Purification and Properties of the Native Form of the Purple Acid Phosphatase from Bovine Spleen[†]

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ABSTRACT: The purple acid phosphatase (PAP) from bovine spleen has been shown to exist as a single ca. 36-kDa polypeptide in intact spleen tissue. The previously isolated microheterogeneous complex of 15-kDa and 23- or 21-kDa subunits appears to arise from proteolytic cleavage of an exposed, highly variable loop in the polypeptide chain. Small amounts of a single polypeptide form, presumed to be the native form of the enzyme, have been obtained; this has permitted its optical and EPR spectra and fundamental kinetic properties to be determined. The most notable difference between the native and two-subunit forms of PAP is a ca. 3-fold higher enzymatic activity for the latter, which is due to a simple increase in V_{max}. The two forms are very similar spectroscopically and chemically and appear to differ only in the loss of a highly antigenic ca. five amino acid segment of the polypeptide between positions 155 and 160 but not in NH_2-terminal sequence or in carbohydrate content. Analysis of published sequence data suggests that the existence of an exposed highly antigenic loop at positions corresponding to 155–161 of the spleen PAP sequence is a relatively general feature of PAP's. Trypsin and chymotrypsin cleave both bovine spleen PAP and uteroferrin, apparently in this region, with significant enhancement of enzymatic activity.

The purple acid phosphatases (PAP's)[1] are a group of basic glycoproteins that contain a binuclear mixed-valent iron center and catalyze the hydrolysis of a variety of phosphate monoesters (Antanaitis & Aisen, 1983; Doi et al., 1988). Apparently similar enzymes have now been reported from a variety of mammalian tissues, including porcine uterine fluids (uteroferrin or Uf) (Roberts & Bazer, 1980; Schlosnagle et al., 1974; Campbell et al., 1978; Antanaitis et al., 1983), bovine spleen (Campbell et al., 1978; Glomset & Porath, 1960; Campbell & Zerner, 1973; Davis et al., 1981), human spleen (Robinson & Glew, 1980; Ketcham et al., 1985), rat spleen (Hara et al., 1984) and rat bone (Ek-Rylander et al., 1991b), while potentially related enzymes have been reported from rat molar enamel organs (Anderson et al., 1982), rat liver (Paigen, 1958; Paigen & Griffiths, 1959), and bovine kidney cortex (Fujimoto et al., 1984). The recent recognition that tartrate-resistant acid phosphatases (TRAP's) are identical to PAP's (Vincent & Averill, 1990a) has resulted in the addition of examples from human lung (Efstradiatis & Moss, 1985), human bone (Lau et al., 1985; Allen et al., 1989), bovine bone (Lau et al., 1987), rat epidermis (Hara et al., 1985), and human osteoclastoma tissue (Hayman et al., 1989; Stephan et al., 1990) to the list of known PAP's. All of these show similar molecular masses (35–40 kDa), are highly basic, and are resistant to inhibition by tartrate, suggesting the existence of substantial similarities in structure. This conclusion is reinforced by available sequence data on the PAP from bovine spleen (Hunt et al., 1987), Uf (Hunt et al., 1987; Simmen et al., 1989), the human placental enzyme (Simmen et al., 1989; Ketcham et al., 1989), and the type 5 acid phosphatase from human macrophages (Lord et al., 1990) and rat bone (Ek-Rylander et al., 1991a), which reveal 85–90% identity between the individual sequences, and by spectroscopic studies on the two best characterized enzymes, the PAP from bovine spleen (BS PAP) and Uf (Doi et al., 1988; Vincent & Averill, 1990; Vincent et al., 1990; Que & True, 1990).

One persistent difference between PAP's is the fact that certain enzymes are reported to consist of two subunits rather than a single polypeptide chain, although the native molecular weight does not vary substantially. Examples include the PAP's from bovine spleen (Davis et al., 1981) and from the spleen of a patient with Gaucher's disease (Robinson & Glew, 1980), and possibly the TRAP from human osteoclastoma tissue (Hayman et al., 1989). Limited proteolysis of the bovine spleen PAP has been invoked to account for the two-subunit structure (Davis et al., 1981). Inasmuch as bovine spleen PAP is the prototypical example of an enzymatically active PAP (the other well-studied example is Uf, which is apparently enzymatically inactive in vivo and instead serves a role in iron transport (Roberts & Bazer, 1984)), the origin and effect of the two-subunit structure become potentially important points in understanding the relationship between structure, function, and spectroscopic properties. This paper presents evidence that the native form of bovine spleen PAP consists of a single polypeptide chain, describes some properties of the native enzyme, and characterizes its relationship to the two-subunit form of the enzyme previously studied.[2] The most important conclusions are that proteolytic cleavage results in a ca. 3–4-fold increase in enzymatic activity and in loss of the most highly antigenic sites on the protein. In addition, a general

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[1] Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; endo F, endoglycosidase F; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; HPAE PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAP, purple acid phosphatase; PMSF, phenylmethanesulfonyl fluoride; PNGase F, peptide *N*-glycosidase F; pNPP, *p*-nitrophenyl phosphate; PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TRAP, tartrate-resistant acid phosphatase; Tris, tris(hydroxymethyl)aminomethane.

[2] Throughout this paper the 36-kDa single-subunit form of bovine spleen PAP will be operationally defined as the native form.

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model for the location of proteolytic cleavage, glycosylation, and antigenic sites in PAP's is presented.

EXPERIMENTAL PROCEDURES

Materials. Cellulose phosphate (P-11) and (carboxymethyl)cellulose (CM-52) were obtained from Whatman; phenyl Sepharose and the gel filtration media were obtained from Pharmacia. Ultrafiltration was performed on Amicon Model 8050, 8MC, or Centricon units equipped with PM-10 membranes; dialysis utilized Spectrapor cellulose membrane tubing. Freund's complete and incomplete adjuvant, pNPP, Triton X-100, and molecular weight protein markers for SDS–PAGE were purchased from Sigma. Reagents for gel electrophoresis, prestained molecular weight protein markers, protein A gold reagent, nitrocellulose membranes, and the silver enhancement kit were obtained from Bio-Rad. Immobilon P poly(vinylidene difluoride) (PVDF) membranes were purchased from Millipore. Methanol was of HPLC grade. BCA protein assay reagent was obtained from Pierce. α-Chymotrypsin and trypsin were obtained from Sigma and Worthington Biochemical Co., respectively. Peptide *N*-glycosidase F, endoglycosidase F, lectin GNA, and other glycoconjugate analysis reagents were obtained from Boehringer Mannheim. Beef spleens were obtained from Dinner Bell Meat Products, Inc., Lynchburg, VA, within 2 h of slaughtering. Uteroferrin was a generous gift from Professors P. Aisen, Albert Einstein College of Medicine, and L. Que, Jr., University of Minnesota. All other chemicals were of reagent grade and were used without further purification.

Enzyme Purification. Acid phosphatase was purified from bovine spleens by two methods. The first utilizes a pH 3.5 extraction ("low-pH preparation") (Davis et al., 1981). A revised purification of the enzyme was carried out according to the same method with the following modifications ("high-pH preparation"). The homogenization buffer consisted of 100 mM Tris, 250 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM sodium azide, and 1 mM EDTA adjusted to pH 7.5 with 1 M acetic acid. The enzyme was eluted from P-11 with 100 mM Tris, pH 7.5, containing 25% ammonium sulfate. Hydrophobic chromatography was performed using phenyl Sepharose equilibrated in the same buffer, using a linear 25–0% ammonium sulfate gradient. Other chromatography steps were performed in 100 mM Tris–acetate buffer, pH 5.8. All purification steps were carried out at 4 °C. Enzyme activity was assayed using the Fe^{2+}/ascorbate/*p*-nitrophenyl phosphate method (Campbell & Zerner, 1973) in 166 mM MES buffer at pH 6.0 at room temperature.

Protein Determination. Protein concentrations were determined by BCA protein assay reagent (Smith et al., 1985) or by assuming an extinction coefficient of 1.0 at 280 nm for a protein concentration of 1 mg/mL when exact concentrations were not required (Suelter, 1985; Stoscheck, 1990).

Gel Electrophoresis. SDS–PAGE was performed on 12.5% or 15% slab gels as described (Laemmli, 1970), with the following proteins as molecular mass markers: phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), lysozyme (14.4 kDa), and α-lactoglobulin (14 kDa). Discontinuous nondissociating gel electrophoresis (native) was performed on 10% slab gels (Reisfeld et al., 1962). Staining and destaining (Weber et al., 1972), and silver staining (Wray et al., 1981) were performed as described.

Molecular Weight Determination. The molecular weight of bovine spleen PAP was estimated by gel filtration chromatography using Sephacryl S-200, with bovine serum albumin, egg albumin, α-chymotrypsinogen, and cytochrome *c* as standards.

Antibody Production. Bovine spleen PAP (obtained from the low-pH preparation; see Results and Discussion) was further purified by native gel electrophoresis and removed from the gel by dialysis against 50 mM acetate buffer, pH 5.0. Antiserum to the protein was raised in a New Zealand white rabbit by injection of 100 μg of antigen in Freund's complete adjuvant in the initial immunization and 10 μg antigen in Freund's incomplete adjuvant in each of two subsequent booster injections spaced 2 weeks apart. The rabbit was bled at 1-week intervals. Booster injections were also given when antibody titers declined. Antiserum to Uf was also raised as described above.

Antibody Probing of Spleen Homogenates by Western Blotting. At 0-, 0.5-, 1.0-, 2.0-, and 3-h intervals after slaughtering, a 1-g section of tissue from a fresh beef spleen was excised and homogenized in 10% TCA using a 15-mL Potter-Elvehjem tissue grinder. Homogenates were centrifuged at 5000*g* for 10 min, and pellets were washed with H_2O repeatedly to remove acid. Precipitates were resuspended in 3 mL of 50 mM Tris–HCl, 5% mercaptoethanol, 1% SDS, 10% sucrose, and 0.005% bromophenol blue, pH 6.8. Solubilization was facilitated by heating at 100 °C for 1 h. Electrophoresis was performed on 100-μL samples using a 1.5 mm thick 15% gel for 18 h at 50 V.

Electrophoretic transfer of separated proteins from the gel to nitrocellulose paper was for 1 h at 1000 mA (Towbin et al., 1979). After transfer, blocking, and washing, the membrane was transferred into a solution of 100 mL of 1% blocking solution containing 1 mL of antiserum and shaken for 5 h or overnight. To remove unbound antibody, the membrane was again washed twice with Tris-buffered saline containing 0.05% Tween-20. Protein A gold and a silver stain (Bio-Rad Laboratories) were employed to detect the protein bands.

Proteolytic Cleavage of Native PAP and Uf with Trypsin and Chymotrypsin. Two serine proteases, chymotrypsin and trypsin, were used to study the cleavage of Uf and native BS PAP. The effects of proteolysis were monitored by means of phosphatase activity assays and SDS–PAGE. The cleavage of Uf was performed by incubating increasing concentrations of protease with the phosphatase for a constant period of time. Chymotrypsin and trypsin were dissolved in 10 mM Tris–acetate buffer, pH 7.0, at 200 and 180 units/mL, respectively. Increasing amounts (0–100 μL) of either protease were added to a solution of uteroferrin (1.45 μg in 3 μL of 1 mM Tris–acetate buffer, pH 5.8); a constant volume of 103 μL was maintained with 10 mM Tris–acetate buffer. The serine protease and the phosphatase were incubated for 45 min at room temperature. Termination of the reaction was achieved either by placing the sample on ice and immediately assaying for phosphatase activity or by heating with SDS–PAGE sample buffer for electrophoresis. Phosphatase assays were performed as described above. Electrophoresis and Western blot analysis were performed as described above.

Proteolytic activation of native PAP (3.73 μg in 10 mL of 10 mM acetate buffer, pH 5.8) was performed as described above for Uf by monitoring phosphatase activity. Samples for gel SDS–PAGE were prepared for N-terminal sequence analysis. Native PAP samples (7.46 μg in 20 μL of 10 mM acetate, pH 5.8) were incubated with trypsin (5% w/w) for varying periods of time (0–48 h) in 100 mM Tris–acetate, pH 7.5. The reactions were quenched by boiling in SDS–PAGE sample buffer. Electrophoresed samples were transferred onto

Immobilon P membrane and stained with Coomassie blue. Protein bands corresponding to the heavier polypeptide of the two bands at 15 kDa were excised, and N-terminal analyses were performed to determine the cleavage site.

Amino Acid Composition. The amino acid composition was determined at the Sequence Analysis Facility at the University of Virginia Medical School using a modified procedure (Bidlingmeyer et al., 1984) and 3–8 μg of protein for each determination; two different native PAP samples were run in triplicate. BSA was used as a standard. Tryptophan content was determined as described (Edelhoch, 1967).

Amino-Terminal Sequence. The N-terminal sequence analysis was performed on a sample of native PAP enzyme that had been immobilized on a membrane support. Twenty-five microliter samples (9.3 μg of enzyme) were subjected to SDS–PAGE and transferred onto Immobilon P membrane (LeGendre & Matsudaira, 1988). Protein bands were stained with 0.1% (w/v) Coomassie blue in 7% (v/v) acetic acid–45% (v/v) methanol solution for 5–10 min, followed by destaining with acetic acid–methanol. Bands corresponding to the 40-kDa native protein (or the ca. 15-kDa subunit) were excised from the membrane. The N-terminal amino acid sequence analysis was performed on an Applied Biosystems 470A sequencer by the Sequence Analysis Facility at the University of Virginia Medical School using the phenyl isothiocyanate (PITC) method of derivatization, followed by phenylthiohydantoin (PTH) derivatization.

Monosaccharide Composition. Samples of both native and two-subunit BS PAP's and uteroferrin were analyzed by the Carbohydrate Analysis Facility at the University of Virginia Department of Pathology (Hardy et al., 1988). Monosaccharide compositions were determined by hydrolyzing 500 pmol of each protein in 2 M trifluoroacetic acid, followed by high-performance anion-exchange chromatography (HPAE) at high pH and pulsed amperometric detection (PAD). Glucose was used as an internal standard for quantitation. Amino sugar composition was determined by hydrolyzing 500 pmol of each protein with 6 N HCl, followed by HPAE and PAD. *N*-Glycanase was employed to determine the linkage of the oligosaccharide chain to the protein (Townsend et al., 1989). Sialic acid analysis was also performed by hydrolysis in 0.1 M HCl for 1 h at 80 °C.

Carbohydrate cleavage was performed at room temperature by incubation of 250 μL of 1.5 mg/mL BS PAP with 0.02% (mol/mol) endo F (2 M KCl, 0.05 M sodium acetate, pH 5) or 0.0075% PNGase F (2 M KCl, 0.10 M HEPES, pH 7). The pH differences reflect the different pH optima of the two glycosidases. Aliquots (50 μL) were removed each day for 4 days and frozen immediately.

Detection of glycoproteins was done after transfer of protein bands to nitrocellulose membrane. After blocking, the membrane was incubated with digoxiginin-labeled lectin GNA, which is specific for terminal mannose linkages in N-linked glycans. This was followed by incubation with anti-digoxiginin–alkaline phosphatase conjugated antibodies and a color reaction with phosphatase substrate.

Circular Dichroism Spectroscopy. CD spectroscopy was performed on a Jasco J-600 spectrophotometer by the Sequence Analysis Facility at the University of Virginia Medical School. Spectra were obtained in the 195–250-nm region and analyzed by a standard method (Yang et al., 1986), as modified by K. Williams (Medical College of Virginia).

Optical Spectroscopy. Visible spectra were obtained with a Cary 219 spectrophotometer at room temperature. Two separate enzyme samples (in 50 mM acetate buffer, pH 5.8, containing 150 mM NaCl) were used for the generation of oxidized and reduced spectra. The first sample was oxidized with 4.4×10^{-5} mol of H_2O_2 [5 μL of 30% (v/v) H_2O_2] (Debrunner et al., 1983). The second enzyme sample was reduced with 100 mM ascorbate containing 6 mM ferrous ammonium sulfate (Keough et al., 1982). Phosphatase assays were performed after oxidation and reduction and showed minimal (<20%) loss of activity in the standard assay.

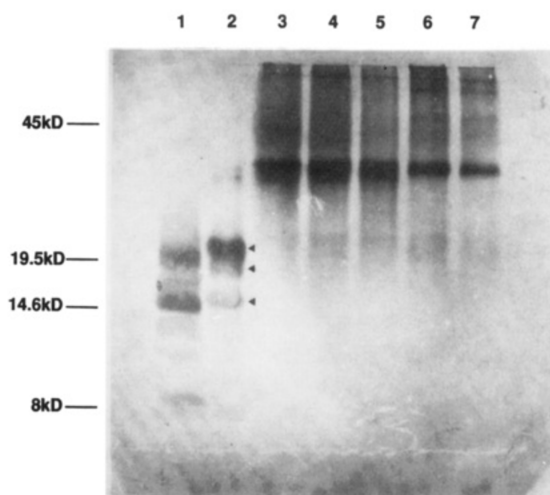

FIGURE 1: Probe of a Western blot of bovine spleen homogenates with anti-PAP antibodies as described under Experimental Procedures. Lane 1 is prestained molecular mass standards. Lane 2 is the two-subunit form of BS PAP (Davis et al., 1981); arrowheads indicate the location of the 23-, 21-, and 15-kDa subunits. Lanes 3–7 are TCA homogenates of spleen tissue maintained at 0 °C for 0, 0.5, 1, 2, and 3 h, respectively.

Electron Paramagnetic Resonance Spectroscopy. EPR spectroscopic studies on native PAP were performed at 4–7 K using a Bruker ESR 300 spectrometer equipped with an Oxford ESR 900 continuous flow cryostat and an Oxford ITC 4 temperature controller. A standard solution of Cu^{2+} EDTA was used for the quantitation of spins. Data were collected and manipulated using the EPR Data Acquisition System version 2.2 software package.

RESULTS AND DISCUSSION

Subunit Structure of PAP. Previous work has demonstrated that bovine spleen PAP has an overall molecular mass of ca. 40 kDa and consists of two dissimilar subunits of molecular mass ca. 15 and 23 kDa (Campbell et al., 1978; Davis et al., 1981). This is in distinct contrast to the well-studied uteroferrin, which is isolated as a single polypeptide chain that has approximately the same overall molecular mass, has >85% amino acid sequence identity, and exhibits remarkably congruent spectroscopic and catalytic properties. In view of these similarities and the fact that most TRAP's isolated to date are single polypeptide proteins, the possibility that the two-subunit form of PAP is the result of posttranslational modification or of proteolytic cleavage during the isolation process was explored.

Electrophoresis of PAP obtained via the earlier preparation clearly shows the presence of the two subunits reported previously (Campbell et al., 1978; Davis et al., 1981). It also demonstrates the presence of microheterogeneity, in that the larger of the two subunits is heterogeneous, exhibiting two bands (Figure 1, lane 2). Apparent molecular masses are 15 kDa for the light subunit and 23 and 21 kDa for the two forms of the heavy subunit. The overall molecular masses of the two forms are thus 38 and 36 kDa, in reasonable agreement

with the value of 36.5 kDa obtained by gel permeation chromatography on Sephacryl S-200 for a mixture of the two forms (data not shown). There are several possible explanations for the existence of microheterogeneity within a two-subunit protein, including the presence of isozymes, partial proteolytic degradation, and the cleavage of an acid-sensitive peptide bond during the pH 3.5 extraction. The microheterogeneity in the heavier subunit appears to be due to partial deglycosylation (see below).

In order to determine whether the unsymmetrical dimers are the *in vivo* form of the enzyme, samples of freshly obtained spleen tissue were homogenized with 10% trichloroacetic acid at varying times (in the abattoir for early time points). This treatment precipitates all proteins present at a given time and inhibits proteolysis by denaturation of the proteases. The results of such an experiment are shown in Figure 1, lanes 3–7; they clearly demonstrate that the major protein in spleen tissue that cross-reacts with anti-bovine PAP antibodies is a ca. 36-kDa polypeptide that exhibits some degree of microheterogeneity. At longer times, the amount of this material steadily decreases, presumably via proteolysis, yet only barely detectable amounts of a 19-kDa band are present. These results indicate that the form of bovine phosphatase in intact spleens is a single polypeptide chain (actually a mixture of several very similar polypeptide chains) and that the unsymmetrical dimers observed in the purified enzyme arise during the extraction and/or purification procedure.

Attempts to suppress the presumed proteolytic cleavage(s) responsible for producing the two-subunit form were carried out using a battery of specific protease inhibitors (pepstatin, aprotinin, ovomucoid α-1-antitrypsin, PMSF, EDTA, and *p*-hydroxymercuribenzoic acid) at concentrations 4–30 times those normally employed; in addition, a nonspecific protease inhibitor, α_2-macroglobulin, was examined. None resulted in a significant increase in the amount of the single polypeptide species, and none altered the relative ratio of the 21- and 23-kDa subunits (data not shown). Thus, if proteolytic conversion is responsible, the protease appears to be an atypical one. PMSF has been previously used to minimize the formation of two dissimilar subunits in the preparation of PAP from the spleen of a patient with Gaucher's disease (Robinson & Glew, 1981), suggesting that proteolytic cleavage is responsible in human tissue at least. These results are not consistent with our own on the bovine enzyme, and the reported specific activity of 1200 units/mg for the Gaucher's enzyme is more consistent with the value we observe for the two-subunit form of bovine PAP.

Purified Uf and the native form of PAP (see below) were also incubated with fresh spleen extracts for up to 3 days at room temperature. Analysis by SDS–PAGE followed by Western blots showed no evidence for proteolytic cleavage to dissimilar subunits (data not shown). Since both Uf and the native form of PAP are substrates for serine proteases at least (see below), this result suggests that a very active protease that is active for only a short period of time in spleen homogenates may be responsible for the observed cleavage.

Finally, in order to test for the presence of an acid-labile peptide bond, both uteroferrin and the native form of PAP (see below) were incubated with 0.5 M HCl at room temperature for 1 h, followed by SDS–PAGE and Western blot analysis. Although this treatment denatures the enzyme and results in loss of the bound iron, no difference in mobility or molecular weight was detected (data not shown). This suggests that the presence of an unusually acid-sensitive bond in the native form of PAP is not responsible for the cleavage observed during the isolation process.

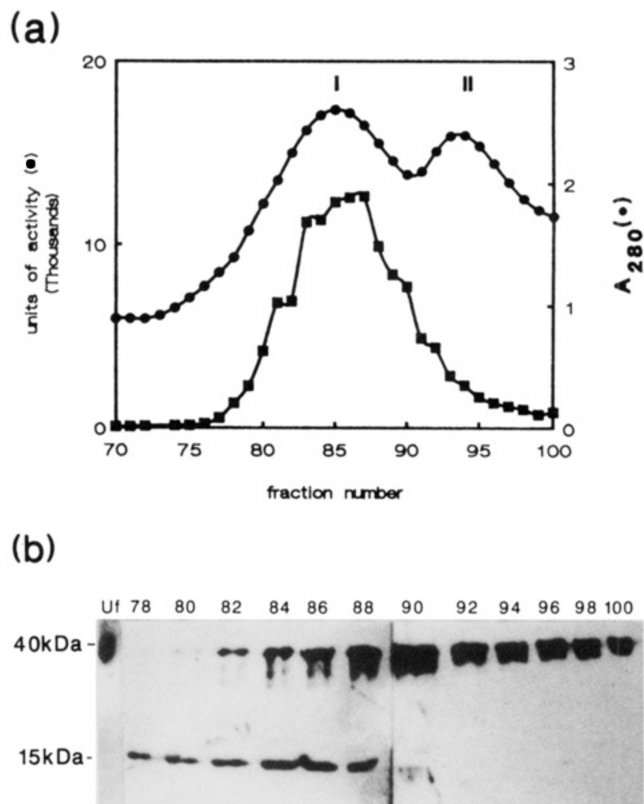

FIGURE 2: (a) Phenyl Sepharose column chromatography of P-11 eluant. The column was equilibrated in 100 mM Tris–acetate, pH 7.5, containing 25% (w/v) ammonium sulfate. A 500-mL linear gradient elution was performed using 25–0% (w/v) ammonium sulfate in 100 mM Tris–acetate, pH 7.5. Fractions (5 mL) were collected and analyzed for absorbance at 280 nm (●) and acid phosphatase activity (■). (b) Western blot analysis of successive fractions from phenyl Sepharose column chromatography using anti-Uf antibodies. Lane 1, 0.5 μg of Uf as a reference; lanes 2–13, successive fractions as indicated (50-μL samples). Fractions 78–90 correspond to peak I and fractions 91–100 correspond to peak II in (a).

Purification of the Native Form of PAP. Western blot analysis of PAP obtained via the low-pH preparation always showed the presence of a small amount of ca. 36-kDa cross-reacting material. Phenyl Sepharose chromatography at pH 7.5 was found to separate the single polypeptide chain form of PAP from the two-subunit form, since the former binds more tightly to the hydrophobic support. Figure 2 shows a typical phenyl Sepharose elution profile monitored by A_{280} and enzymatic activity (Figure 2a) and Western blot analysis (Figure 2b). The first protein peak possessed the bulk of the phosphatase activity and contained the two-subunit form almost exclusively. (As is discussed below, the 23- or 21-kDa subunit is difficult to detect by Western blot analysis using anti-Uf antibodies and hence is not visible in Figure 2b.) The second peak contained a relatively small amount of phosphatase activity that correlated with the presence of the native form of PAP, along with a substantial amount of cytochrome-containing contaminant. Further purification to homogeneity employed CM-52 ion-exchange chromatography and a second phenyl Sepharose column. A typical purification is summarized in Table I, which indicates that the native form of the enzyme can be obtained in amounts ca. 5% of those of the two-subunit form previously studied (ca. 1 mg/kg spleen). The native enzyme was isolated in its reduced (pink) form with λ_{max} of 518 nm, exhibited an A_{280}/A_{518} ratio of 13.9, comparable to the 13–14 reported for Uf (Antanaitis et al.,

Table I: Purification of Native and Two-Subunit Forms of PAP from Bovine Spleen Using a pH 7.5 Extraction

	activity (units)	sp act. (units/mg)	% recovery	total protein (mg)
detergent extraction (1.1 kg of spleen)	111 000	3.12	100	3560[a]
cellulose phosphate batch adsorption (P-11)	108 000	75.0	97	1440[a]
phenyl Sepharose column				
(1) native	15 000	45	13.6	334[c]
(2) two subunit	45 000[b]	153	40.5	294[c]
(carboxymethyl)cellulose (CM-52) column				
(1) native	2 900	126	2.6	23[c]
(2) two subunit	31 000[b]	470	27.9	66[c]
phenyl Sepharose column				
(1) native	653	408	0.6	1.6[c]
(2) two subunit	31 000[b]	1210	29.7	27.3[c]

[a] Protein concentrations were determined by BCA protein assay reagent. [b] Represents a mixture of both native and two-subunit forms of the enzyme. [c] Protein concentrations were determined from absorbance at 280 nm.

1983), and was >99% homogeneous by SDS–PAGE (not shown). The specific activity was 340–410 units/mg, comparable to the 350 units/mg reported for uteroferrin (Keough et al., 1982) but significantly lower than the value of 1200–1300 reported for the two-subunit form of bovine spleen PAP (Davis et al., 1981). Noteworthy points of the purification include the initial extraction conditions (pH 7.5, 250 mM NaCl, 1% Triton X-100), which result in a ca. 5-fold increase in the amount of enzyme solubilized vs the previous pH 3.5 extraction (Davis et al., 1981), and the use of a relatively high pH (7.5) phenyl Sepharose chromatography step, since the two forms of the enzyme are not separated in the pH 5.0 buffers previously used.

Spectroscopic Properties of Native PAP. Although the limited quantities of single polypeptide chain PAP that are available make extensive studies of the type performed on Uf and the two-subunit form of bovine spleen PAP unfeasible, characterization of the basic spectroscopic and kinetics properties of the native enzyme was essential to ensure that previous conclusions are valid for it as well.

Visible absorption and EPR spectra of native bovine spleen PAP are shown in panels a and b of Figure 3, respectively. The visible spectrum of the reduced enzyme in the presence of ascorbate and ferrous iron (spectrum a in Figure 3a) is essentially identical to that of the enzyme as isolated (not shown), with a λ_{max} of 518 nm; this form of the enzyme is pink. Upon oxidation with stoichiometric amounts of H_2O_2, the absorption maximum shifts to 550 nm. These results indicate that the native enzyme is closer in properties to Uf (Debrunner et al., 1983; Beck et al., 1984) and the recently reported high-salt, two-subunit form of bovine spleen PAP (Vincent et al., 1991a) than to the two-subunit form of PAP obtained from the previous low-pH purification (Campbell et al., 1978; Davis et al., 1981). Most telling is the facile oxidation by H_2O_2 in the absence of phosphate; in contrast, the form of bovine spleen PAP originally examined is readily bleached by H_2O_2 (Davis, 1982). The λ_{max} of the reduced form of the native enzyme is intermediate between that of the low-salt (510 nm) and high-salt (538 nm) two-subunit forms of the enzyme and falls on a smooth curve relating visible maxima and percent random coil as determined by CD spectroscopy (Vincent et al., 1991a).

The EPR spectrum of the reduced enzyme at ≤30 K (Figure 3b) shows a rhombic signal with g values of 1.83, 1.72, and

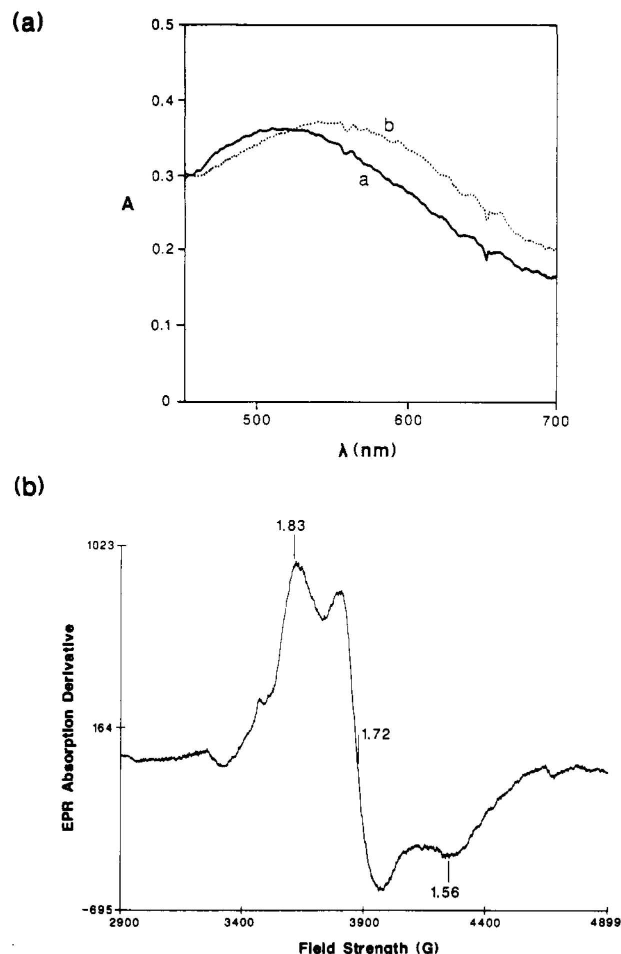

FIGURE 3: (a) Electronic absorption spectrum of the native form of BS PAP in the visible region. Spectrum a represents PAP reduced with 10 mM ascorbate and 0.03 mM ferrous ammonium sulfate; λ_{max} ≈ 518 nm. Spectrum b represents oxidation of the sample from spectrum a with H_2O_2, λ_{max} = 550 nm. (b) Electron paramagnetic resonance spectrum at 5 K of the native form of BS PAP. The reduced (pink) form was produced by treating the enzyme with 100 mM ascorbate and 6 mM $(NH_4)_2Fe(SO_4)_2$, pH 5.0. Conditions: microwave frequency, 9.30 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; receiver gain, 400; scan time, 16 min; temperature, 5 K; sample concentration, 0.5 mM.

1.56, values that are essentially identical with those of either of the two-subunit forms of PAP examined [cf. low-salt two-subunit PAP, 1.85, 1.73, 1.58 (Davis & Averill, 1982; Antanaitis & Aisen, 1982) and high-salt two-subunit PAP, 1.87, 1.71, 1.57 (Crowder et al., 1992)] and very similar to those observed for Uf (1.93, 1.75, 1.59) (Doi et al., 1988b). These data are consistent with the presence of analogous mixed-valence, binuclear iron centers in all of these systems.

Kinetics Properties of Native PAP. Lineweaver–Burk plots (not shown) demonstrate that the native form of PAP obeys Michaelis–Menten kinetics with *p*-nitrophenyl phosphate as a substrate of pH 5.0; the values of K_m and V_{max} are 2.25 mM and 116 s^{-1}, respectively. Phosphate exhibits mixed competitive/noncompetitive inhibition with a K_i of 1.7 mM, while molybdate exhibits essentially noncompetitive inhibition with a K_i of 3.5 μM. These findings and the values of the K_m and K_i parameters are very similar to those reported for the two-subunit form of PAP (Vincent et al., 1991a), suggesting that cleavage of the polypeptide chain has little effect on the enzyme's affinity for phosphate esters or tetraoxyanions. In contrast, the value of V_{max} for the native form is reduced by a factor of 5 vs the two-subunit form of PAP [116 vs 580 s^{-1}

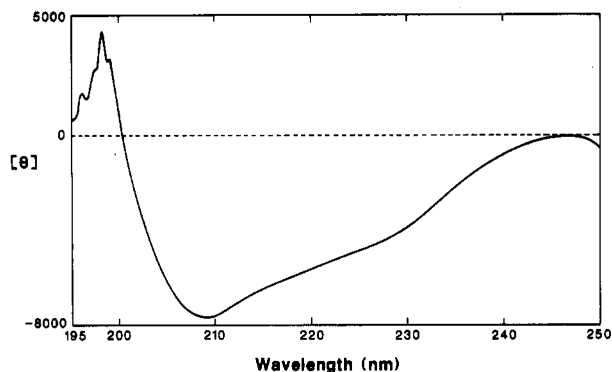

FIGURE 4: Circular dichroism spectrum of the reduced (λ_{max} = 524 nm) native form of BS PAP in the far-UV region. The ordinate is optical rotation in degrees. Conditions: protein concentration, 2.5 μM; buffer, 50 mM acetate, pH 5.0, containing 150 mM KCl; light path, 0.1 cm.

(Davis et al., 1981)], consistent with the limiting specific activities observed (400 vs 1200 units/mg, respectively). The lower value is consistent with those reported for other TRAP's and Uf, which consist of single polypeptide chains. It seems clear that proteolytic cleavage of native PAP results in a substantial increase in V_{max} (see below); the reasons for this phenomenon are not clear. A few TRAP's [those from Gaucher's spleen (Robinson & Glew, 1981), rat spleen (Hara et al., 1984), and one preparation of the rat bone enzyme (Kato et al., 1986)] also exhibit specific activities in the 1200–1400 units/mg range, similar to that observed for two-subunit PAP, suggesting the possibility of at least partial proteolysis in these systems. On the basis of the kinetics parameters, the native form of bovine spleen PAP appears to be a typical TRAP.

Molecular Properties of Native PAP. The amino acid compositions of the native and two-subunit forms of bovine spleen PAP are summarized in Table IS (supplementary material) along with results reported for several other TRAP's. The normalized results predict a polypeptide chain of 305 residues with a molecular mass of 34 349 Da, not including iron and carbohydrate (see below). The results obtained for the two-subunit form of the enzyme are generally in good agreement with those calculated from the published partial (>90%) amino acid sequence (Hunt et al., 1987). The overall composition and inferred molecular mass are quite similar to those of other TRAP's, with the greatest similarity to the type 5 human macrophage (Lord et al., 1990) and human placenta (Ketcham et al., 1989) enzymes.

The amino-terminal amino acid sequence was determined for the native form of PAP in order to ascertain whether the native and two-subunit forms differed by cleavage at the NH_2 terminus in addition to that resulting in the two-subunit structure. The results for the native form of PAP are TPAPML•FVA, where • indicates an undetected residue. These results are in good agreement with those obtained earlier for the two-subunit form of PAP [TPAPM•XRFVA, where X = I, L (Hunt et al., 1987)] and suggest that the missing residue in the native sequence is R, which is often difficult to detect with samples immobilized on PVDF (Hsu & Soban, 1982). The LRFVA portion of the NH_2-terminal sequence is conserved among all known TRAP's (Stephan et al., 1990; Hunt et al., 1987; Ketcham et al., 1989; Lord et al., 1990; Hsu & Soban, 1982; Ek-Rylander et al., 1991a).

The secondary structure of the native form of PAP was examined by circular dichroism spectroscopy; the spectrum of the reduced (pink) form is shown in Figure 4. The most obvious features are a sharp positive ellipticity band at 198 nm and a broad negative ellipticity band at 209 nm with shoulders at 216 and 228 nm. The general features are similar to those reported for the reduced high-salt two-subunit form of PAP (Vincent et al., 1991a), except that the long wavelength shoulder (attributable to α-helical structure) is slightly less intense in the spectrum of the native enzyme.

Table II: Average Secondary Structure of PAP and Uf Estimated from CD Data

sample	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
native PAP_{ox}[a]	20	30	20	30
low-salt two-subunit PAP_{ox}[b]	0	0	0	100
high-salt two-subunit PAP_{red}[b]	22.5	67.5	2.5	7.5
high-salt two-subunit PAP_{ox}[b]	37.5	40	2.5	15
reduced Uf[c]	18	11	NR[d]	71
oxidized Uf[c]	17	11	NR	74

[a] This work. [b] Vincent et al. (1991a). [c] Antanaitis et al. (1983). [d] NR = not reported.

Analysis of the CD spectrum (Yang et al., 1986) gave the results shown in Table II, which are compared to published data on the corresponding reduced forms of Uf and the high-salt two-subunit form of PAP. The results suggest that the native form of PAP isolated via the protocol described above exhibits significantly higher random coil and β-turn contents and a correspondingly lower β-sheet content than does the high-salt two-subunit form of PAP. This result is somewhat surprising, but previous work (Vincent et al., 1991) using identical instrumentation and methods for spectral analysis has provided clear evidence that a range of conformations are available for bovine spleen PAP, ranging from highly ordered α/β to nearly 100% random coil structures. In addition, CD data from another laboratory suggest an unusually high (70–80%) random coil content for Uf prepared under low-salt conditions (Antanaitis et al., 1983). The high random coil content reported for Uf and the low-salt two-subunit form of PAP is not consistent with secondary structure predictions based on the Chou–Fasman or Garnier sequence analysis methods (Vincent et al., 1991a) and is difficult to rationalize at this time. The results for the native form of PAP are, however, intermediate between those for the high-salt two-subunit (ordered) and the low-salt two-subunit (random) forms of PAP (Vincent et al., 1991a), consistent with the position of λ_{max} of the reduced form and the previously observed correlation between λ_{max} and percent random coil structure (Vincent et al., 1991a). We note that no attempt was made to maintain the native PAP in a high-salt environment at all times, and earlier work on the two-subunit form of PAP indicates that an irreversible transition to a much less ordered form occurs upon incubation at low ionic strength (Vincent et al., 1991a).

In addition, hydrophobicity profiles were calculated for the type 5 macrophage TRAP using two published algorithms (Kyte & Doolittle, 1982; Hopp & Woods, 1981), with a window of seven residues for each. [The human type 5 macrophage TRAP sequence (Lord et al., 1990) was utilized because the bovine spleen PAP sequence is only ca. 90% determined, and the former enzyme exhibits the greatest compositional and sequence homologies (>90%) to native PAP.] The former predicts surface-accessible sites at regions 63–67, 153–158, and 250–254, while the latter predicts surface accessibility at positions 63–68, 78–80, 155–157, and 249–255. The Hopp–Woods algorithm also predicts a total of five antigenic sites, corresponding to maximum hydrophilicity, at positions $157 \gg 155 \approx 251 \gg 79 \approx 63$, in order of decreasing

antigenicity. Sites 157 and 155 are within a region for which sequence information is lacking in the two-subunit form of PAP (Hunt et al., 1987) and which is presumably lost during proteolytic cleavage (see below). Residue 251 is in the smaller (15-kDa) subunit, while residues 79 and 63 correspond to the larger (23-kDa) subunit of the two-subunit form of PAP.

This analysis provides a possible explanation for previously puzzling observations regarding the relative antigenicity observed for Uf and native PAP vs the two-subunit form of PAP on Western blots using anti-Uf polyclonal antibodies. As can be observed in Figure 2, native PAP gives rise to an intense band on Western blots even though it is present in only relatively minor amounts, while the 23-kDa subunit is virtually undetectable by Western blots under conditions where the 40-kDa single polypeptide and 15-kDa subunit give intense stains (see Figure 6b). This behavior is *not* due to poor adsorption of the 23-kDa subunit to the membrane, since staining for protein gives comparable intensities for the 23- and 15-kDa subunits (data not shown). Quantitative studies of Western blot intensity vs protein amount show that 0.2 mg of native PAP gives essentially the same intensity as 1 mg of the isolated 15-kDa subunit, while the 23-kDa subunit can be detected only at ≥20 mg of protein. The affinity of anti-Uf antibodies for Uf and native PAP is virtually identical, based on the intensity of Western blots using 0.2 mg of each (data not shown). These experimental results are in excellent agreement with the Hopp–Woods antigenicity predictions, inasmuch as the two most antigenic sites in native PAP are in a region that is apparently removed during the proteolytic cleavage(s) that result in the two-subunit form.

Carbohydrate Content. The amino acid sequences of several TRAP's, including Uf (Ketcham et al., 1989), the type 5 human macrophage enzyme (Lord et al., 1990), and the human placental enzyme (Ketcham et al., 1989), show the presence of two potential Asn-Val-Ser glycosylation sites, and Uf has been shown to contain at least one (Saunders et al., 1985) and probably two (Baumbach et al., 1991) N-linked oligosaccharide chains that are either high-mannose (Saunders et al., 1985) or a mixture of high-mannose and complex types (Baumbach et al., 1991). Esterification of a mannose 6-phosphate on the high-mannose oligosaccharide to a covering *N*-acetylglucosamine (Baumbach et al., 1984) has been suggested to result in the secretion of Uf rather than its localization in lysosomes after biosynthesis. The potential glycosylation sites in the type 5 macrophage enzyme, which is the most similar to bovine spleen PAP, are at positions 97 and 128. The former is present and glycosylated in the sequence of the two-subunit form of PAP, while the latter is apparently missing (Hunt et al., 1987). It should be noted, however, that the complete Uf sequence (Ketcham et al., 1989) showed errors in this region in the original report (Hunt et al., 1987), including a misplaced small intervening peptide, and the sequence of the two-subunit form of PAP in this region is supported by only a single peptide with weak overlaps with the surrounding sequence. Consequently, the carbohydrate composition of the native and two-subunit forms of PAP was examined and compared to that of a Uf sample.

Digestion with *N*-glycanase revealed that the oligosaccharide chains of both Uf and PAP were N-linked to asparagine residues, as expected. The composition of the oligosaccharide portion of the native form of PAP is compared to that of Uf in Table IIS (supplementary material). The results on both the native and two-subunit forms of PAP show the presence of 12–13 mannose, 6–7 *N*-acetylglucosamine, and ca. 0.5 galactose residues per protein molecule. No other sugars were detected. Given the basic branched structure of both high-mannose and complex-type oligosaccharides, which differ primarily in whether the branches attached to the chitobiose core contain only mannose or mannose plus *N*-acetylglucosamine residues, these results are most consistent with the presence of two oligosaccharide chains per PAP molecule, suggesting that the published amino acid sequence is indeed in error in the vicinity of residue 128. The individual chains are likely to be either hybrid-type oligosaccharides, containing *N*-acetylglucosamine on one of the branches (if they are identical), or a mixture of high-mannose and complex-type oligosaccharides as observed for cultured Uf (Baumbach et al., 1991).

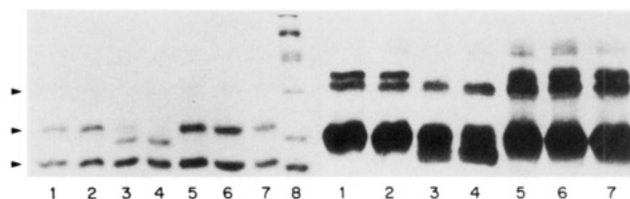

FIGURE 5: Effects of PNGase F and endo F on the two-subunit form of BS PAP. (Left) SDS–PAGE analysis with Coomassie blue staining. Lanes 1 and 2, BS PAP, pH 7; lanes 3 and 4, BS PAP incubated with 0.0075% (mol/mol) PNGase F, 1 day and 4 days, pH 7; lane 5, BS PAP, pH 5; lanes 6 and 7, BS PAP incubated with 0.02% (mol/mol) endo F, 1 day and 4 days, pH 5; lane 8, molecular weight markers. (Right) Western blot of the above gel, using anti-Uf antibodies and stained for N-linked glycoproteins with terminal mannose linkages. Arrowheads indicate the positions of the 40-, 23-, and 15-kDa bands due to native and proteolytically cleaved enzyme.

Figure 5 shows the results of attempts to remove the carbohydrate side chains using peptide *N*-glycosidase F and endoglycosidase F, which cleave prior to or after the first N-linked *N*-acetylglucosamine unit, respectively. It is clear from these results that one of the two carbohydrate chains can be removed from the heavier subunit with PNGase F but not with endo F, resulting in a decrease in apparent molecular mass of ca. 3 kDa [22.8 ± 0.7 kDa for the heavier subunit ($n = 11$) vs 19.6 ± 0.7 kDa ($n = 7$) after deglycosylation]. These gels demonstrate the presence of two glycosylation sites on the heavier subunit. The highly sensitive Western blot detection of terminal mannose residues also reveals the presence of trace amounts of the native form of PAP, which appears to be a mixture of species containing one or two oligosaccharide chains and is also converted to what appears to be a species containing a single oligosaccharide chain upon treatment with PNGase F. The total molecular mass calculated from the gels [after correction for shifts in apparent molecular mass due to the carbohydrate chains (Segrest & Jackson, 1973) and subtracting two $(Gal)(GlcNAc)_4(Man)_5$ chains] is 33.2 kDa, in good agreement with the values of 33.1 kDa calculated from the amino acid composition data in Table IS and 33 kDa reported for Uf (Ketcham et al., 1988).

Effects of Proteases on the Native Form of PAP. It has been previously reported that serine proteases cleave Uf into 20- and 15-kDa polypeptides with retention of enzymatic activity (Ketcham et al., 1985). In addition, it has been shown that the human Gaucher's spleen TRAP was proteolytically cleaved to give dissimilar subunits during the purification and that such cleavage could be minimized, but not prevented, by inclusion of PMSF in the homogenization buffer (Robinson & Glew, 1981). These reports and the difference in specific activity for the native vs two-subunit forms of PAP prompted an examination of the effects of serine proteases on the former.

The effects of trypsin on native PAP are shown in Figure 6. As shown in Figure 6a, the activity of the enzyme increases

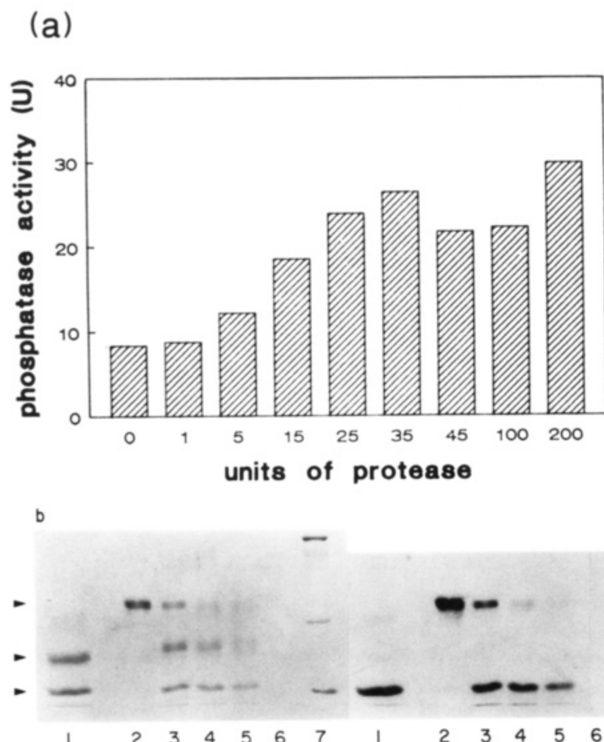

FIGURE 6: Effects of trypsin on the native form of PAP. (a) Phosphatase activity versus trypsin concentration. (b) (Left) SDS–PAGE analysis with Coomassie blue staining of native BS PAP incubated with 10% (w/w) trypsin for increasing amounts of time. Lane 1, 16 μg of two-subunit BS PAP; lane 2, 3.5 μg of native BS PAP; lanes 3–5, 35 μg of native BS PAP incubated with 10% (w/w) trypsin at 3, 24, and 48 hours, respectively; lane 6, 3.5 μg of trypsin; lane 7, molecular weight standards. (Right) Western blot of gel at the left, using anti-Uf antibodies, protein A gold, and silver staining. Arrowheads indicate the positions of the 40-, 23-, and 15-kDa bands due to native and proteolytically cleaved enzyme.

ca. 4-fold, to approximately the same limiting value observed for the two-subunit form of PAP, upon incubation with increasing amounts of trypsin for a fixed period of time. As shown in Figure 6b, tryptic cleavage results in the formation of two dissimilar polypeptides with apparent molecular masses of 23 and 15 kDa; these are, however, electrophoretically distinct from the subunits observed for the two-subunit enzyme obtained from spleen tissue, as expected since the exact site(s) of cleavage probably differ(s). Figure 6b also illustrates the relatively low antigenicity of the heavier (21- or 23-kDa) subunit, whether produced by cleavage by trypsin or by an endogenous protease. The 36-kDa form and the smaller, ca. 15-kDa subunit show comparable intensity in both Comassie blue stained gels and Western blots, but the 23- or 21-kDa subunit is virtually undetectable on the latter (as alluded to earlier).

In order to determine the site of tryptic cleavage, the NH_2-terminal sequence was determined for the 15-kDa fragment and was found to be NLAMARTQ. This corresponds to positions 157–164 of the human macrophage type 5 TRAP (Lord et al., 1990) and contains only four residues more than the smaller subunit of the two-subunit form of PAP, which begins with ARTQ (Hunt et al., 1987). This region of the polypeptide chain is a highly variable one in the known TRAP sequences, with the human macrophage type 5 sequence (Lord et al., 1990) having an extra two amino acids, and lies between the SQQPER and ARTQ—regions that are conserved in all TRAP's sequenced to date (sequence data are missing for the former region in the two-subunit PAP sequence, however, perhaps suggesting that it has been completely excised by proteolysis). The hydrophobicity profile analysis discussed above strongly suggests that residues 156–161 in the human macrophage type 5 TRAP form an exposed loop that exists as a turn (with Pro at position 156) or random coil structure. The similarities between the human macrophage type 5 TRAP and bovine spleen PAP suggest the presence in both of such an exposed loop, containing an additional R or K residue at position 156 in the latter, consistent with facile proteolysis in this region by either trypsin or an endogenous protease. Similar results were obtained with chymotrypsin (data not shown) but were not explored in detail.

Table III: Effect of Chymotrypsin on Specific Activity of Uteroferrin[a]

chymotrypsin (units)	Uf sp act. (units/mg)	chymotrypsin (units)	Uf sp act. (units/mg)
0	319	25	1207
1	2220	35	859
5	4180	100	340
15	1550	200	230

[a] Uf (1.35 μg) was incubated with the indicated amounts of chymotrypsin in 10 mM Tris–acetate buffer, pH 7.0, for 45 min as described under Experimental Procedures, and 10-μL aliquots were assayed using the standard assay.

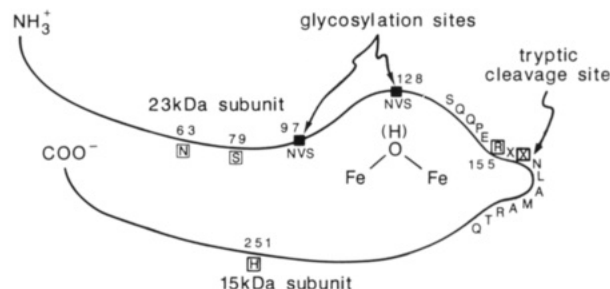

FIGURE 7: Schematic representation of the relationship between the native and two-subunit forms of BS PAP, showing the glycosylation sites and the proposed tryptic cleavage site. The numbering scheme is taken from the human macrophage type 5 TRAP sequence (Lord et al., 1990); X indicates unidentified residue. Predicted antigenic sites are boxed; the darkness of the lines corresponds to increasing predicted antigenicity.

Finally, the effect of serine proteases on Uf was also examined. Assays show that the enzymatic activity of Uf increases dramatically (by ≥10-fold) upon incubation with chymotrypsin (Table III), and SDS–PAGE analysis reveals that this phenomenon is accompanied by asymmetric cleavage of the polypeptide chain into ca. 23- and 15-kDa subunits (data not shown). Again, similar results were obtained with trypsin (data not shown) but with only a ca. 2-fold increase in specific activity.

The results presented above strongly suggest that an exposed loop in the residue 155–161 region of the peptide chain that is susceptible to proteolytic cleavage is a general feature of the structure of TRAP's. A schematic view of the structure of the native form of PAP, indicating both the glycosylation sites and the proposed tryptic cleavage site, is shown in Figure 7. The origin of the significant rate enhancements observed upon proteolysis is not obvious on the basis of the data available. [Similar proteolytic cleavage with accompanying activation has been reported to occur for other, apparently unrelated, acid phosphatases as well (Lau et al., 1992).] It should be noted, however, that the region susceptible to proteolytic cleavage is not in close proximity to the amino acid residues that have been suggested to act as ligands to the diiron center, which lie between positions 193 and 273 in bovine spleen PAP

(Vincent et al., 1990; Vincent & Averill, 1990b). This, of course, does not rule out the possibility that residues in either of the two conserved regions surrounding the exposed loop participate in the catalytic mechanism, although the absence of histidine in both regions means that they cannot contain the essential histidine that has been suggested to be phosphorylated during turnover (Vincent & Averill, 1991b).

Conclusions. Bovine spleen PAP has been shown to exist as a ca. 40-kDa polypeptide in spleen tissue *in vivo*, and a purification protocol that affords the pure, single-polypeptide form of PAP (presumably the native form), albeit in low yield, has been devised. Native PAP consists of a single polypeptide chain with amino acid composition and NH_2-terminal sequence similar to those of other TRAP's, contains two oligosaccharide chains, probably of the hybrid-type, and exhibits virtually identical reactivity toward anti-Uf antibodies as does Uf. Available sequence data suggest that native PAP probably contains an exposed, highly antigenic loop at positions 155–161 that is very susceptible to proteolytic cleavage at one or more sites. Proteolysis of both native PAP and Uf in this region results in substantial increases in enzymatic activity: ca. 3–4-fold for native PAP, resulting in a specific activity similar to that of the two-subunit PAP studied previously, and 2–10-fold for Uf. Whether proteolytic activation of native PAP actually occurs *in vivo* is unclear; if so, this could be an example of a control mechanism that functions during, e.g., phagocytosis. Spectroscopic studies have shown that the native and two-subunit forms of PAP are virtually identical. By all criteria examined to date, native PAP is a typical TRAP; as by far the spectroscopically best characterized example of this class of important enzymes, it should serve as the prototype to which other examples may be compared.

SUPPLEMENTARY MATERIAL AVAILABLE

Two tables of the amino acid composition of native and two-subunit bovine spleen PAP and other TRAP's and of the carbohydrate composition of native and two-subunit bovine spleen PAP vs Uf (2 pages). Ordering information is given on any current masthead page.

REFERENCES

Allen, S. H., Nuttleman, P. R., Ketcham, C. M., & Roberts, R. M. (1989) *J. Bone Miner. Res. 4,* 47–55.

Anderson, T. R., Toverud, S. U., Price, J. B., Hamrick, D., Hogan, D., & Braswell, L. (1982) *Arch. Oral Biol. 27,* 255–259.

Antanaitis, B. C., & Aisen, P. (1982) *J. Biol. Chem. 257,* 5330–5332.

Antanaitis, B. C., & Aisen, P. (1983) *Adv. Inorg. Biochem. 5,* 111–136.

Antanaitis, B. C., Aisen, P., & Lilienthal, H. R. (1983) *J. Biol. Chem. 258,* 3166–3172.

Baumbach, G. A., Saunders, P. T. K., Bazer, F. W., & Roberts, R. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81,* 2985–2989.

Baumbach, G. A., Saunders, P. T. K., Ketcham, C. M., Bazer, F. W., & Roberts, R. M. (1991) *Mol. Cell. Biochem. 105,* 107–117.

Beck, J. L., Keough, D. T., deJersey, J., & Zerner, B. (1984) *Biochim. Biophys. Acta 791,* 357–363.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336,* 93–104.

Campbell, H. D., & Zerner, B. (1973) *Biochem. Biophys. Res. Commun. 54,* 1498–1503.

Campbell, H. D., Dionysius, D. A., Keough, D. T., Wilson, B. E., deJersey, J., & Zerner, B. (1978) *Biochem. Biophys. Res. Commun. 82,* 615–620.

Crowder, M. W., Vincent, J. B., & Averill, B. A. (1992) *Biochemistry 31,* 9603–9608.

Davis, J. C. (1982) Ph.D. Thesis, Michigan State University, East Lansing, MI.

Davis, J. C., & Averill, B. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79,* 4623–4627.

Davis, J. C., Lin, S. S., & Averill, B. A. (1981) *Biochemistry 20,* 4062–4067.

Debrunner, P. C., Hendrich, M. P., deJersey, J., Keough, D. T., Sage, J. T., & Zerner, B. (1983) *Biochim. Biophys. Acta 745,* 103–106.

Doi, K., Antanaitis, B. C., & Aisen, P. (1988a) *Struct. Bonding (Berlin) 70,* 1–26.

Doi, K., McCracken, J., Peisach, J., & Aisen, P. (1988b) *J. Biol. Chem. 263,* 5757–5763.

Edelhoch, H. (1967) *Biochemistry 6,* 1948–1954.

Efstratiadis, T., & Moss, D. W. (1985) *Enzyme 33,* 34–40.

Ek-Rylander, B., Bill, P., Norgård, M., Nilsson, S., & Anderson, G. (1991a) *J. Biol. Chem. 266,* 24684–24689.

Ek-Rylander, B., Bergman, T., & Anderson, G. (1991b) *J. Bone Miner. Res. 6,* 365–373.

Fujimoto, S., Urata, Y., Nakagawa, T., & Ohara, A. (1984) *J. Biochem. (Tokyo) 96,* 1079–1088.

Glomset, J., & Porath, J. (1960) *Biochim. Biophys. Acta 39,* 1–8.

Hara, A., Sawada, H., Kato, T., Nakayama, T., & Matsumoto, Y. (1984) *J. Biochem. (Tokyo) 95,* 67–74.

Hara, A., Kato, T., Sawada, H., Fukuyama, K., & Epstein, W. L. (1985) *Comp. Biochem. Physiol. 82B,* 269–274.

Hardy, M. R., Reid, R., & Lee, Y. C. (1988) *Anal. Biochem. 170,* 54–62.

Hayman, A. R., Warburton, M. J., Pringle, J. A. S., Coles, B., & Chambers, T. J. (1989) *Biochem. J. 261,* 601–609.

Hopp, T. P., & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78,* 3824–3828.

Hsu, S.-M., & Soban, E. (1982) *J. Histochem. Cytochem. 30,* 1079–1082.

Hunt, D. F., Yates, J. R., III, Shabanowitz, J., Zhu, N.-Z., Zirino, T., Averill, B. A., Durat-Larroque, S. T., Shewale, J. G., Roberts, R. M., & Brew, K. (1987) *Biochem. Biophys. Res. Commun. 144,* 1154–1160.

Kato, T., Hara, A., Nakayama, T., Sawada, H., Hanatake, M., & Matsumoto, Y. (1986) *Comp. Biochem. Physiol. 83B,* 813–817.

Keough, D. T., Beck, J. L., deJersey, J., & Zerner, B. (1982) *Biochem. Biophys. Res. Commun. 108,* 1643–1648.

Ketcham, C. M., Baumbach, G. A., Bazer, F. W., & Roberts, R. M. (1985) *J. Biol. Chem. 260,* 5768–5776.

Ketcham, C. M., Roberts, R. M., Simmen, R. C. M., & Nick, H. S. (1989) *J. Biol. Chem. 264,* 557–563.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157,* 105–132.

Laemmli, U. K. (1970) *Nature 227,* 680–685.

Lau, K.-H. W., Farley, J. R., & Baylink, D. J. (1985) *J. Biol. Chem. 260,* 4653–4660.

Lau, K.-H. W., Freeman, T. K., & Baylink, D. J. (1987) *J. Biol. Chem. 262,* 1389–1397.

Lau, K.-H., Thomas, A. B., Yoo, A., Nguyen, T. S. A., & Wergedal, J. E. (1992) *Int. J. Biochem. 24,* 1815–1824.

LeGendre, N., & Matsudaira, P. (1988) *BioTechniques 6,* 154–159.

Lord, D. K., Cross, N. C. P., Bevilacqua, M. A., Rider, S. H., Gorman, P. A., Groves, A. V., Moss, D. W., Sheer, D., & Cox, T. M. (1990) *Eur. J. Biochem. 189,* 287–293.

Paigen, K. (1958) *J. Biol. Chem. 233,* 388–394.

Paigen, K., & Griffiths, S. K. (1959) *J. Biol. Chem. 234,* 299–303.

Pyrz, J. W., Sage, J. T., Debrunner, P. G., & Que, L., Jr. (1986) *J. Biol. Chem. 261,* 11015–11020.

Que, L., Jr., & True, A. E. (1990) *Prog. Inorg. Chem. 38,* 97–200.

Reisfeld, R. A., Lewis, N. J., & Williams, D. E. (1962) *Nature 195,* 281–283.

Roberts, R. M., & Bazer, F. W. (1980) in *Steroid Induced Uterine Proteins* (Beato, M., Ed.) pp 133–149, Elsevier Scientific Publishing Co., Amsterdam.

Roberts, R. M., & Bazer, F. W. (1984) *BioEssays 1,* 8–11.

Robinson, D. B., & Glew, R. H. (1980) *J. Biol. Chem. 255,* 5864–5870.

Robinson, D. B., & Glew, R. H. (1981) *Arch. Biochem. Biophys. 210,* 186–199.

Saunders, P. T. K., Renegar, R. H., Raub, T. J., Baumbach, G. A., Atkinson, P. H., Bazer, F. W., & Roberts, R. M. (1985) *J. Biol. Chem. 260,* 3658–3665.

Schlosnagle, D. C., Bazer, F. W., Tsibris, J. C. M., & Roberts, R. M. (1974) *J. Biol. Chem. 249,* 7574–7579.

Segrest, J. P., & Jackson, R. L. (1973) *Methods Enzymol. 28,* 54–63.

Simmen, R. C. M., Srinivas, V., & Roberts, R. M. (1989) *DNA 8,* 543–554.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150,* 76–85.

Stephan, J. J., Lau, J.-H. W., Mohan, N. S., Singer, F. R., & Baylink, D. J. (1990) *Biochem. Biophys. Res. Commun. 168,* 792–800.

Suelter, C. M. (1985) *A Practical Guide to Enzymology,* p 28, Wiley-Interscience, New York.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76,* 4350–4354.

Townsend, R. R., Handy, M. R., Cumming, D. A., Carver, J. P., & Bendiak, B. (1989) *Anal. Biochem. 182,* 1–8.

Vincent, J. B., & Averill, B. A. (1990a) *FASEB J. 4,* 3009–3014.

Vincent, J. B., & Averill, B. A. (1990b) *FEBS Lett. 263,* 265–268.

Vincent, J. B., Olivier-Lilley, G. L., & Averill, B. A. (1990) *Chem. Rev. 90,* 1447–1467.

Vincent, J. B., Crowder, M. W., & Averill, B. A. (1991a) *Biochemistry 30,* 3025–3034.

Vincent, J. B., Crowder, M. W., & Averill, B. A. (1991b) *J. Biol. Chem. 266,* 17737–17740.

Weber, K., Pringle, J. R., & Osborne, M. (1972) *Methods Enzymol. 26,* 3–27.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118,* 197–203.

Yang, J. T., Wu, C.-S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130,* 208–269.