Comparative studies of rat recombinant purple acid phosphatase and bone tartrate-resistant acid phosphatase

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The tartrate-resistant acid phosphatase (TRAP) of rat osteoclasts has been shown to exhibit high (85–94 %) identity at the amino acid sequence level with the purple acid phosphatase (PAP) from bovine spleen and with pig uteroferrin. These iron-containing purple enzymes contain a binuclear iron centre, with a tyrosinate-to-Fe(III) charge-transfer transition responsible for the purple colour. In the present study, production of rat osteoclast TRAP could be achieved at a level of 4.3 mg/litre of medium using a baculovirus expression system. The enzyme was purified to apparent homogeneity using a combination of cation-exchange, hydrophobic-interaction, lectin-affinity and gel-permeation chromatography steps. The protein as isolated had a purple colour, a specific activity of 428 units/mg of protein and consisted of the single-chain form of molecular mass 34 kDa, with only trace amounts of proteolytically derived subunits. The recombinant enzyme had the ability to dephosphorylate bone matrix phosphoproteins, as previously shown for bone TRAP. Light absorption spectroscopy of the isolated purple enzyme showed a λ_{max} at 544 nm, which upon reduction with ascorbic acid changed to 515 nm, concomitant with the transition to a pink colour. EPR spectroscopic analysis of the reduced enzyme at 3.6 K revealed a typical μ-hydr(oxo)-bridged mixed-valent Fe(II)Fe(III) signal with g-values at 1.96, 1.74 and 1.60, proving that recombinant rat TRAP belongs to the family of PAPs. To validate the use of recombinant PAP in substituting for the rat bone counterpart in functional studies, various comparative studies were carried out. The enzyme isolated from bone exhibited a lower K_m for *p*-nitrophenyl phosphate and was slightly more sensitive to PAP inhibitors such as molybdate, tungstate, arsenate and phosphate. In contrast with the recombinant enzyme, TRAP from bone was isolated predominantly as the proteolytically cleaved, two-subunit, form. Both the recombinant enzyme and rat bone TRAP were shown to be substituted with N-linked oligosaccharides. A slightly higher apparent molecular mass of the monomeric form and N-terminal chain of bone TRAP compared with the recombinant enzyme could not be accounted for by differential N-glycosylation. Despite differences in specific post-translational modifications, the recombinant PAP should be useful in future studies on the properties and regulation of the mammalian PAP enzyme.

INTRODUCTION

Purple acid phosphatases (PAPs) constitute a family of enzymes, with pig uteroferrin and bovine spleen PAP as the most well characterized members [1–5]. These two PAPs contain an antiferromagnetically spin-coupled binuclear iron centre that exists in two stable interconvertible states: pink, reduced, EPR-visible and enzymically active, with a mixed-valent Fe(II)–Fe(III) cluster; and purple, oxidized, EPR-silent and catalytically inactive, with the binuclear pair as Fe(III)–Fe(III) [3,6,7]. Furthermore, the PAP members are related to a superfamily of μ-(hydr)oxo-bridged binuclear iron proteins, including haemerythrin, the R2-subunit of ribonucleotide reductase, methane mono-oxygenase hydroxylase and others [8]. All members of this superfamily of iron–oxygen proteins contain a binuclear iron centre, but they have different functions. Among the PAPs, uteroferrin is thought to serve a physiological role as an iron-transport protein in the fetal pig [9,10], whereas the role of spleen PAP is less well understood. The localization of splenic PAP to macrophage lysosomes [11] as well as the catalytic generation of hydroxyl radicals by the enzyme [12] may point to a role in the oxygen-dependent degradation of phagocytosed material.

Type 5 tartrate-resistant acid phosphatases (TRAPs) (EC 3.1.3.2) are iron-containing cationic glycoproteins with molecular masses of around 35 kDa and a monomeric peptide structure [6,13]. In the rat, TRAP has been detected in a wide variety of tissues as a minor acid phosphatase isoenzyme; however, it is highly expressed in the osteoclasts of growing bone, where bone is rapidly resorbed [14]. The deduced amino acid sequence of osteoclast TRAP shows a high degree of identity with members of the PAP family [14]; furthermore, only a single gene encoding this acid phosphatase has been detected in the human, mouse and pig genomes [15–17]. Recently the three-dimensional structure of a PAP from kidney beans was determined [18], and shows a close structural similarity to the mammalian PAP enzyme uteroferrin in the positioning of the amino acid residues ligating the bimetal centre [19]. Furthermore, the mammalian protein phosphatase calcineurin (type 2B) [20] and a protein phosphatase type 1 [21] contain a binuclear metal centre and a $\beta\alpha\beta\alpha\beta$ motif in the active site similar to that in the plant PAP enzyme. These two latter enzymes are serine/threonine protein phosphatases, suggesting that PAP also functions as a protein phosphatase.

Although widely used as a marker for osteoclasts, the physiological role of TRAP in bone resorption is still not known. Bone TRAP can dephosphorylate certain bone matrix phosphoproteins, such as osteopontin [22], which is known to be an anchor for the binding of the osteoclast via integrin receptors to the bone [23,24], and bone sialoprotein [22]. Dephosphorylation of osteopontin was shown to impair the capacity of this protein to promote osteoclast attachment *in vitro* [22], suggesting that

Abbreviations used: CK II, casein kinase II; Endo H, endoglycosidase H; PAP, purple acid phosphatase; pfu, plaque-forming units; Sf9 cells, *Spodoptera frugiperda* cells; TRAP; tartrate-resistant acid phosphatase.

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one potential physiological function of TRAP could be to regulate osteoclast attachment to, or motility on, bone.

In order to explore further the physiological role and the structural features of the bone TRAP enzyme, a baculovirus system was chosen in which to express the protein in large amounts. Using this system, Hayman and Cox [12] produced human TRAP from a cDNA cloned from a human placenta library. The isolated 35 kDa enzyme exhibited a purple colour and contained 2 iron atoms/molecule. However, spectroscopic evidence that their TRAP enzyme belonged to the bi-iron PAPs was not presented. In the present study, infection of *Spodoptera frugiperda* (Sf9) insect cells with a recombinant baculovirus-containing cDNA encoding TRAP from rat bone osteoclasts resulted in the generation of large amounts of active TRAP in the medium, which, like uteroferrin and PAP from bovine spleen, exhibit spectroscopic properties consistent with the presence of an iron centre typical of the PAPs.

MATERIALS AND METHODS

Expression of recombinant PAP in Sf9 cells

Vector construction

A 1397 bp cDNA fragment encoding TRAP from rat bone [14], ligated into the *Eco*RI site of plasmid pT7T3 19U (Pharmacia LKB Biotechnology Inc.), was excised from the vector with restriction endonuclease *Eco*RI, isolated from a low-melting-point agarose gel and ligated into the *Eco*RI site of baculovirus transfer vector pVL1392 (Invitrogen), to give pVL/TRAP.

Generation of recombinant virus

Recombinant virus was generated by co-transfection with plasmid pVL/TRAP and linearized *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold; Pharmingen) into semi-confluent Sf9 cells employing Lipofectin (BRL) transfection. Virus-containing medium was collected at 72 h post-transfection and used for plaque assay. After two rounds of plaque purification, candidate plaques were used to infect Sf9 cells. The medium and Sf9 cell extracts were assayed for TRAP activity at 48 h post-infection, and a recombinant virus clone capable of producing a high level of medium TRAP was selected.

Virus amplification

The selected recombinant virus clone was amplified by infection of approx. 5×10^5 Sf9 cells/ml with 0.1 plaque-forming unit (pfu)/cell in spinner cultures using CCM3 serum-free medium (HyClone) for 6 days at 27 °C. After the amplification step, the virus titre was determined to 10^9 pfu/ml by plaque assay.

Infection of Sf9 suspension cultures

To determine optimal infection conditions, Sf9 insect cells (25 ml) were grown to 1 or 3×10^6 cells/ml in 125 ml Erlenmeyer flasks at 27 °C rotating at 140 rev./min. CCM3 and Sf900II (Gibco) media were used, and the cells were infected with either 0.5 (low titre) or 16 (high titre) pfu/cell.

Large-scale expression of recombinant PAP

For large-scale expression of recombinant PAP, 3×10^5 Sf9 insect cells/ml were inoculated in a Belach fermentor containing 11 litres of CCM3 medium plus 50 mg/l gentamicin (Sigma), 0.1 % Pluronic F-68 (Sigma) and 40 p.p.m. Antifoam C (Sigma) and grown at 27 °C to 3×10^6 cells/ml, followed by infection with approx. 0.5 pfu/cell. At day 5, the cell suspension was centrifuged at 1000 ***g*** for 35 min and the supernatant was collected.

Purification of recombinant PAP

Except where indicated, all operations were performed at 4 °C. The harvested medium (10 litres) from day 5 after infection of Sf9 cells was concentrated to 1.2 litres using a Prep/Scale cassette system (cut-off 10 kDa; 0.23 m^2; Millipore). Protamine sulphate (5 %) was then added dropwise under continuous stirring to a final concentration of 0.5 %. The suspension was adjusted to pH 6.5 with acetic acid and, after overnight precipitation of insoluble material, centrifuged at 1000 ***g*** for 10 min. The supernatant was pumped on to a CM-cellulose (1600 ml; CM-52; Whatman) column previously equilibrated with 0.1 M sodium acetate buffer, pH 6.5. The column was washed with 3 column vol. of equilibration buffer before applying a linear gradient (from 0.1 to 0.5 M) of sodium acetate buffer, pH 6.5. The fractions containing TRAP activity were pooled, followed by the addition of solid ammonium sulphate to a final concentration of 1.1 M. The preparation was subsequently loaded on a phenyl-Sepharose column (35 ml) equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 1.1 M ammonium sulphate. The column was washed with 100 ml of equilibration buffer before starting a 550 ml linear gradient from 1.1 M ammonium sulphate in 0.05 M sodium acetate buffer, pH 5.0, to 0.05 M sodium acetate buffer, pH 5.0. Fractions containing TRAP activity were pooled and, after addition of Triton X-100 to a final concentration of 0.01 %, loaded on a concanavalin A–Sepharose 4B column (25 ml) equilibrated with 0.025 M sodium acetate buffer, pH 6.5, containing 0.5 M KCl. The column was washed with 3 vol. of equilibration buffer before elution with 0.5 M methyl α-mannoside and 30 % ethylene glycol in equilibration buffer at room temperature. TRAP-containing fractions were pooled and concentrated using an Amicon ultrafiltration cell equipped with a YM 10 filter before loading the sample on a Sephacryl S-200 column (93 cm × 1.6 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.8, containing 0.1 M KCl. The TRAP enzyme pool was concentrated by ultrafiltration to 1 ml. Protein concentrations were estimated after trichloroacetic acid precipitation by a modified Lowry method [25].

TRAP was purified from rat bone as described previously [26].

Assay procedures

TRAP activity was assayed in 96-well plates using *p*-nitrophenyl phosphate as substrate in an incubation medium (150 μl) containing (final concentrations): 10 mM *p*-nitrophenyl phosphate (di/tris salt; Sigma), 0.1 M sodium acetate buffer, pH 5.8, 0.2 M KCl, 0.1 % 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*-nitrophenolate by the addition of 100 μl of 0.1 M NaOH, and the absorbance at 405 nm was read using a Titre-Tek Multiscan Plus (Flow Laboratories) spectrophotometer. One unit of TRAP activity corresponds to 1 μmol of *p*-nitrophenol liberated per min at 37 °C.

The phosphoprotein phosphatase activity of purified recombinant PAP was assayed using purified bovine sialoprotein or bovine osteopontin as substrate. The sialoproteins were purified as described [27], and were kindly provided by Professor Dick Heinegård, University of Lund, Sweden. The dephosphorylation reaction was performed in an incubation medium (25 μl) containing (final concentrations) 10 μg of phosphoprotein substrate, 1 unit of recombinant PAP, 0.1 M sodium acetate buffer, pH 5.8, 0.2 M KCl, 0.1 % Triton X-100, 10 mM sodium tartrate, 1 mM

ascorbic acid and 0.1 mM $FeCl_3$, for 4 h at 37 °C. Then the substrates were rephosphorylated by human recombinant casein kinase II (CK II) (Boehringer-Mannheim) for 1 h at 37 °C by adding 0.2 m-unit of CK II together with 250 nmol of [γ-^{33}P]ATP (2000 Ci/mmol) in a phosphorylation buffer (200 μl) containing 20 mM phosphate buffer, pH 7.4, 80 mM KCl, 5 mM $MgCl_2$ and 5 mM dithioerythritol. ^{33}P incorporated into phosphoprotein was separated from free ^{33}P on a PD-10 column (Pharmacia) pre-equilibrated with phosphorylation buffer. Fractions (250 μl) were collected and radioactivity was counted in a β-scintillation counter.

Electrophoresis and blotting

SDS/PAGE was carried out essentially by the method of Laemmli [28]. Proteins were electroblotted to nitrocellulose filters and protein-stained with Colloidal Gold (Bio-Rad) using the protocol of the manufacturer. In some experiments the filters were stained with polyclonal antibodies raised in rabbits (see below). The primary antibodies were diluted (1:100) and incubated with blotted proteins for 2 h at room temperature. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) diluted 1:500 was used as secondary antibody. Rainbow markers (Amersham) were used as molecular mass standards.

Antibody preparation

New Zealand rabbits (3 months old; AntiCimex) were used. Immunization was initiated by the injection of 100 μg of recombinant PAP emulsified in Freund's complete adjuvant at multiple subcutaneous sites in the neck. Booster injections were given at 4-week intervals with 100 μg of antigen emulsified in Freund's incomplete adjuvant. Animals were bled 2 weeks after the booster injections, and serum was collected.

Protein deglycosylation

Deglycosylation of purified recombinant PAP (75 ng and 2 μg for monomeric and two-subunit forms respectively) or purified bone TRAP (150 ng and 200 ng for monomeric and two-subunit forms respectively) with 300 m-units of recombinant N-Glycanase (Genzyme) was initiated by boiling the sample for 5 min in 0.1 M Tris buffer, pH 7.6, 0.5% SDS and 50 mM β-mercaptoethanol. After addition of Nonidet P-40 to a final concentration of 1.25% and 300 m-units of N-Glycanase, the mixture was incubated at 37 °C for 16 h. Before incubation with endoglycosidase H (Endo H), purified recombinant PAP or bone TRAP was boiled for 3 min in the presence of 0.05% SDS and 60 mM β-mercaptoethanol, followed by the addition of 1 m-unit of recombinant Endo H (Genzyme) and sodium citrate buffer, pH 5.7, to a final concentration of 50 mM, and incubation for 16 h at 37 °C.

Determination of iron content

The iron content of the purified recombinant PAP was determined by atomic absorption spectroscopy using a Varian Spectra AA 800 GTA 100 atomic absorption spectrometer at 248.3 nm. Correction for background was performed according to the Zeeman technique. The detection limit was 1 ng of Fe/ml. The analysis was performed by Analytica AB (Taby, Sweden).

Optical characterization

Visible spectra were obtained on a Perkin–Elmer lambda 2 spectrophotometer using PECSS software. The recombinant PAP protein was reduced with 1 mM ascorbic acid and 0.1 mM $FeCl_3$ at room temperature. The treatment was stopped after 2 h when no further shift in light absorption maximum was observed and the colour had changed from purple to pink.

EPR characterization

The sample analysed for light absorption spectra was subsequently frozen in liquid N_2 in 4 mm EPR tubes and used for EPR characterization. Other samples were reduced with higher concentrations of reductants and subsequently subjected to gel filtration on G-25 Sephadex at 4 °C and then frozen in EPR tubes. EPR spectra were obtained at 3.6 K with a Bruker ESP300 spectrometer with an Oxford ESR 900 helium flow cryostat at 9.32 or at 9.62 GHz, 100 KHz, 0.1 mT modulation of amplitude. Quantification of the PAP samples incubated with 1 mM ascorbate and 0.1 mM iron was carried out under non-microwave saturation conditions relative to 1 mM Cu and corrected by the method of Aasa and Vänngård [29].

RESULTS AND DISCUSSION

Expression of recombinant PAP in Sf9 cells

When recombinant baculovirus containing cDNA encoding rat bone TRAP was used to infect Sf9 insect cells, catalytically active TRAP could be detected in the serum-free CCM3 medium 2 days after infection, reaching a maximum level 6 days after infection. No difference in production efficiency could be detected using different serum-free media (CCM3 and Sf 900II) or on infection with high or low titres (16 or 0.5 pfu/cell respectvely) of recombinant virus. A linear correlation between medium TRAP activity and cell density was achieved up to 3×10^6 cells/ml. Media from non-infected or wild-type virus-infected Sf9 cells did not contain any TRAP activity. Production was stopped at 5 days post-infection, when no viable cells were present, to minimize proteolytic degradation of the enzyme.

Purification of recombinant PAP

TRAP activity was purified from 10 litres of medium containing the recombinant baculovirus-infected Sf9 cells. The purification procedure involved as major steps protamine sulphate precipitation, ion-exchange chromatography (CM-cellulose), hydrophobic binding chromatography (phenyl-Sepharose), affinity chromatography (concanavalin A–Sepharose) and finally gel filtration (Sephacryl S-200). Table 1 shows the total units, specific activity and yield of iron- and ascorbate-activated TRAP activity (mean values of two preparations) after the different purification

Table 1 Purification of recombinant PAP expressed in a baculovirus system

One unit of iron- and ascorbate-stimulated TRAP activity is defined as 1 μmol of *p*-nitrophenol formed/min.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Medium (10 litres)	18300	10800	1.7	100
Protamine sulphate	17000	6400	2.7	93
CM-cellulose	13800	405	34	75
Phenyl-Sepharose	13800	89	155	75
Concanavalin A–Sepharose	9200	25	370	50
Sephacryl S-200	7700	18	428	42

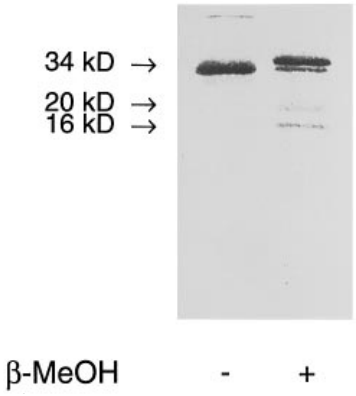

Figure 1 SDS/PAGE of purified recombinant PAP

Samples of 2 μg of purified recombinant medium PAP were subjected to SDS/15%-PAGE. After electrophoresis, the gel was blotted on to a nitrocellulose filter and stained for protein with Colloidal Gold. The samples were boiled in SDS-containing sample buffer in the absence (−) or in the presence (+) of β-mercaptoethanol (β-MeOH). Molecular mass standards (kDa) were electrophoresed in separate lanes.

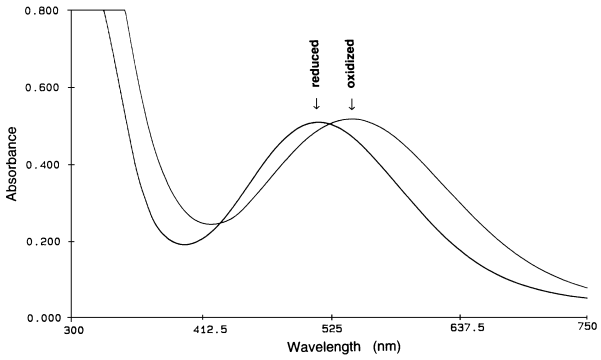

Figure 2 Light absorption spectra of oxidized and reduced recombinant PAP

Electronic absorption spectra are shown of 6 mg/ml (175 μM) purified recombinant PAP in 0.1 M sodium acetate buffer, pH 5.8, and 0.1 M KCl. The absorption maximum at 544 nm shifted gradually after treatment with 1 mM ascorbic acid and 0.1 mM $FeCl_3$ at 25 °C. After 2 h of reducing treatment, no further shift was observed (515 nm).

steps using the standard TRAP assay, where 1 unit corresponds to 1 μmol of *p*-nitrophenol liberated per min at 37 °C. The specific activity of the purified enzyme after the final gel-filtration step was 428 units/mg of protein, with a yield of 42%. This is comparable with the value of 408 units/mg reported for the native, single-chain, form of bovine spleen PAP [30], and that of 320–350 units/mg for pig uteroferrin [10,30,31]. Since a total of 18 mg of enzyme protein was recovered, the expression level was estimated to 4.3 mg/litre of medium.

Characterization of recombinant PAP

SDS/PAGE

To examine the purity of the TRAP preparation and for determination of the molecular mass of the purified protein, a 2 μg aliquot was subjected to SDS/15%-PAGE in the absence or presence of β-mercaptoethanol as the disulphide reductant. The proteins were transferred to nitrocellulose sheets and stained for protein using Colloidal Gold (Figure 1). The preparation contained, under non-reducing conditions, a single band with a molecular mass of 34 kDa. This is comparable with the theoretical molecular mass of 34350 Da calculated from the deduced amino acid sequence of the rat enzyme [14]. A faint band around 64 kDa was observed under non-reducing conditions, but was not detectable after reduction. In the reduced sample, the major band was slightly retarded, corresponding to a molecular mass of 37 kDa, and two faint bands at 20 and 16 kDa were also present. In addition, a faint band was observed at the position of the major 34 kDa band of the non-reduced sample, presumably indicating incomplete disulphide reduction.

It was evident that only a minor proportion of the recombinant PAP molecules existed as two smaller 'subunits', at 20 and 16 kDa, upon disulphide reduction. These subunits correspond to the N-and C-terminal parts of the protein respectively [32]. This is in contrast with TRAP purified from rat bone [26] or bovine spleen [6,30], where the majority of the purified enzyme molecules exist in the two-chain form, presumably as a result of limited proteolytic cleavage [30]. Thus the recombinant rat osteoclast PAP is maintained as the monomeric, non-cleaved, molecule.

Determination of iron content

The iron content of purified recombinant TRAP, as determined by atomic absorption spectroscopy, was 2.5 μg of iron/mg of protein. Assuming a molecular mass of 37 kDa for the TRAP enzyme, this corresponds to 1.7 mol of Fe/mol of enzyme.

Light absorption spectroscopy

TRAP isolated from the medium of baculovirus-infected cells is a violet/purple protein, like the PAPs. The A_{280}/A_{515} ratio was calculated as 13.8, in agreement with that for other PAP forms [7,30,33]. The PAPs so far characterized, e.g. bovine spleen PAP and uteroferrin, commonly exhibit a visible absorption maximum at 545–550 nm and a purple colour in the oxidized/inactive form, which shifts to 505–515 nm and a pink colour in the reduced/active form [2,4,6,9,30,31,34–39]. The light absorption spectra of isolated TRAP (Figure 2) exhibit an intense absorption maximum at 544 nm ($\epsilon = 3.1\ cm^{-1} \cdot mM^{-1}$). It has been shown for the PAPs, using resonance Raman techniques [1–3,34,40] and from the three-dimensional structure [18], that the origin of this strong absorption band is a charge-transfer transition from a tyrosinate to Fe(III). Therefore we conclude by analogy that recombinant TRAP also contains such an iron centre.

After incubation with 0.1 mM $FeCl_3$ and 1 mM ascorbic acid, the enzyme gradually became pink over a 2 h period at room temperature and the light absorption maximum shifted to 515 nm, with one stable isosbestic point at 524 nm (Figure 2). This is a typical reaction for the PAPs and is associated with the reduction of the diferric enzyme to its mixed-valent Fe(II)Fe(III) form (see below). Interestingly, the results of Hayman and Cox [12] in their studies of human placental TRAP produced in a baculovirus expression system are not entirely consistent with this common pattern of light absorption characteristics. In their study, the human TRAP protein (0.6 mg of enzyme) exhibited a λ_{max} at 537 nm that shifted to 531 nm after reduction by 15 mM ascorbic acid, with the protein retaining a purple colour even after reduction. Although it is difficult at this point to define the structural basis for the discrepancy between the two preparations, it remains possible that factors important for the structure of the iron centre are different between the two baculovirus-produced enzymes. In conclusion, the purple and

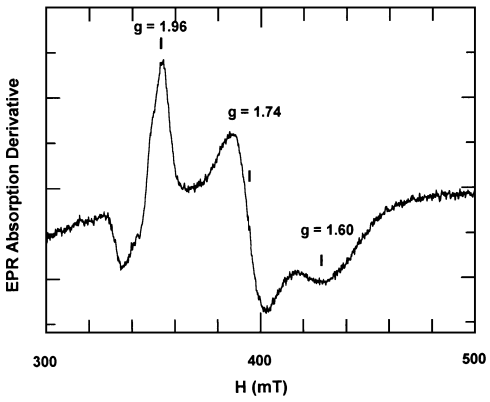

Figure 3 EPR spectrum of reduced recombinant PAP

The EPR spectrum is shown (at 3.9 mW, 9.62 GHz, 4 accumulations, 100 KHz, 3.6 K, 0.1 mT modulation of amplitude) of 6 mg/ml purified recombinant PAP after 2 h of treatment with 0.1 mM $FeCl_3$/1 mM ascorbic acid and analysis of visible spectra.

pink colours of osteoclast TRAP demonstrate that this enzyme is an iron-tyrosinate protein typical of the PAPs.

Low-temperature EPR spectroscopy

Low-temperature EPR spectroscopy is very useful in the study of iron-containing proteins, since the presence and nature of clusters and different redox states of the paramagnetic centre can be demonstrated. It has been especially useful in the study of PAPs [1–3,8,34,40], where the presence of a signal in the mixed-valent state [Fe(II)Fe(III)] with all three g-values below 2.0 is taken as proof of the presence of a di-iron cluster with a μ-(hydr)oxy bridge [3,8,41–43]. Low-temperature EPR spectra of isolated TRAP at 3.6–77 K showed the presence of only trace amounts of unspecific Fe(III) and some Cu(II) (results not shown). After incubation of TRAP at pH 5.5 with 1 mM $FeCl_3$ and 10 mM ascorbate at room temperature for 40 min, and subsequent removal of excess iron and ascorbate by filtration on Sephadex G-25 or immediately after incubation with 1 mM ascorbate and 0.1 mM iron as in Figure 3, the appearance of a mixed-valent Fe(II)Fe(III) EPR signal with g-values at 1.96, 1.74 and 1.60 at 3.6 K, and the presence of the above-mentioned unspecific iron and copper signals, was observed. After incubation of reduced recombinant TRAP with 1 mM hydrogen peroxide at room temperature for 10 min, 90% of the signal disappeared, indicating oxidation of the mixed-valent state to the diferric state. At 3.6 K the EPR signal from reduced recombinant TRAP was difficult to saturate by microwave power with $P_{\frac{1}{2}}$ [40] over 25 mW, as commonly observed with these types of signals. The TRAP signal was detectable at 8 K, as expected for a weak antiferromagnetically coupled Fe(II)Fe(III) system, but the signal was too weak to be used to determine the value of the exchange coupling. Quantification of the TRAP samples incubated with 1 mM ascorbate and 0.1 mM iron showed that the EPR-active mixed-valent cluster corresponded to 0.9 ± 0.1 spins per protein, as expected for one iron–oxygen cluster per protein.

In conclusion, the EPR analysis shows that recombinant TRAP as isolated does not have any EPR signal and is presumably in an Fe(III)Fe(III) state, as indicated by its light absorption spectrum. After reduction, a mixed-valent Fe(II)Fe(III) state is formed, which can be oxidized by hydrogen peroxide. This EPR signal is similar to the mixed-valent EPR signals from the acid form of bovine PAP [2], uteroferrin [1,34,40] and plant PAP [18]. The observation of a mixed-valent EPR signal, as observed for other reduced PAPs, is the first proof for the formation of a di-iron μ-(hydr)oxy-bridged cluster in recombinant TRAP.

Thus expression of a recombinant TRAP enzyme from the cloned cDNA template of rat osteoclast TRAP resulted in a product that exhibited light absorption and EPR spectroscopic characteristics typical of the PAPs. These two spectroscopic approaches yielded sufficient evidence that recombinant rat osteoclast TRAP produced in a baculovirus system is a PAP, and thus can be included together with bovine spleen PAP and uteroferrin as a fully established member of the PAP family.

Table 2 Recombinant PAP has phosphoprotein phosphatase activity

Dephosphorylation of bone sialoprotein and osteopontin by 1 unit of recombinant PAP was monitored by the level of rephosphorylation by CK II using [γ-^{33}P]ATP as substrate. Data represent means $\pm$ S.D. from 3–4 experiments. The signifiance of differences between means was analysed by Students t test: $^{*}P < 0.05$; $^{**}P < 0.005$.

	Rephosphorylation (c.p.m./10 μg)	
	− PAP	+ PAP
Osteopontin	2380 ± 100	3860 ± 630*
Bone sialoprotein	4010 ± 800	7280 ± 550**

Phosphoprotein phosphatase activity

Analysis of the phosphorylation pattern of bovine osteopontin has demonstrated the presence of phosphorylated serine residues located in consensus sequences corresponding to a CK II motif [44,45]. As an indirect measure of dephosphorylation of bone sialoprotein and osteopontin by recombinant PAP, the number of phosphorylation sites available to CK II was monitored by determining the level of rephosphorylation when purified CK II was added subsequent to incubation with recombinant PAP. The bone matrix phosphoproteins were phosphorylated even without prior incubation with the recombinant enzyme, but significantly more radioactive label was incorporated after prior incubation with recombinant PAP (Table 2). The rephosphorylation could be inhibited by molybdate (results not shown), which was not inhibitory to the CK II enzyme under these conditions. In one experiment, rephosphorylated bone sialoprotein was subjected to SDS/PAGE and blotted to a nitrocellulose filter. The radioactive label migrated in an identical manner to unlabelled bone sialoprotein, as demonstrated by PhosphoImager analysis (not shown). The results were corroborated by densitometric quantification of the radiolabelled bands from the PhosphoImager analysis, which gave the same ratios as the radioactive counts from the incubations.

Comparison of rat recombinant PAP and rat bone TRAP

Since one of our intentions in producing TRAP as a recombinant protein was to use it to substitute for the TRAP enzyme purified from rat bone, primarily in structural and functional studies, we felt obliged to carry out a comparative analysis of some characteristic features between the recombinant PAP and native TRAP enzymes.

Comparison of catalytic properties

In order to examine the catalytic properties of recombinant PAP, the IC_{50} values for four well known PAP inhibitors were

Table 3 Effects of different inhibitors on the activity of rat recombinant PAP and bone TRAP

Values are means ± S.D. of two experiments.

Inhibitor	IC_{50} (μM)	
	Recombinant PAP	Bone TRAP
Molybdate	26 ± 7	8.0 ± 1.0
Tungstate	70 ± 24	29 ± 9.0
Arsenate	590 ± 150	310 ± 21
Phosphate	5000 ± 1800	3400 ± 1300

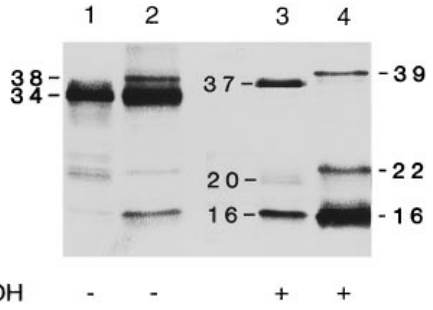

Figure 4 Western blot analysis of rat recombinant PAP and bone TRAP

Equal TRAP activities of rat recombinant PAP (lanes 1 and 3) and bone TRAP (lanes 2 and 4) were applied to an SDS/15%-polyacrylamide gel in the absence (−) or the presence (+) of β-mercaptoethanol (β-MeOH) as reducing agent, blotted to nitrocellulose and incubated with rabbit polyclonal antibodies to recombinant PAP. Prestained molecular mass standards (kDa) were electrophoresed in separate lanes.

determined (Table 3). The IC_{50}s, derived from linear regression analysis, were in the same concentration ranges as published previously for PAPs [6,9,30,31,40,46,47], with the rank order of molybdate > tungstate > arsenate > phosphate. The sensitivity of the TRAP enzyme isolated from rat bone was slightly greater, e.g. a 1.5–3-fold lower concentration of the inhibitors was needed. The affinity constant of the bone enzyme for hydrolysis of *p*-nitrophenyl phosphate was 0.4 mM, compared with 1.6 mM for the recombinant enzyme. Although minor differences were detected in inhibitor sensitivity and affinity for the substrate, it is concluded that the catalytic properties of the recombinant enzyme and the endogenous enzyme are basically similar.

Structural comparison

Polyclonal antibodies were generated in rabbits using recombinant PAP as the immunogen. Western blot analysis (Figure 4), subsequent to SDS/PAGE under reducing and non-reducing conditions, demonstrated that the antibody preparation recognized both recombinant PAP (Figure 4, lanes 1 and 3) and purified bone TRAP (lanes 2 and 4). A notable and consistent difference between the preparations was that only a minor proportion of the recombinant PAP appeared as smaller fragments, whereas bone TRAP was isolated predominantly as the fragmented (or two-subunit) form. This difference could be a reflection of exposure to different cellular environments for the endogenous and recombinant enzymes. It was suggested by Orlando et al. [30] that the two subunits in the cleaved form of

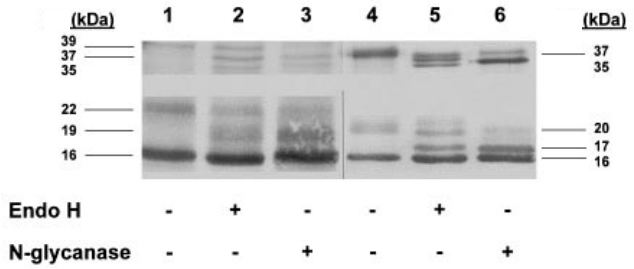

Figure 5 N-linked carbohydrate analysis of recombinant PAP and bone TRAP

Recombinant PAP purified from the medium (lanes 4–6) and purified bone TRAP (lanes 1–3) were treated with Endo H or N-Glycanase as indicated. Following disulphide reduction, the samples were electrophoresed on an SDS/15%-polyacrylamide gel. After electrophoresis, the proteins were blotted on to nitrocellulose and visualized with polyclonal antibodies to recombinant PAP.

bovine spleen PAP are truncated in a highly antigenic, surface-exposed, region. However, polyclonal antibodies generated towards the intact recombinant PAP recognized the two-subunit form of native rat bone TRAP, suggesting that the major antigenic determinants are common to recombinant PAP and native TRAP. A notable difference was seen when comparing the monomeric forms of the two preparations. The unreduced monomeric form of recombinant PAP had an apparent molecular mass of 34 kDa, which shifted to 37 kDa in the reduced sample. Interestingly, the monomeric bone TRAP, presumably represented by the distinct 38 kDa band in unreduced samples, exhibited an apparent molecular mass of 39 kDa following disulphide reduction. These size differences were also evident when comparing the immunoreactive fragments of the two preparations. The larger of the two fragments of recombinant PAP migrated as a 20 kDa species, whereas the larger of the bone TRAP fragments migrated slightly more slowly, corresponding to an apparent molecular mass of 22 kDa. The smaller of the two fragments in the two preparations migrated to similar positions in the gel, corresponding to a molecular mass of 16 kDa.

N-linked oligosaccharide analysis

Two potential N-glycosylation sites, at Asn-118 and Asn-149, can be inferred from the deduced amino acid sequence of rat bone TRAP, both being present in the N-terminal 20 kDa subunit [14,30,32]. To examine whether the size differences observed between recombinant PAP and bone TRAP could be due to differences in N-glycosylation, an analysis of N-linked oligosaccharides in recombinant PAP and purified bone TRAP was conducted using the endoglycosidases Endo H and N-Glycanase (Figure 5). Incubation of recombinant 37 kDa monomeric PAP with Endo H resulted in the formation of novel bands at 36 and 35 kDa, as well as leaving some 37 kDa species unmodified (Figure 5, lanes 4–6). N-Glycanase treatment shifted most of the recombinant PAP to the 35 kDa species (lane 6). These results show that recombinant PAP contains oligosaccharide chains N-linked to the protein core. Furthermore, a portion of the purified protein contains oligosaccharides that are sensitive to Endo H, indicating a high-mannose type of oligosaccharide [48]. In comparison, with monomeric 39 kDa TRAP purified from bone (Figure 5, lanes 1–3), partial conversion into a 37 kDa species and the presumably unglycosylated 35 kDa variant was achieved using Endo H (lane 2). After treatment with

N-Glycanase (lane 3), complete conversion into the 37 kDa and 35 kDa bands was observed. These results suggest that TRAP isolated from the medium is more heterogeneous than bone TRAP with respect to substitution with N-linked oligosaccharides, and that a structural component corresponding to a size increase of approx. 2 kDa, which is unrelated to N-glycosylation, is present on the bone TRAP enzyme.

The glycosylated 20 kDa (recombinant PAP) and 22 kDa (bone TRAP) fragments showed similar shifts in mobility after deglycosylation as observed for the corresponding monomeric forms (Figure 5). A size difference corresponding to approx. 2 kDa was again evident in the larger of the two fragments after N-Glycanase treatment (Figure 5, lanes 3 and 6), confirming that the molecular mass difference between recombinant PAP and isolated bone TRAP is not a result of differences in N-glycosylation.

In conclusion, the recombinant TRAP enzyme expressed from a cloned cDNA template of rat osteoclast TRAP using a baculovirus system exhibits spectroscopic characteristics typical of the PAPs. Thus osteoclast TRAP can be included together with bovine spleen PAP and uteroferrin as a fully established member of the PAP family. Similar to TRAP isolated from bone, the recombinant enzyme can act as a protein phosphatase. Comparative studies with the isolated bone TRAP demonstrate structural differences generated post-translationally. However, since the enzymic properties of the recombinant PAP and the bone TRAP are similar, the recombinant PAP enzyme should be useful in studies aimed at elucidating the properties and mechanisms of regulation of the mammalian PAP enzyme. Since the amino acids that co-ordinate the iron cluster in recombinant rat PAP can now be deduced from the three-dimensional structure of plant PAP and sequence alignment, the recombinant PAP enzyme is suitable for site-directed mutagenesis studies, which will help to clarify the structural determinants of the different phosphatase activities of the PAPs.

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