masses of the large and small fragments are 17896.2 and 16452.8, respectively; thus the small fragment is not modified. The difference of 335.6 Da between the native and non-reduced proteolytically cleaved protein is therefore due to modification of the large fragment. It can be explained by a second cleavage between Arg151 and Asp152, resulting in the loss of the last 3 amino acids of the C-terminus of the large fragment (D-V-K, calculated mass 342.4 Da).

During Edman degradation of the RP-LC eluate (non-reduced), two N-terminal sequences were observed. The first sequence, A-T-P-A, corresponded to the N-terminus of the enzyme after post-translational processing. The second sequence was L-A-R-T-Q, corresponding to the amino acids starting at position 161. C-terminal sequence analysis of the sample indicated only one (very weak) sequence, (Leu?)-Val-Lys (0.01%). The C-terminal sequence of the small subunit was not observed since it ends with a Pro, a residue that precludes C-terminal sequence analysis (44). After denaturation and reduction neither of the fragments yielded any sequence information. This is in agreement with the occurrence of a second cleavage event, since Arg as C-terminal amino acid also precludes C-terminal sequence analysis.

**Enzymatic parameters of intact and proteolytically cleaved recHPAP**

Previously reported specific activities for recHPAP vary from 190 U/mg at pH 5.6 (33) to 350 U/mg at pH 4.9 (29). To gain insight into the origin of the differences between the intact and proteolytically cleaved protein, $k_{cat}$ versus pH profiles were measured. The $k_{cat}$ versus pH profile of the intact protein showed a symmetric bell-shaped curve with an optimum at pH 5.5. At 22°C the intact mixed valent recHPAP had a specific activity of 520 U/mg, with a $K_m$ of 4.8 mM for the substrate $p$-NPP at its optimal pH (Figure 2.2). At the often used pH of 6.0, the activity was lower (440 U/mg) and the $K_m$ higher (8.6 mM). The pH dependencies were analyzed according to a rapid equilibrium diprotic model (45, 46). The following expressions were derived for the observed values of $k_{cat}$ defined as in equation (a) and $k_{cat}/K_m$ as defined in equation (b), assuming that all equilibria are fast compared to $k_{cat}$.

\[
k_{cat(\text{obs})}=k_{cat}/(1+[H^+] / K_{es,1} + [H^+] / K_{es,2})
\]

\[
k_{cat(\text{obs})}/K_m(\text{obs})=k_{cat}/K_S(1+[H^+] / K_{e1} + [H^+] / K_{e2})
\]

Fitting of the $k_{cat}$ dependencies resulted in the $pK_a$ values given in Figure 2.2. The oxidized protein typically had a residual activity of 5-10%. The $K_m$ and pH optimum were similar to those of the intact mixed valent protein.

Cleavage with trypsin resulted in an almost 5 fold increase in activity at pH 6 (420 versus 2100 U/mg) with $p$-NPP as substrate. Other substrates showed the same dependency on cleavage: $k_{cat}$ increases with a concomitant increase in $K_m$ (Table 2.2), and there is no dependence on the $pK_a$ of the leaving group. In addition, the pH optimum shifted from 5.5 to 6.2 upon cleavage with trypsin. Analysis of the data indicated that $pK_{e,1}$ increased from 4.6 to 5.5 while the effect on the $pK_{e,2}$ value was less pronounced (6.7 versus 6.9). The $k_{cat}/K_m$ versus pH data could be fitted using a single $pK_a$ ($pK_{e,2}$), but the lack of data at low pH suggests that this result should be interpreted with some care. Nonetheless, it is clear that a significant shift in $pK_{e,2}$ occurs (5.0 versus 5.6). The $K_m$ for
the proteolytically cleaved protein is also lower than that of the intact protein at pH 6.0 (4.8 versus 8.5 mM).

Spectroscopic characterization of intact and proteolytically cleaved recHPAP

In order to determine if the change in kinetics properties upon cleavage could be caused by a change in secondary structure, circular dichroism (CD) measurements were performed on the intact and proteolytically cleaved forms of recHPAP. The spectra of both forms exhibit strong negative ellipticities with shoulders at 210 and 218 nm. The CD spectra of the intact and proteolytically cleaved proteins are virtually identical to one another in both the mixed-valent and oxidized states, suggesting that neither proteolytic cleavage nor oxidation of the dinuclear iron center is accompanied by significant structural rearrangements (Figure 2.3). Quantitation of the secondary structure shows that approximately 15% random coil is present in all forms studied. No significant differences between intact and proteolytically cleaved recHPAP were found, although the latter apparently had slightly lower \( \alpha \)-helix (35 versus 27%) and higher \( \beta \)-turn (15 versus 25%) contents. In the oxidized state, however, virtually identical \( \alpha \)-helix, \( \beta \)-sheet and turn, and random coil contents were observed for both the intact and proteolytically cleaved forms.

Figure 2.3: CD spectra of reduced (A) and oxidized (B) single polypeptide (E) and proteolytically cleaved (■) recHPAP. All spectra were obtained in 0.05 mM TRIS, pH 6.3, at 25.0 °C in an 1 mm cuvet. Concentrations were 0.1002 mg/ml (recHPAP\(_{\text{mono,red}}\)), 0.1004 mg/ml (recHPAP\(_{\text{cleaved,red}}\)), 0.1001 mg/ml (recHPAP\(_{\text{mono,ox}}\)), and 0.1006 mg/ml (recHPAP\(_{\text{cleaved,ox}}\)). Results of quantitation of the secondary structure are given in the table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( \alpha )-helix (%)</th>
<th>( \beta )-sheet (%)</th>
<th>( \beta )-turn (%)</th>
<th>random coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>recHPAP(_{\text{mono,red}})</td>
<td>520</td>
<td>35 (3)</td>
<td>35 (6)</td>
<td>15 (7)</td>
<td>15 (5)</td>
</tr>
<tr>
<td>recHPAP(_{\text{cleaved,red}})</td>
<td>520</td>
<td>27 (3)</td>
<td>33 (8)</td>
<td>25 (3)</td>
<td>15 (12)</td>
</tr>
<tr>
<td>recHPAP(_{\text{mono,ox}})</td>
<td>550</td>
<td>32 (2)</td>
<td>35 (5)</td>
<td>17 (5)</td>
<td>16 (3)</td>
</tr>
<tr>
<td>recHPAP(_{\text{cleaved,ox}})</td>
<td>555</td>
<td>34 (4)</td>
<td>37 (9)</td>
<td>19 (6)</td>
<td>10 (7)</td>
</tr>
</tbody>
</table>

(1) Numbers in parentheses are standard deviation values.
Figure 2.4: Monitoring tryptic cleavage of recHPAP by EPR. Spectra were recorded at a specific activity of 365 (A), 965 (B), 1280 (C) and 1500 (D) U/mg. The amplitudes were corrected for instrument gain and protein concentration. EPR conditions: microwave power 20 mW; microwave frequency, 9.42 GHz; modulation, 12.7 G at 100 kHz; T, 4.3 K. The insert shows the increase in specific activity in time.

In addition, the optical spectra of both intact and proteolytically cleaved recHPAP were identical, showing a tyrosine-to-Fe$^{3+}$ charge transfer band at 520 nm which shifted to lower energy (550 nm) upon oxidation with H$_2$O$_2$.

The large changes in pK$_a$ upon cleavage observed in the kinetics studies indicate that there must be a significant difference between the intact and proteolytically cleaved proteins. Because EPR spectroscopy is a very sensitive method that specifically probes structural changes at the diiron site, EPR spectra of both the intact and proteolytically cleaved proteins were obtained. The intact mixed-valent recHPAP exhibited a $S = \frac{1}{2}$ rhombic spectrum, with apparent g-values of 1.94, 1.73 and 1.58 (Figure 2.4A). This spectrum is virtually identical to that reported for Uf (47), which is isolated as an intact 36 kDa polypeptide. In contrast, the proteolytically cleaved mixed-valent protein gave a rhombic spectrum with apparent g-values of 1.86, 1.73 and 1.58 (Figure 2.4D). This spectrum is very similar to that of BSPAP, which is isolated in a proteolytically cleaved form.

In order to confirm that the changes in EPR spectra were indeed due to cleavage and correlated with changes in specific activity, a large-scale proteolysis experiment was monitored by EPR spectroscopy (Figure 2.4). The spectra clearly show that the $g = 1.94$ feature disappears with concomitant appearance of a signal at $g = 1.86$, and that the spectral changes parallel the increase in specific activity.
**Discussion**

Like recHPAP, Uf and recRPAP are isolated as intact polypeptides with a molecular mass of ca. 36 kDa. Compared to PAPs such as BSPAP and the rat bone and spleen PAPs, which are isolated as proteins consisting of two fragments of molecular mass of 20 and 16 kDa, the former exhibit: (i) lower specific activity ($k_{cat}$ ca. 300 s$^{-1}$); (ii) a more acidic pH optimum; and (iii) a more rhombic EPR spectrum, with apparent g-values of ca. 1.94, 1.73 and 1.58 (4, 13, 29, 33, 42, 43, 47-49).

The large differences in specific activity reported for mammalian PAPs appear to be related to the presence or absence of proteolytic cleavage. Treatment of single polypeptide BSPAP, Uf and recRPAP with proteases such as trypsin, papain, or cathepsin has been reported to result in the formation of a two-subunit protein with a significant increase in activity (26) and a shift in pH optimum (27). In the present study, we attempted to clarify the relationship between proteolytic cleavage, kinetics properties, and spectroscopic characteristics using a representative recombinant mammalian PAP.

Treatment of recHPAP with trypsin results in a cleaved protein with an increased specific activity, which is comparable to that of recRPAP treated with cysteine proteases (27). Although cleavage by trypsin is highly unlikely to be physiologically relevant, trypsin was used in these experiments because of the presence of well-defined trypsin cleavage sites and the previously reported activation of BSPAP and Uf by trypsin (26). Physiologically relevant proteases are the cathepsins, cysteine proteases that are present in the same cells as PAP (27). For recRPAP and BSPAP, the N-terminal sequence of the small subunit is D-L-G-V-A-R-T-Q, and N-L-A-M-A-R-T-Q, respectively, while the N-terminal sequence of the small subunit of the recombinant human protein contains three amino acids fewer, L-A-R-T-Q. The precise site of cleavage has an important effect on the specific activity. As shown in Table 2.3, cleavage at the end of the loop, in or near the sequence A-R-T-Q, seems to produce proteins with higher specific activity than cleavage upstream of this sequence (26, 27). Mass spectrometry indicates that 3 amino acids at the C-terminus of the large fragment in recHPAP are removed by trypsin. The mass of the small fragment produced by trypsin cleavage is the same as the calculated mass of 16452 Da. The difference of 335.6 Da found between the single polypeptide and non-reduced proteolytically cleaved recHPAP must therefore be due to proteolytic processing of the larger subunit. Within experimental error, this difference is equal to the mass of D-V-K (calculated mass 342.7 Da), which are the C-terminal amino acids of the larger fragment. A V-K C-terminus was found, but corresponded to only 0.01% of the

<table>
<thead>
<tr>
<th>Species</th>
<th>N-Terminal Sequence of Small Subunit</th>
<th>N-Terminal Sequence of Large Fragment</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>D-L-G-V-A-R-T-Q</td>
<td>D-V-K</td>
<td>145 (26)</td>
</tr>
<tr>
<td>Rat</td>
<td>N-L-A-M-A-R-T-Q</td>
<td></td>
<td>11 J45 (10)</td>
</tr>
<tr>
<td>Human</td>
<td>D-L-G-V-A-R-T-Q</td>
<td>D-V-K</td>
<td>1200</td>
</tr>
</tbody>
</table>

| Table 2.3: Sequence alignment of mammalian PAPs including their reported cleavage site and corresponding specific activity (U/mg) after digestion with T: trypsin, P: papain, N: as isolated |

From ref. (27) and ref. (10) and this work, from ref. (26) and ref. (18).
The C-termini of both the small and large subunits are known to preclude C-terminal sequence analysis, and therefore they were not detected (44). Removal of these three amino acids should weaken or abolish the interaction of the loop region with the active site residues which results in a concomitant increase in activity. This would explain why almost no increase in activity is found upon trypsin cleavage of recRPAP, for which only one cleavage site is available; presumably the resulting amino acid “tail” is held in place by non-covalent forces. When papain is used, however, 6 amino acids between Glu154 and Gly160 (Table 2.3) are removed (27), leading to complete loss of the interaction of the exposed loop with the active site and an increase in enzymatic activity. The variability of cleavage products with proteases is supported by the finding of Ljusberg et al., that SDS-PAGE of trypsin- and papain-digested recRPAP show different N-terminal fragment masses (27).

The EPR spectrum of intact recHPAP resembles those of Uf and intact recRPAP. All of these proteins consist of a single polypeptide chain of molecular mass ± 36 kDa, and in their mixed-valent state all exhibit rhombic EPR spectra with features around 1.94, 1.73 and 1.58 (43, 48). Upon proteolytic cleavage, $g_z$ shifts to 1.86, resulting in a spectrum which resembles that of BSPAP (26, 50, 51). The visible spectrum of recHPAP is not affected by proteolytic cleavage, which suggests that cleavage does not affect the position or orientation of the tyrosine ligand. A change in the interaction of the metal ion(s) with a water molecule or in the interaction of the metal ligands with other amino acids upon cleavage could, however, account for the observed differences in EPR spectra. High salt BSPAP (i.e., BSPAP prepared under high salt conditions; $\lambda_{max} = 536$ nm) constitutes an apparent contradiction to the change in the EPR spectrum, in that the EPR spectrum reported for intact BSPAP is similar to that of the proteolytically cleaved form (26).

Compared to Uf, which exhibits a bell-shaped pH profile with an optimum at 4.9 and $pK_{a,app}$ values of about 4 and 5.2 (52), BSPAP has a higher pH optimum (6.2) with $pK_a$ values of 5.4 and 7.5 (46). Merkx et al., suggested that the reported differences in pH optima between the mammalian PAPs are artifacts due to the use of non-saturating substrate concentrations in most studies of the pH dependence of the kinetics; this leads to a pH profile with an apparent lower pH optimum and shifted $pK_a$ values (46). We therefore measured $k_{cat}$ as a function of pH for the recombinant human protein before and after proteolytic cleavage. The resulting pH profiles clearly show that the observed difference in pH optima is not artifactual, but reflects real differences in the properties of the enzyme. Fits of the $k_{cat}$ versus pH data to a rapid equilibrium model allowed extraction of the corresponding $pK_a$ values for the two forms. Both $pK_a$ values increase upon cleavage, $pK_{es,2}$ by only 0.2 pH units, and $pK_{es,1}$ by almost a full pH unit. In addition, the $k_{cat}/K_M$ versus pH profile shows that $pK_{e,2}$ increases by 0.6 units upon cleavage. $pK_{e,2}$ probably corresponds to a group in the free enzyme that must be protonated in order for the enzyme to bind substrate, possibly one of the conserved histidine residues near the active site. The acidic $pK_a$ in the $k_{cat}$ versus pH profile has been attributed to a water molecule coordinated to the Fe$^{3+}$ ion in the enzyme-substrate complex (18, 46, 53), but the identity of the group giving rise to $pK_{es,2}$ remains unclear. The magnitude of the observed change in $pK_{es,1}$ suggests that proteolytic cleavage causes a significant change in the interactions between the protein and a coordinated water molecule. This conclusion is in agreement with the change in EPR spectrum,
Proteolytic activation

In addition to the effect of proteolytic cleavage upon the pH dependence of the catalytic properties, the intrinsic activity of the enzyme at its optimum pH is also affected by proteolysis. Our results show that proteolysis of recHPAP with trypsin increases $k_{\text{cat}}$ by a factor of about 4, while $K_M$ is not affected. For recRPAP, however, a decrease in $K_M$ was observed upon proteolysis; consequently, the efficiency of the proteolytically cleaved enzyme (as judged by the $k_{\text{cat}}/K_M$ criterion) is higher. In this case, however, the assays were performed at a single standard pH, not at the optimal pH for each species (27).

Why proteolytic cleavage of an exposed loop of polypeptide on the surface of the protein should have such a large effect upon the catalytic properties of the enzyme constitutes an important problem. The simplest explanation for the increase in enzymatic activity upon proteolysis is a steric one, in which cleavage or removal of the loop increases the accessibility of the active site to the substrate. This explanation is unlikely to be correct, however, because there is no evidence in the X-ray structures that the loop blocks access to the active site. Further, this explanation would predict a larger effect on $K_M$ than on $k_{\text{cat}}$, which is the reverse of that observed with several substrates. Finally, it is difficult to see how an increase in active site accessibility would result in the similar changes in $pK_a$'s and EPR spectra that are observed for all PAPs examined to date.

In principle, proteolysis could also induce a protein conformational change, which causes the observed changes in activity. CD spectroscopy, however, indicates that negligible changes in secondary structure occur upon cleavage (Figure 2.4). These results do not, however, exclude the possibility that smaller differences in protein structure occur in the vicinity of the active site. Superposition of the active sites of KBPAP, Uf and recRPAP, however, shows only minimal differences in the active site region, suggesting that it is both highly conserved and not very flexible. An additional result of the present work is the finding that the correlation proposed by Vincent et al. (42) between $\lambda_{\text{max}}$ and percentage random structure, as found for "high-salt" and "low-salt" BSPAP (26, 42, 54, 55), is apparently not applicable to recHPAP.

The crystal structures of two mammalian PAPs in which the loop region is present and well ordered have recently been reported: Uf at 1.5 Å resolution (24), and one form of rat bone PAP at 2.2 Å resolution (30). Both structures show that certain residues in the loop between positions 143 and 160 interact with the active site, and suggest a molecular basis for the changes in catalytic properties that occur upon proteolytic cleavage. Particularly noteworthy are residues Ser145 and Asp146, which are situated near the active site with their side chains directed towards the binuclear metal center. In the following discussion, distances cited are for the Uf crystal structure (24), which has the highest resolution of the mammalian PAP structures, while the residue numbers correspond to the human sequence (10). Within experimental error, there are no significant differences in the rat bone structure in which the residues of the loop region are resolved (30). The interaction between Ser145 and Asn91 seems unlikely to be important in the activation process, however, this residue is resolved in the same position in both RPAP structures, with and without the loop region (25, 30). Furthermore, the orientation of the serine hydroxyl group is not favorable for hydrogen bonding to the NH$_2$ group of the Asn side chain. Hence, the following discussion focuses on the role of Asp146.

The carboxylate group of Asp146 is in very close proximity to Asn91, with one oxygen
atom only 2.9 Å from the amido nitrogen, which is well within hydrogen bonding distance. The side chain of Asn91, which is conserved in all PAP and PP sequences, provides one of the ligands to the divalent metal site. Although mutagenesis studies aimed at elucidating the role of this residue have not yet been reported for any PAP, such studies have been performed on the closely related PP's (56-58), and the results are consistent with an important role for Asn91 in both substrate binding and catalysis. As shown in Figure 2.5, Asp146 should interact strongly with Asn91. In valence bond terms, the interaction between Asp146 and Asn91 stabilizes the amide resonance structure containing a C-N double bond, thereby polarizing the carbonyl oxygen and making it a better metal ligand. This will in turn decrease the Lewis acidity of the Fe$^{2+}$ ion, which is presumed to bind and activate the phosphate ester substrate. Ser145 can also interact with Asn91, thereby enhancing this effect. Cleavage of the loop and concomitant removal of these residues would eliminate these interactions.

The carboxylate group of Asp146 can also interact with His92, with a carboxylate oxygen-imidazole nitrogen distance of only 3.6 Å. Similarly, in PP1 and PP2B the histidine corresponding to His92 is within 5 Å of both metals (20-23); consequently, it has been proposed to be important in catalysis. Site-directed mutagenesis of phage λPP (59), in which this residue is also conserved, suggested that this histidine plays a role in base catalysis (59-61), but this conclusion has not yet been confirmed by isotope effect studies (62). A nearby Asp residue is also believed to be involved in catalysis, because site directed mutagenesis of this residue to Asn or Ala (56, 58, 60) resulted in a 36 or 71 fold decrease, respectively, in catalytic activity. It has been proposed that the interaction of this histidine/aspartate pair with a solvent molecule and the metal ion could be thought of as a His-Asp-M$^{n+}$-$\text{H}_2\text{O}$ "catalytic tetrad". In single polypeptide recHPAP, Asp146 is the only carboxylate in the vicinity of His92, other than the bridging ligand Asp52. It could, therefore, be part of a similar "catalytic tetrad". Upon cleavage of the loop, the interaction between His92 and Asp146 would be disrupted, decreasing the basicity of the His92 side chain. As a result, His92 will interact less strongly with the water bound to the ferric ion. The changed interaction between Fe$^{3+}$ and bound water.

Figure 2.5: The interaction of the residues Asn91, His92, and Asp146 of the mammalian purple acid phosphatase Uf (1Ute) with distances in Å (24).
would also be expected to affect the EPR spectrum.

The picture that emerges from this and other studies of PAPs is that of enzymes that, as synthesized, contain specific interactions that diminish their catalytic activity. Because PAPs are generally located in organelles that are rich in proteases, the possibility arises that at least some organisms and/or tissues utilize these proteases to release the full intrinsic activity of PAPs. For example, cathepsins B, H, K, and L are capable of cleaving PAP and are present in osteoclasts (27), which are known to contain PAP. In addition, purification of human osteoclast PAP in the presence of protease inhibitors resulted in a two-subunit PAP (63) similar to BSPAP, suggesting that cleavage was not an artifact of the purification (26). To date, however, there is no compelling evidence that proteolysis constitutes an important mechanism for controlling PAP activity in vivo. In fact, TCA precipitation of fresh samples of bovine spleen tissue followed by electrophoresis and staining showed that the major form of BSPAP in vivo was the intact 36 kDa polypeptide (26). These results do not, however, preclude the possibility that differentially processed forms of PAP may be present in different tissues (7).

In summary, our results strongly suggest that the kinetics and spectroscopic differences reported thus far for mammalian PAPs from different sources are due to variations in the site and extent of proteolytic removal of an exposed loop near the active site of the protein. The interaction between a conserved Asp residue in the loop region and active site residues is suggested to be responsible for the differences in catalytic and spectroscopic properties observed between PAPs isolated as single polypeptide chains and those isolated in proteolytically cleaved form. Site-directed mutagenesis studies of residues in the loop region (e.g., Asp146) are in progress.

Acknowledgements

We thank Dr. A.R. Hayman and Prof. T.M. Cox from the Medical School of the University of Cambridge (UK) for providing the recombinant baculovirus, M. de Vocht from the University of Groningen for his help and advice with CD spectroscopy, Dr. W.J. de Grip for providing facilities to produce protein on a large scale, S. Knol for the determination of some kinetics constants, and Dr. M. Merkx for helpful advice.

References

Chapter 2

Proteolytic activation