Tartrate-resistant purple acid phosphatase is synthesized as a latent proenzyme and activated by cysteine proteinases

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Purple acid phosphatases (PAPs) are binuclear acid metallohydrolases also referred to as tartrate-resistant acid phosphatases (TRAPs) or type 5 acid phosphatases. The cDNA sequences of TRAP/PAP enzymes from different species and organs indicate that these enzymes are translated as monomeric polypeptides of approx. 35 kDa, contrasting with the predominantly two-subunit structure observed in purified enzyme preparations. In the present study we have compared certain structural and enzyme-kinetic properties of recombinant rat PAP (monomeric) with those of the native rat bone TRAP/PAP enzyme (two-subunit), and examined effects on these parameters by cleaving the monomeric recombinant PAP with the serine proteinase trypsin or the cysteine proteinases papain or cathepsin B. Cleavage with trypsin resulted in a moderate activation of the recombinant enzyme and shifted the pH optimum to a slightly more basic value (5.0–5.5). Cleavage with papain resulted in complete activation and conferred similar properties to those of the bone PAP variant with regard to pH optimum (5.5–6.0) and sensitivity to reducing agents, as well as in the sizes of the subunits. Substrate specificity studies showed that the two-subunit bone PAP was considerably more active than the monomeric recombinant rat PAP towards a variety of serine-, threonine- and tyrosine-phosphorylated substrates. Of these substrates, bovine milk osteopontin seemed to be the most readily dephosphorylated substrate. In conclusion, the results suggest that the monomeric form of PAP represent a latent proenzyme with low enzymic activity towards both tyrosine- and serine/threonine-containing phosphorylated substrates. Besides being implicated in the catabolism of the extracellular matrix, members of the cysteine proteinase family might also exert a regulatory role in degradative processes involving the PAP enzymes by converting the newly synthesized PAPs to enzymically active and microenvironmentally regulated species.

Key words: macrophages, osteoclasts, protein processing, type 5 acid phosphatases, uteroferrin.

INTRODUCTION

Purple acid phosphatases (PAPs) are acid metallohydrolases that contain a binuclear $Fe^{3+}M^{2+}$ centre in their active site, where M is Fe or Zn [1–3]. In mammals, these enzymes are also referred to as tartrate-resistant acid phosphatases (TRAPs) (EC 3.1.3.2) or type 5 acid phosphatases [4]. TRAPs are iron-containing, monomeric glycoproteins with a molecular mass of 35–39 kDa [5]. The deduced amino acid sequences of human, rat and mouse TRAPs show a high degree of similarity to the mammalian members of the PAP family, e.g. uteroferrin and bovine spleen PAP [6–9]. Recently, EPR spectroscopic analysis of rat recombinant TRAP [10] has provided compelling evidence that this enzyme also belongs to the PAP family.

Mammalian PAPs contain a FeFe centre, whereas a related PAP from red kidney beans ('KBPAP') has instead a FeZn centre [11]. Moreover, the mammalian protein phosphatases calcineurin (protein phosphatase 2B) [12] and protein phosphatase type 1 ('PP-1') [13] contain binuclear metal centres and also possess a striking similarity to the plant PAP enzyme in the co-ordination environment of the active site. Protein phosphatase type 1 and calcineurin are serine/threonine protein phosphatases, suggesting that PAPs might also function as protein phosphatases. PAP enzymes exhibit a rather broad specificity because these enzymes can dephosphorylate both serine- and tyrosine-bound phosphate moieties in certain phosphopeptides and phosphoproteins [10,14–19]. In humans and rats, PAP enzymes are highly expressed in certain cells of the monocyte–macrophage lineage, such as the bone-resorbing osteoclasts and certain macrophages in spleen, liver and lung [20–23]. Given the broad phosphoamino acid substrate specificity of PAP enzymes, it is conceivable that other factors, such as local availability and proper compartmentation of PAPs with their potential substrates, are other important factors in determining the physiological action of PAPs in biological systems.

The cDNA sequences of TRAP/PAP enzymes from different species and organs all indicate that these enzymes are translated as monomeric polypeptides of 35–37 kDa [7–9,24]. This contrasts with their predominantly two-subunit structure, consisting of a 20–23 kDa N-terminal domain linked through a disulphide bond to a 15–17 kDa C-terminal domain, observed in purified enzyme preparations from a variety of sources including human and rat bone [25,26], giant cell tumours [27] and normal and pathological spleen [28–30]. In contrast, uteroferrin purified from endometrial secretions mostly occurs as the single-subunit form [28,31], as are the recombinant PAPs generated by overexpression with the baculovirus system [10,17,32]. Orlando et al. [29] managed to separate the monomeric and two-subunit variants of PAP from bovine spleen and demonstrated a markedly higher specific enzyme activity associated with the two-subunit form. Moreover, digestion of the single-subunit form with the serine proteinase trypsin generated the 23 and 15 kDa disulphide-linked fragments characteristic of the two-subunit form together with a 3-fold enhancement of enzyme activity. Similar nicking and activation

Abbreviations used: baculo PAP, baculovirus-produced recombinant PAP; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PAP, purple acid phosphatase; PNPP, *p*-nitrophenyl phosphate; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; TRAP, tartrate-resistant acid phosphatase.

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of the non-cleaved purified recombinant human and mouse PAPs were noted after prolonged storage [17].

In the present study we have compared certain structural and enzyme-kinetic properties of recombinant rat PAP (single-subunit) with those of the native TRAP/PAP enzyme (two-subunit) and examined the effects of cleaving the monomeric recombinant PAP with the serine proteinase trypsin or the cysteine proteinase papain. Cysteine proteinases were chosen because enzymes belonging to this family seem to be important in resorptive and degradative processes in cells of the monocyte–macrophage–osteoclast lineage [33–35]. Moreover, the activity of rat recombinant and bone PAP with several different phosphorylated substrates was compared, to assess whether proteolytic modification of PAP influenced substrate specificity.

MATERIALS AND METHODS

Materials

Phosphothreonine (pT), phosphoserine (pS), phosphotyrosine (pY) and *p*-nitrophenyl phosphate (PNPP) were purchased from Sigma. The phosphopeptides RRA(pT)VA, END(pY)INASL and DADE(pY)LIPQQG were from Promega. FRI(pS)HELDS (F9S) and EDEE(pS)EDEE were synthesized by Neosystem Laboratoire (Strasbourg, France). Osteopontin was purified from bovine milk by the procedure described below. DEAE-Sephacel and Phenyl-Sepharose CL-4B were purchased from Pharmacia Biotech (Uppsala, Sweden). Proteinases and proteinase inhibitors were purchased from the following suppliers: papain–agarose (7 units/ml; Pierce), trypsin–agarose (50 units/ml; Sigma), cathepsin B (14.4 units/mg of protein; Anawa, Wangen Zürich, Switzerland), proteinase inhibitor cocktail Complete, Pefabloc, Pepstatin A and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (Boehringer Mannheim, Mannheim, Germany). Materials used for Western blot analysis were: immuno-PVDF membranes (Bio-Rad), colloidal gold (Bio-Rad), alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma) and Nitro Blue Tetrazolium chloride/5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt (Bio-Rad).

Purification of PAP

Purification of baculovirus-produced recombinant PAP (baculo PAP) was performed as described previously [10]. Separation of the monomeric from the cleaved form present in this preparation as a minor constituent was achieved by using a Resource S cation-exchange chromatography column attached to an Äkta Explorer 10XT HPLC system (Amersham–Pharmacia Biotech.). Details of this procedure are available from the authors.

Bone PAP was purified from the long bones of forty 3-week-old Sprague–Dawley rats. All operations were performed at 4 °C. The dissected bones were freed from soft tissue and cartilage ends, cut into small pieces and placed in homogenization solution [0.15 M KCl/0.1 % (v/v) Triton X-100] (3 ml/g of bone) with the proteinase inhibitors Pefabloc (1 mg/ml), Pepstatin A (10 μg/ml) and E-64 (10 μg/ml), and 5 mM EDTA. A Polytron homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.) was used for homogenization at 4 °C for 10 s, with 1 min intervals, until a homogeneous suspension was achieved. The homogenate was cleared by centrifugation at 3200 ***g*** for 30 min. Protamine sulfate (5 %) was added dropwise to the supernatant, with continuous stirring, to a final concentration of 0.5 %; stirring was continued for a further 30 min. The suspension was centrifuged for 30 min at 3200 ***g*** and the supernatant was adjusted to pH 6.5. The supernatant was loaded on a CM-cellulose column; subsequent purification steps in the presence of proteinase inhibitors were performed as described previously [10].

Proteolytic digestion of PAP

Baculo PAP or bone PAP (25 μg) was digested with 100 μl (0.7 unit) of papain–agarose in 500 μl of incubation solution [10 mM sodium acetate (pH 4.6)/0.1 % (v/v) Triton X-100/2 mM dithiothreitol]. Incubation was performed at room temperature for 24 h, with constant mixing of the gel. Baculo PAP or bone PAP (25 μg) was digested with 100 μl (5 units) of trypsin–agarose in 500 μl of incubation solution [10 mM Tris/HCl (pH 7.0)/0.1 % (v/v) Triton X-100]. Incubation was performed at room temperature for 1 h, with constant mixing. The proteolytic digestions above were stopped by centrifugation and the cleavage products of PAP in the supernatant were analysed further. The digestion of baculo PAP or bone PAP with cathepsin B was performed with (final concentrations) 10 ng/μl PAP, 0.4 m-unit/μl cathepsin B, 2 mM dithiothreitol, 50 mM sodium acetate and 1 mM EDTA, pH 5.5. The incubation was performed at 37 °C for 24 h and digestion was stopped with proteinase inhibitor cocktail Complete in accordance with the instructions of the manufacturer.

Purification of milk osteopontin

Osteopontin was purified from bovine milk essentially as published [36]. In brief, 1 litre of fresh raw milk was centrifuged for 15 min at 1250 ***g***; the non-fatty part was collected and mixed with DEAE-Sephacel and rotated overnight at 4 °C. The slurry was first washed by centrifugation with 1.1 litre of 0.2 M NaCl in 10 mM phosphate buffer, pH 7.4, and then with 600 ml of 0.25 M NaCl in the same buffer. The gel was poured into a column and eluted with 0.3 M NaCl in 10 mM sodium phosphate buffer, pH 7.4. The protein peak was pooled and adjusted to 4 M NaCl before being applied to a Phenyl-Sepharose column (30 ml). After a wash with 4 M NaCl in 10 mM sodium phosphate buffer, pH 7.4, protein was eluted with 2 M NaCl in the same buffer. The protein peak was pooled, adjusted to 5 M NaCl and applied to a smaller (5 ml) Phenyl-Sepharose column. After a rigorous wash with equilibration buffer, the protein was eluted with 2 M NaCl in 10 mM sodium phosphate buffer, pH 7.4. The protein peak was pooled and the elution buffer was replaced with TBS [137 mM NaCl/2 mM KCl/25 mM Tris/HCl (pH 7.4)] by ultrafiltration with an Amicon cell equipped with a YM 10 filter. Approximately 5 mg protein/litre of milk was recovered, as determined by amino acid analysis. The identity of the isolated protein with osteopontin was confirmed by N-terminal sequence analysis; purity was estimated to be 85–90 %.

PAP activity assays

p-Nitrophenyl-phosphophatase activity was assayed in 96-well plates with PNPP as substrate in an incubation medium (150 μl) containing (final concentrations): 10 mM PNPP, 0.1 M sodium acetate, pH 5.8, 0.15 M KCl, 0.1 % (v/v) Triton X-100, 10 mM sodium tartrate, 1 mM ascorbic acid and 0.1 mM $FeCl_3$. The *p*-nitrophenol liberated after 1 h of incubation at 37 °C was converted into *p*-nitrophenylate by the addition of 100 μl of 0.3 M NaOH, and the absorbance was read at 405 nm with a Spectramax 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.).

One unit of *p*-nitrophenyl-phosphatase activity is defined as 1 μmol of *p*-nitrophenol (or PO_4) liberated per minute at 37 °C.

The phosphatase assay with the substrates pT (1–10 mM), pS (1–15 mM), pY (0.25–6 mM), RRA(pT)VA (0.1–1 mM), END-(pY)INASL (0.04–0.1 mM), DADE(pY)LIPQQG (0.04–0.1 mM), FRI(pS)HELDS (0.02–0.1 mM), EDEE(pS)EDEE (0.05–0.1 mM) and osteopontin (0.002–0.02 mM) was performed essentially as described [37]. For all substrates, the enzyme concentration was adjusted to give a linear increase in product formation over the incubation period. The substrates were dissolved in incubation medium containing the same buffer as in the PNPP assay, with the exception that pH was lowered to the optimal pH 5.0 for END(pY)INASL, DADE(pY)LIPQQG, EDEE(pS)EDEE and osteopontin. After 1 h of incubation (final volume 50 μl) at 37 °C, the assay was stopped by the addition of 50 μl of colour reagent [0.12% Malachite Green in 3 M H_2SO_4/7.5% ammonium molybdate/11% (v/v) Tween-20 (10:2.5:0.2, by vol.)]. A phosphate standard curve (0–2 nmol) was always run in parallel. After colour development for 10 min, the absorbance at 630 nm was measured with a Spectramax 250 spectrophotometer.

Immunoblot analysis

SDS/PAGE was performed under reducing conditions essentially as described by Laemmli [38]. Proteins were blotted to immuno-PVDF membranes. Colloidal gold was used for protein staining. Immunoblots were probed with polyclonal antiserum (diluted 1:100) raised in rabbits with the use of rat recombinant PAP as the immunogen [10] and with alkaline-phosphatase-conjugated goat anti-rabbit IgG (diluted 1:500) as the secondary antibody. Development was performed with Nitro Blue Tetrazolium chloride/5-bromo-4-chloroindol-3-yl phosphate *p*-toluidine salt. All operations were performed in accordance with the protocols of the manufacturers.

N-terminal amino acid sequence analysis

After electroblotting to PVDF membrane and detection via staining with Coomassie Blue, the protein bands were cut and applied to a Procise cLC sequencer (PE-Applied Biosystems) for Edman degradation. Between 10 and 25 μg of the different PAP preparations were used for sequence analysis.

RESULTS

Purification of rat recombinant PAP and bone PAP

Recombinant PAP was purified from the culture supernatant of recombinant baculovirus-infected cells, as described previously [10]. This preparation contained as a minor constituent a two-subunit form [10], which from densitometric analysis was estimated to account for less than 5% of colloidal-gold-stained protein. We therefore introduced, as a final step, an additional cation-exchange HPLC step to separate the monomeric PAP from the small amount of cleaved form. The purified monomeric PAP used in the experiments exhibited a specific activity of 185 units/mg of protein (Table 1). SDS/PAGE under reducing conditions, electroblotting and immunostaining of blots with a polyclonal antibody generated in rabbits by using the purified recombinant rat PAP as the immunogen [10] showed a single band corresponding to a molecular mass of 37 kDa (Figure 1). The bone PAP was purified from long bones of 3-week-old rats by essentially the same procedure as for the recombinant PAP. In this preparation (Figure 1), which had a specific activity of 1640 units/mg of protein (Table 1), the two-subunit form was the predominant species; the bands at 23 and 16 kDa corresponded to the disulphide-linked fragments contained in the two-subunit form [10]. Thus the PAP enzyme from rat bone was mainly in the fragmented, two-subunit form and exhibited approx. 9-fold higher catalytic activity as well as a lower K_m for PNPP than the monomeric species contained in the recombinant PAP preparation.

Table 1 Enzyme activity of recombinant PAP, recombinant PAP treated with proteinases and bone PAP

Assays were conducted with 50–100 pM PAP enzymes and between 0.1 and 10 mM concentrations of pNPP at pH 5.8. Cleavage with agarose-conjugated papain or agarose-conjugated trypsin was performed as described in the Materials and methods section. Results are means for two or three experiments.

Enzyme	Protease treatment	K_m (mM)	Specific activity (μmol/min per mg of protein)
Baculo PAP	None	3.1	185
	Trypsin	2.1	320
	Papain	1.0	1410
	Cathepsin B	0.9	1700
Bone PAP	None	0.5	1640

Proteolytic cleavage *in vitro* of the monomeric recombinant PAP

It has previously been demonstrated that the monomeric form of bovine spleen PAP can be converted to the two-subunit form by limited proteolytic cleavage with either of the serine proteinases trypsin or chymotrypsin, with a significant increase in enzyme activity [29]. By using trypsin-conjugated agarose beads, a complete conversion of the monomeric recombinant PAP was achieved, with novel bands at 25 (T1), 24 (T2) and 16 (T3) kDa (Figure 1). The cysteine proteinases papain and cathepsin B also resulted in conversion to two-subunit forms with sizes of 25 (P1, C1), 24 (P2), 23 (C2), 21 (P3), 20 (C3) and 16 (P4 and C4) kDa. For comparison, the rat bone PAP preparation contained only the band centred on 23 kDa (B1) together with the 16 kDa band (B2).

To determine the cleavage site(s), an N-terminal sequence analysis of bands cut out from PVDF membranes was performed. In the recombinant monomeric PAP fraction, a single N-terminal sequence TAPAST, corresponding to amino acid residues 1–6 in the mature protein, was detected [9]. In the rat bone PAP, the N-terminal sequence of the 23 kDa B1 fragment was APAST and for the 16 kDa B2 fragment it was RTQLSW, the latter corresponding to residues 163–168 [9]. We have previously noted the discrepancy between the predicted sequence of the mature protein and the actual N-terminal sequence of the purified rat bone enzyme, lacking a N-terminal threonine residue [9,26]. Fragments T1 and T2 generated by trypsin cleavage of the monomeric recombinant PAP started with TAPAS, indicating that these fragments were N-terminal of the cleavage site. The T3 fragment started with DLGVA, corresponding to residues 158–162. The papain-generated fragments P1, P2 and P3 also started with the N-terminal TAPAS sequence, whereas the lighter P4 fragment started with VARTQL, corresponding to residues 161–166.

Effects of proteolytic cleavage *in vitro* on PAP enzymic parameters

Cleavage of recombinant PAP with the proteinases trypsin and papain was associated with an enhancement of enzymic activity with PNPP as the substrate (Table 1). For the trypsin-cleaved PAP, the increase in specific activity was approx. 1.7-fold, with a small decrease in the K_m for PNPP. No further activation was

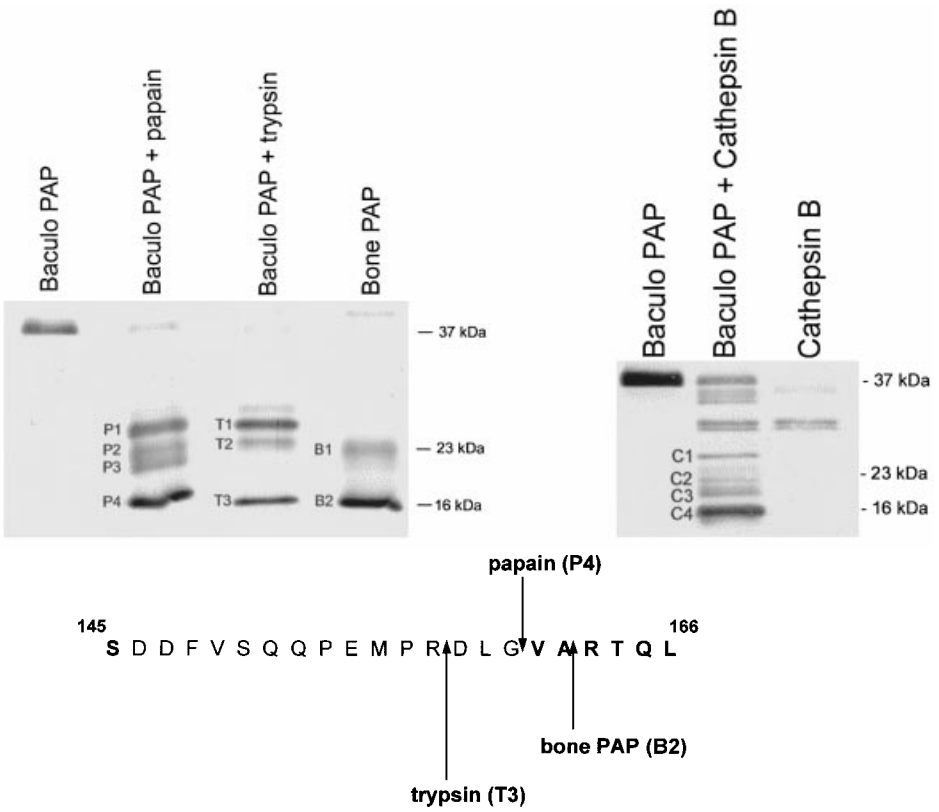

Figure 1 Fragmentation pattern after proteolytic digestion of recombinant PAP compared with that of bone PAP

Baculo PAP was digested with agarose-conjugated papain, agarose-conjugated trypsin or soluble cathepsin B and compared with undigested baculo PAP and bone PAP. PAP (150 ng) was subjected to SDS/PAGE [12% (w/v) gel] under reducing conditions. The proteins were blotted to a PVDF membrane and probed with anti-PAP polyclonal antibodies as described in the Materials and methods section. The figures at the right of the top two panels are the estimated molecular masses of the major PAP bands. The arrows in the partial sequence of rat PAP (lower panel) indicate the start of the N-terminal sequence of the 16 kDa fragment of the different preparations.

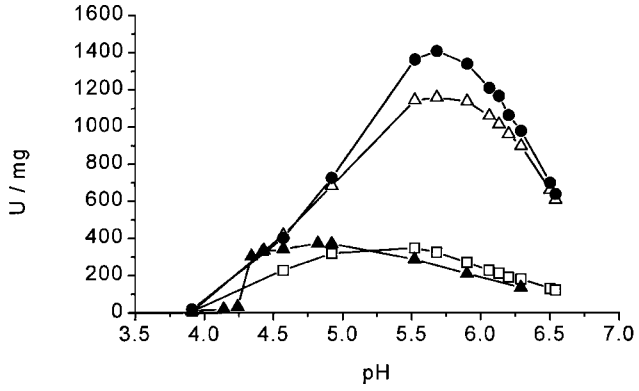

Figure 2 pH dependence of PNPP hydrolysis by intact and proteolytically cleaved PAP

The activity of baculo PAP digested with papain (△) or trypsin (□) was compared with that of undigested baculo PAP (▲) and bone PAP (●) in 0.1 M sodium acetate buffer at various pH values. PAP activity was measured with PNPP as substrate, as described in the Materials and methods section. U, units.

observed by increasing the ratio of trypsin to recombinant PAP to 100 units/mg of PAP (results not shown). Cleavage with papain was associated with a larger increase in PAP activity. This was due to a 7–8-fold increase in the specific activity and a decrease in the K_m to one-third. In addition to papain, another member of the cysteine proteinase family, cathepsin B, was able to activate the recombinant PAP to a similar extent as papain. No additional increase in enzyme activity was detected after the incubation of bone PAP with papain or cathepsin B (results not shown).

The PAPs usually exhibit a pH optimum for the hydrolysis of phosphomonoesters in the range 5.5–6.0 [39]. The recombinant PAP as isolated exhibited a rather broad pH optimum between 4.5 and 5.0, i.e. 1 pH unit lower than that of the rat bone PAP (Figure 2). Interestingly, cleaving the monomeric recombinant PAP with papain or trypsin caused a shift in the optimal pH of PNPP hydrolysis to more basic values: for trypsin this was 5.0–5.5 and for papain 5.5–6.0. This suggests that the protonation of amino acid residues involved in catalysis is affected by limited proteolytic cleavage, presumably induced by conformational changes.

The di-iron-containing PAPs are redox-sensitive enzymes owing to a redox-active M2 site, which when present as the ferrous ion yields a catalytically active enzyme [3]. Consequently, in an oxygen environment the PAPs are present mainly in an inactive diferric form, which can be rapidly activated by the addition of reducing agents such as ascorbate. Whereas the enzyme activity of both monomeric and cleaved forms of PAP was strongly increased by the addition of 1 mM ascorbic acid and 0.1 mM $FeCl_3$ under standard assay conditions (results not

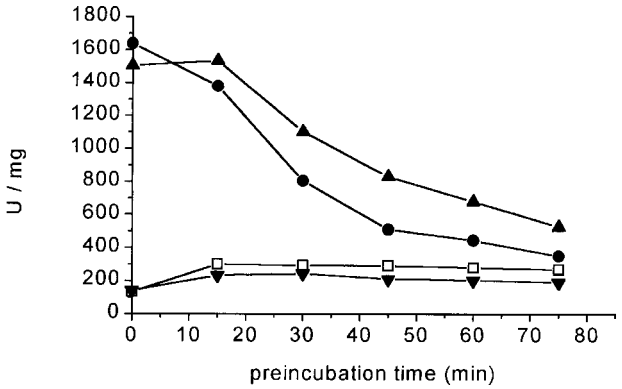

Figure 3 Sensitivity of intact and proteolytically cleaved PAP to reducing agents

Baculo PAP digested with papain (▲) or trypsin (▼) was compared with undigested baculo PAP (□) and bone PAP (●). Ascorbic acid and $FeCl_3$ were used as reducing agents during the preincubation of PAP for the indicated durations with (final concentrations): 1 mM ascorbic acid, 0.1 mM $FeCl_3$, 0.15 M KCl, 10 mM sodium tartrate and 0.1% (v/v) Triton X-100. PAP activity was measured after incubation with PNPP for 10 min as described in the Materials and methods section. U, units.

shown), it was observed (Figure 3) that preincubation (in the absence of the substrate PNPP) with 1 mM ascorbate and 0.1 mM $FeCl_3$ led to a time-dependent inactivation of the two-subunit PAP from rat bone and of recombinant PAP cleaved with papain. In contrast, neither the monomeric recombinant PAP nor two-subunit trypsin-cleaved recombinant PAP exhibited this pattern of inactivation under the same conditions (Figure 3). The inactivation of PAP could possibly be due to a conversion of the mixed-valency active enzyme to an inactive $Fe^{2+}Fe^{2+}$ species, with the concomitant dissociation of iron [40].

Thus proteolytic cleavage with papain might induce structural changes in the PAP molecule that affect the redox sensitivity of the di-iron metal centre.

Substrate specificities of rat recombinant PAP and bone PAP

To establish whether the structural alterations induced by proteolytic cleavage of the monomeric PAP were generally affecting known substrates for PAP and were not restricted to the non-physiological PNPP substrate, we surveyed a number of different phosphoamino acids and phosphopeptides and the phosphoprotein osteopontin as substrates for the PAPs (Table 2). Among the phosphoamino acids, both pS and pT were poor substrates for both forms of PAP, with k_{cat}/K_m values in the range 10^2–10^3 $M^{-1} \cdot s^{-1}$. A similar value was observed with the phosphothreonyl peptide RRA(pT)VA, containing the consensus sequence for protein kinase A [41]. The acidic phosphoseryl peptide EDEE(pS)EDEE, with the consensus sequence for casein kinase II [42], and also the osteopontin peptide FRI(pS)HELDS [43,44] were slightly more effective substrates. In contrast, pY and two different phosphotyrosyl peptides were equally effective as PNPP as substrates with a k_{cat}/K_m ratio between 10^4 and 10^5 $M^{-1} \cdot s^{-1}$. However, the most effective of all substrates tested was the acidic phosphoseryl protein osteopontin from bovine milk. With all substrates, the rat bone enzyme was more active, varying in activity for different substrates between 17-fold and 71-fold that of the recombinant PAP.

DISCUSSION

Mammalian PAPs in different species and organs are encoded by a single mRNA species, suggesting that these proteins are translated as single polypeptides of approx. 35 kDa [5]. In contrast, purified PAP enzyme preparations generally exhibit a more complex protein pattern, with additional protein bands of 20–23 and 15–17 kDa. These bands of lower molecular mass are presumed to be the result of proteolytic nicking in an exposed and highly antigenic loop domain of a mid-portion of the protein, and owing to an intermolecular disulphide bond the resulting fragments remain associated [29]. It is conceivable that such limited proteolysis could occur during enzyme purification but this seems less likely because the inclusion of inhibitors of the main proteinase family members during the purification of PAP from rat bone (the present study) did not prevent the formation of the two-subunit variant.

The study by Orlando et al. [29], where the monomeric and two-subunit forms of PAP from bovine spleen were separated, demonstrated that the two-subunit form exhibited a higher enzyme activity and that cleavage of the monomeric form with the serine proteinase trypsin resulted in a two-subunit form

Table 2 Substrate specificity of recombinant PAP compared with that of PAP isolated from bone

Results are means for two to four experiments for each substrate.

	Baculo PAP		Bone PAP		
Substrate	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	Ratio	k_{cat}/K_m (10^3 $M^{-1} \cdot s^{-1}$)	Ratio	Ratio, bone PAP to baculo PAP
pT	37	1.0	1.1	1.0	30
RRA(pT)VA	89	2.4	1.7	1.5	19
pS	120	3.5	6.5	5.9	50
FRI(pS)HELDS	450	12	17	15	38
EDEE(pS)EDEE	1100	30	19	17	17
pY	6900	190	390	350	57
END(pY)INASL	12000	320	300	270	25
DADE(pY)LIPQQG	9800	265	700	640	71
pNPP	35000	950	760	690	22
OPN	49000	1320	2600	2360	53

with 2–3-fold higher enzyme activity. The present study confirmed that trypsin (and chymotrypsin; results not shown) can activate the monomeric recombinant rat PAP 2-fold, in association with the formation of a two-subunit variant. However, the cysteine proteinases papain and cathepsin B activated the recombinant rat PAP to a larger extent, i.e. approx. 10-fold, also in conjunction with the formation of a two-subunit variant. This implies that the limited proteolytic cleavages with trypsin compared with the cysteine proteinases are not identical. Notably, the larger N-terminal fragment can be processed to slightly lower molecular mass by digestion with papain than by digestion with trypsin, whereas the smaller C-terminal fragment differs by only three amino acid residues. Because the N-termini of the N-terminal fragments (25–20 kDa) are intact, this pattern could indicate that the largest of these fragments (25 kDa) has undergone further processing from the C-terminal end by papain. The apparent discrepancy between the sum of the molecular masses of the fragments and that of the monomeric form could theoretically be explained by cleavage within the 16 kDa subunit. However, because sequencing analysis yielded only a single N-terminal amino acid from the 16 kDa fragment, and no additional fragment smaller than 16 kDa was detected, additional cleavage within the 16 kDa region is unlikely. Instead, anomalous migration in SDS/PAGE is more likely to be the result of the physicochemical properties of the fragments, leading, for instance, to different binding of SDS in comparison with the uncleaved monomeric precursor. We therefore interpret our results to show that not only proteolytic nicking but also the removal of the whole or part of the loop region must be accomplished for the complete activation of monomeric PAP enzymes. In support of this proposal, part of the putative loop region between Ser^{145} and Val^{161} is undetectable in the crystal structure of the two-subunit rat recombinant PAP [45]. That the loop region is also partly undetectable in the protein sequence of the cleaved form of bovine spleen PAP gives additional support to this proposal [46]. This short stretch of amino acids might act in a repressive manner in communication with the active-site residues.

That the active site was affected by proteolytic cleavage was indicated by the shift in pH optimum and redox sensitivity, noted particularly when papain was used for cleavage. By comparison with two-subunit rat PAP purified from bone, it was apparent that papainolytic cleavage converted the recombinant PAP to express similar properties in these respects. It can only be speculated that conferring such redox behaviour on the biologically relevant PAP from rat bone by endogenous cysteine proteinases such as cathepsin B could be part of a regulatory mechanism, serving to make the PAP enzymes active only under certain redox conditions, for instance in the extracellular resorption lacuna between the osteoclast and bone. However, the mechanistic reasons underlying these alterations of active-site chemistry remain to be elucidated.

Another consequence of PAP enzyme activation could be alteration of the substrate specificity. However, a comparison of the activity of the monomeric recombinant PAP with that of the two-subunit bone PAP towards different substrates did not verify this assumption. In all cases, the bone PAP was considerably more active than the recombinant enzyme. However, whereas pS and, in particular, pT, both as free phosphoamino acids and as part of phosphopeptides, seemed to be rather poor substrates, pY and phosphotyrosyl peptides were much more efficient substrates. This corroborates previous studies showing that PAPs are potent phosphotyrosyl phosphatases *in vitro* [15,17,18,47,48]. It has been suggested that the PAPs function as phosphotyrosyl phosphatases in biological systems [48,49]. In considering this possibility, it is important to realize that the PAPs seem to be synthesized on the endoplasmic reticulum and transported intracellularly in luminal compartments, either destined for secretion, as in osteoclasts, or directed to lysosomes, as in macrophages [5]. The luminal compartmentation of PAP enzymes is strikingly different from that of intracellular phosphotyrosine-containing proteins, which are phosphorylated in the cytoplasmic matrix. In addition, as far as we know, there are no extracellular phosphotyrosine modified proteins.

The most readily dephosphorylated substrate among those tested was the acidic protein osteopontin, which is phosphorylated mainly on serine residues [44]. This observation also suggests that the substrate preference of PAPs not only resides in the specificity for phosphoamino acids but is also dictated by other features such as the ionic properties of proteins or peptides. It has been observed in the studies of Robinson and Glew [19] that Gaucher spleen PAP prefers acidic proteins as substrates. PAPs are highly cationic proteins; the net positive surface charge could therefore facilitate the recognition and docking of anionic proteins by electrostatic interactions. If this hypothesis is correct, the ionic properties of the phosphorylated substrate, in combination with a suitable environment for the activated PAP enzymes, could have profound influences on the biological activity of the PAP enzymes.

Bone resorption occurs in a specialized acidic extracellular compartment, namely the resorption lacuna, between the osteoclast and the bone surface. There the bone matrix is thought to be degraded by the concerted action of cysteine proteinases as well as the matrix metalloproteinases [33]. Several cathepsins, notably B, H, L and K, have been demonstrated in osteoclasts, as well as one member of the matrix metalloproteinase family, MMP-9 [50–55]. Our observation that cathepsin B is capable of activating the latent monomeric form of PAP suggests that, besides being implicated in the degradation of the collagen matrix, cathepsin B and/or other cysteine proteinases expressed by osteoclasts could also have a regulatory role in activating a latent PAP enzyme.

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