Purification and characterization of a tartrate-resistant acid phosphatase from human osteoclastomas

Alison R. HAYMAN,*§ Michael J. WARBURTON,* Jean A. S. PRINGLE,† Brian COLES‡ and Timothy J. CHAMBERS*

* Department of Histopathology, St. George's Hospital Medical School, London SW17 0RE,
† Department of Morbid Anatomy, Royal National Orthopaedic Hospital, Stanmore, Middlesex,
and ‡ Department of Biochemistry, University College and Middlesex School of Medicine, London W1P 6DB, U.K.

Tartrate-resistant acid phosphatase is one of the major enzymes produced and secreted by osteoclasts. To obtain sufficient enzyme for biochemical characterization, we have purified this enzyme from human osteoclastomas by sequential chromatography on SP-Sephadex, CM-Sephadex, hydroxylapatite, Sephadex G-150 and concanavalin A–Sepharose. The purification over the original tumour extract was about 2000-fold, with a yield of 10%. The enzyme appeared to be homogeneous when assessed by SDS/polyacrylamide-gel electrophoresis. Both gel filtration and SDS/polyacrylamide-gel electrophoresis indicated an M_r of about 30000. The reduced and alkylated enzyme consists of two subunits with M_rs of 15000 and 17500. The *N*-terminal amino acid sequence of both subunits indicates that there is a high degree of identity between the osteoclastoma enzyme and similar enzymes purified from spleen and uterus. Using 4-methylumbelliferyl phosphate as substrate, the specific activity of the purified enzyme was 387 units·mg^{-1}, and the K_m was 284 μM. The pH optimum was 5.7. Unlike similar enzymes purified from human and bovine bone, osteoclastoma acid phosphatase is not activated by reducing agents (2-mercaptoethanol or ascorbic acid). The enzyme contains 4.8 mol of $Fe^{2+/3+}$, 0.3 mol of Mn^{2+} and 1.7 mol of Mg^{2+} per mol of enzyme. Although the enzyme loses 50% of its activity in the presence of EDTA, it is not inhibited by the iron chelator 1,10-phenanthroline. However, the enzyme is activated to a small extent by Mn^{2+} and Mg^{2+}. Using a variety of substrates and inhibitors, we demonstrate that there are differences between the osteoclastoma acid phosphatase and the enzyme purified from other sources.

INTRODUCTION

Tartrate-resistant acid phosphatase is either undetectable or barely detectable in normal tissues except bone [1], where it can be identified histochemically in osteoclasts [2,3]. Histochemical demonstration of the enzyme is frequently used for the specific identification of osteoclasts in tissues. During bone resorption, osteoclasts secrete acid hydrolases, including tartrate-resistant acid phosphatase [4,5], in a hormone-responsive manner. Increased concentrations of tartrate-resistant acid phosphatase are detectable in serum in physiological and pathological circumstances associated with increased bone resorption [6–9]. Tartrate-resistant acid phosphatase also appears in circulating white cells and enlarged spleens of patients with hairy-cell leukaemia [10], in spleens and sera of patients with Gaucher's disease [11], and in macrophages after incubation *in vitro* [12]. Although the tartrate-resistant acid phosphatases from patients with Gaucher's disease and leukaemic reticuloendotheliosis have been characterized [11,13–16], the extent to which these resemble the major physiological source of the enzyme (osteoclasts) has not been clearly defined. This is largely because, although osteoclasts contain large amounts of the enzyme, they are numerically a very small cell population, and it has been difficult to obtain sufficient cells for full characterization of the enzyme. The only practicable source of osteoclasts in numbers sufficiently large for enzyme characterization is the giant cell tumour of bone, a very rare neoplasm that can be obtained in gram quantities and which contains relatively large numbers of osteoclasts [17,18]. We used tissue from giant cell tumours to extract and purify the enzyme, to enable comparison with the properties of tartrate-resistant acid phosphatases from other sources. Such information may assist in the identification of the role for this enzyme in bone resorption, and may enable development of more specific and sensitive means of identification of osteoclastic phenotype and assessment of osteoclastic function.

EXPERIMENTAL

Materials

Sephadex G-150, sulphopropyl (SP)-Sephadex, CM-Sephadex and concanavalin A–Sepharose were obtained from Pharmacia Fine Chemicals. Hydroxylapatite and the silver stain kit were from Bio-Rad Laboratories. Cellulose powder was from Whatman. SDS was obtained from Koch–Light, and cirrasol ALN-WF (a non-ionic detergent) from I.C.I. (Specialist Chemicals) Ltd., Leatherhead, Surrey, U.K. All substrates and chemicals were of analytical grade and were obtained from either

Abbreviation used: SP-Sephadex, sulphopropyl-Sephadex.
§ To whom correspondence should be addressed.

BDH Ltd. or Sigma Chemical Co. Osteoclastomas were collected immediately after surgery and were stored at −70 °C.

Determination of acid phosphatase activity

Enzyme activity was measured fluorimetrically. The mixture, containing enzyme, 1.0 mM-4-methylumbelliferyl phosphate, 50 mM-disodium tartrate and 0.1 M-sodium acetate, pH 5.7, in a final volume of 0.2 ml, was incubated at 37 °C for 1–5 min. The reaction was terminated by the addition of 0.8 ml of 0.2 M-sodium phosphate-glycinate buffer, pH 10.4 [5]. The fluorescence of the 4-methylumbelliferone liberated was measured using a Locarte model 8–9 fluorimeter. One unit of enzyme activity corresponds to the hydrolysis of 1 μmol of substrate/min.

The release of phosphate generated from assays involving phosphorylated compounds was determined spectrophotometrically based on the method of Atkinson *et al.* [19]. The mixture, containing enzyme, 0.25–4.0 mM-substrate, 0.1 M-sodium acetate, pH 5.7, in a final volume of 0.2 ml, was incubated at 37 °C for 20 min. The reaction was terminated by the addition of 0.3 ml of 5% aqueous cirrasol ALN-WF/2% ammonium molybdate/0.4 M-acetic acid (20:50:30, by vol.). After mixing, 0.3 ml of 2.5% SDS in 5 mM-EDTA, pH 8.0/5% ascorbic acid/0.4 M-acetic acid (50:20:30, by vol.) was added. The phosphomolybdic acid produced was measured at 700 nm against a water blank after 20–30 min at 25 °C. A standard curve was constructed using potassium dihydrogen orthophosphate. Absorbance was measured after 20 min as for the test samples.

In kinetic experiments, the concentration of substrate ranged from 0.25 to 4.0 mM. The reaction was linear with time and over the range of enzyme concentration used throughout these experiments.

Protein determination

Protein concentrations were determined fluorimetrically [20]. The aqueous protein (0.5 ml), mixed with 0.75 ml of 0.1 M-sodium borate buffer, pH 9.2, was vortexed with 0.25 ml of fluorescamine (0.2 mg·ml^{-1} in acetone), and the products measured fluorimetrically. Bovine serum albumin was used as a standard.

Purification of tartrate-resistant acid phosphatase from osteoclastomas

All procedures were carried out at 4 °C unless stated otherwise. The standard buffer contained 10 mM-sodium acetate, pH 5.5, 0.02% sodium azide and 0.1 mM-phenylmethanesulphonyl fluoride. Acid phosphatase was determined using the fluorimetric method.

Step 1: Homogenization. Frozen tissue (20 g) was thawed and homogenized in a Waring blender with 8 vol. (w/v) of 50 mM-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and 0.1 mM-phenylmethanesulphonyl fluoride. Two vol. of 5% (v/v) Triton X-100 in the same buffer were added to the homogenate which was then stirred overnight. The homogenate was centrifuged for 2 h at 12000 *g*, and the supernatant was dialysed exhaustively against the standard buffer containing 0.1 M-NaCl, followed by further centrifugation for 2 h at 12000 *g*.

Step 2: SP-Sephadex chromatography. The supernatant from step 1 was applied to a 2.6 cm × 35 cm SP-Sephadex column equilibrated in the same buffer. After thorough washing of the column with 500 ml of the equilibration buffer, the tartrate-resistant acid phosphatase was eluted with 2 × 500 ml linear gradients of 0.1–0.5 M- and 0.5–1.0 M-NaCl. Fractions (9 ml) were collected and assayed for acid phosphatase activity. Active fractions were pooled and dialysed against the standard buffer.

Step 3: CM-Sephadex chromatography. The tartrate-resistant enzyme from the previous step was applied to a 1.6 cm × 20 cm CM-Sephadex-C50 column equilibrated in the standard buffer containing 0.1 M-NaCl. A 400 ml linear gradient of 0.1–1.0 M-NaCl in the same buffer was applied to elute the enzyme. Acid phosphatase activity was determined and active fractions were pooled and dialysed against the standard buffer containing 0.1 M-NaCl.

Step 4: Hydroxylapatite chromatography. The enzyme from step 3 was applied to a 1.6 cm × 10 cm column containing hydroxylapatite/cellulose powder (2:1, w/w) equilibrated in the standard buffer containing 0.1 M-NaCl. The column was eluted with 300 ml of a 0–0.5 M linear ammonium sulphate gradient in the same buffer. Acid phosphatase activity was determined and active fractions were pooled and dialysed exhaustively against water.

Step 5: Sephadex G-150 gel-filtration chromatography. Acid phosphatase from step 4 was lyophilized and applied in a 0.5 ml vol. of the standard buffer containing 0.1 M-NaCl to a 1.6 cm × 100 cm Sephadex G-150 column previously equilibrated in the same buffer. The active fractions were pooled.

Step 6: Concanavalin A–Sepharose chromatography. The pooled enzyme from the previous step was adjusted to pH 6.5 with solid sodium acetate and the NaCl concentration increased to 0.5 M. This was applied at 25 °C to a 1 cm × 10 cm column of concanavalin A–Sepharose previously equilibrated in 10 mM-sodium acetate buffer, pH 6.5, containing 0.5 M-NaCl. The enzyme was eluted at 4 °C using the same buffer supplemented with 0.4 M-α-methyl mannoside and 30% (v/v) ethyleneglycol. Fractions containing acid phosphatase activity were pooled, dialysed against water and lyophilized.

The purified enzyme was stored at −20 °C in a solution of the standard buffer containing 0.1 M-NaCl and 30% (v/v) glycerol.

Structural studies

The enzyme (10 μg) was dissolved in 150 μl of acetonitrile/water (1:1, v/v), and ammonium bicarbonate added to 150 mM and guanidine hydrochloride to 6 M. Mercaptoethanol (1 μl) was added and the mixture incubated for 1 h at 37 °C in a sealed tube under N_2. 4-Vinylpyridine (1.5 μl) was added and the incubation was continued for 16 h at 37 °C. The reaction mixture was applied to a Brownlee Aquapore RP300, C-8 reverse-phase h.p.l.c. column (50 mm × 2.1 mm) and eluted with a gradient of 10–65% acetonitrile in water containing 0.05% trifluoroacetic acid. Amino acid sequence analysis of the separated subunits was carried out by Edman

degradation using an Applied Biosystems 470A protein sequencer, and h.p.l.c. analysis of phenylthiohydantoin (PTH)-derivatized amino acids. The average repetitive yield was 85.8% for the A subunit and 96.7% for the B subunit.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Laemmli [21]. SDS/polyacrylamide gels were stained using a silver stain kit obtained from Bio-Rad.

M_r determination by gel filtration

Acid phosphatase was chromatographed on a Sephadex G-150 column as described in purification step 5. The void volume was measured using Dextran Blue and the total volume determined using Page Blue 83. Aldolase (158000), haemoglobin (70000), bovine serum albumin (67000), ovalbumin (43000) and cytochrome *c* (12000) were used as M_r markers. The elution volume of acid phosphatase was determined by enzymic activity measured fluorimetrically, whereas each standard was determined by absorbance at 280 nm.

Metal determination

The metal composition of the enzyme was determined by atomic absorption spectroscopy by Butterworth Laboratories, Twickenham, Middlesex, U.K.

RESULTS

Purification of acid phosphatase

The tartrate-resistant enzyme was purified by sequential chromatography on SP-Sephadex, CM-Sephadex, hydroxylapatite, Sephadex G-150 and concanavalin A–Sepharose by 2000-fold over the tumour homogenate supernatant, with a yield of 10% and a specific activity of 387 units·mg^{-1}. The purification summary is shown in Table 1. The first chromatographic stage on SP-Sephadex removes most of the contaminating proteins including a high-M_r acid phosphatase which is partially tartrate-sensitive. This partly accounts for the low recovery of enzyme activity. The affinity of the enzyme for concanavalin A suggests it is a glycoprotein, although we have no further information on the carbohydrate content. SDS/polyacrylamide-gel electrophoresis of the purified enzyme indicated an M_r of 30000 under non-reducing conditions (Fig. 1). This value was in agreement with that obtained by gel filtration. Under reducing conditions a major band was observed with an M_r of 15000, and a diffuse band with an M_r of about 17500 (Fig. 2).

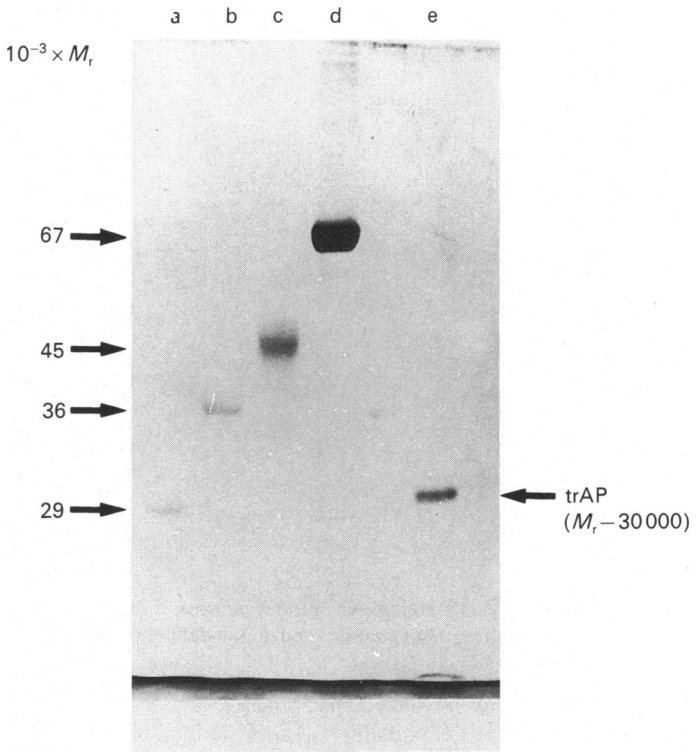

Fig. 1. M_r determination of acid phosphatase by SDS/polyacrylamide-gel electrophoresis

Tartrate-resistant acid phosphatase (trAP) (lane e) was analysed by SDS/polyacrylamide-gel electrophoresis followed by silver staining together with standards: carbonic anhydrase, M_r 29000 (lane a), glyceraldehyde-3-phosphate dehydrogenase, M_r 36000 (lane b), ovalbumin, M_r 45000 (lane c) and bovine serum albumin, M_r 67000 (lane d).

Reverse-phase h.p.l.c. of the purified enzyme revealed a major protein peak (Fig. 3) which coincided with enzyme activity (results not shown). After purification by h.p.l.c. and subsequent reduction and alkylation with 4-vinylpyridine [22], two protein peaks were observed (Fig. 3). SDS/polyacrylamide-gel electrophoresis demonstrated that peak B corresponded to the M_r-17500 subunit and peak A to the M_r-15000 subunit (Fig. 4). Peak B was usually a doublet. Alkylation resulted in complete loss of enzyme activity. *N*-Terminal amino acid analysis of subunits A and B revealed a high degree of identity with the *N*-terminal sequence of the subunits of bovine spleen tartrate-resistant acid phosphatase and with the *N*-terminus and an internal sequence of pig

Table 1. Summary of the purification of tartrate-resistant acid phosphatase

Purification procedure	Protein (mg)	Total activity (μmol·min^{-1})	Specific activity (μmol·min^{-1}·mg^{-1})	Purification (-fold)	Recovery (%)
Extract	848.4	185.8	0.2	–	100
SP-Sephadex	1.805	23.5	13.0	65	12.6
CM-Sephadex	1.037	24.3	23.4	117	13.1
Hydroxylapatite	0.832	39.9	48.0	240	21.5
Sephadex G-150	0.289	18.3	63.3	317	9.8
Concanavalin A–Sepharose	0.046	17.8	387.0	1935	9.6

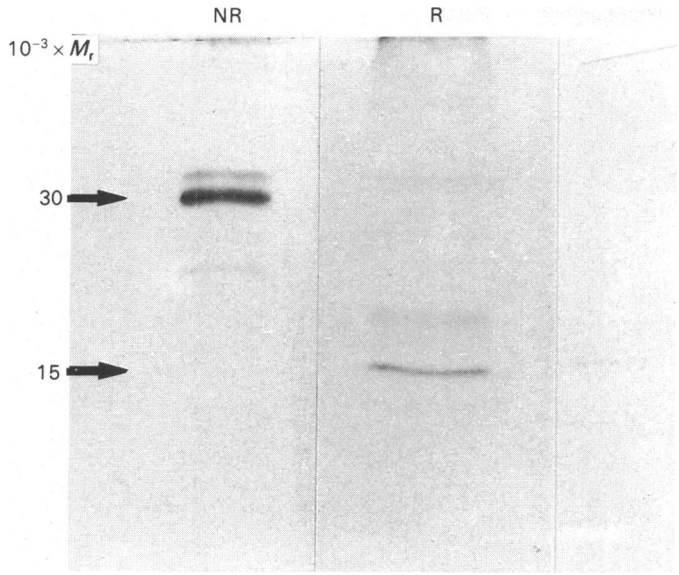

Fig. 2. SDS/polyacrylamide-gel electrophoresis of tartrate-resistant acid phosphatase under non-reducing (NR) and reducing (R) conditions

After reduction, the enzyme is converted into two major subunits, a sharp band with M_r 15000 and a diffuse band with M_r 17500.

uteroferrin (Fig. 5). A search of the National Biological Research Foundation protein sequence data bank also revealed that the *N*-terminus of the B subunit was 41 % identical to a sequence near the *N*-terminus of the proteolipid subunit of maize mitochondrial F_0-ATPase [23] (Fig. 6). *N*-Terminal amino acid sequence analysis of the leading and trailing edge of the B subunit yielded identical sequences.

The metal content of the enzyme was determined by atomic absorption spectroscopy. The purified enzyme contained 4.8 mol of iron, 0.3 mol of manganese and 1.7 mol of magnesium per mol of enzyme. A similar analysis of an acid collagenase also purified from osteoclastomas [24] contained negligible quantities of these three metals.

Substrate specificity

The hydrolysis of natural and synthetic substrates was investigated (Table 2). Preferred substrates were the artificial compounds *p*-nitrophenyl phosphate, 4-methylumbelliferyl phosphate and the nucleoside tri- and diphosphates, whereas the nucleoside monophosphates, pyrophosphate, the phosphoprotein phosvitin and sugar phosphates were poor substrates, activity being undetected in the assay system (Table 2). Values were calculated using Lineweaver–Burk plots. When the results are calculated as $k_{cat.}/K_m$, the most efficiently hydrolysed substrates were ATP and GTP. The optimum pH was 5.5–5.7 in sodium acetate buffer for all substrates. The enzyme was stable in the pH range 4–7 (results not shown).

Inhibition studies

A product inhibition analysis of the purified enzyme was carried out. A Lineweaver–Burk plot at various concentrations of phosphate indicated that it was a competitive inhibitor of the enzyme with a K_i of 0.75 mM

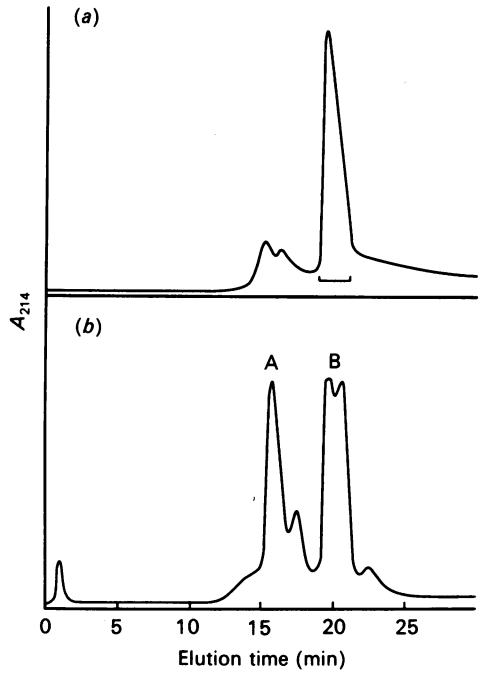

Fig. 3. Reverse-phase h.p.l.c. of tartrate-resistant acid phosphatase either non-reduced (*a*), or reduced and alkylated with 4-vinylpyridine (*b*)

The major protein peak from the non-reduced enzyme (indicated by a bar) yields two major subunits (A and B) after reduction and alkylation.

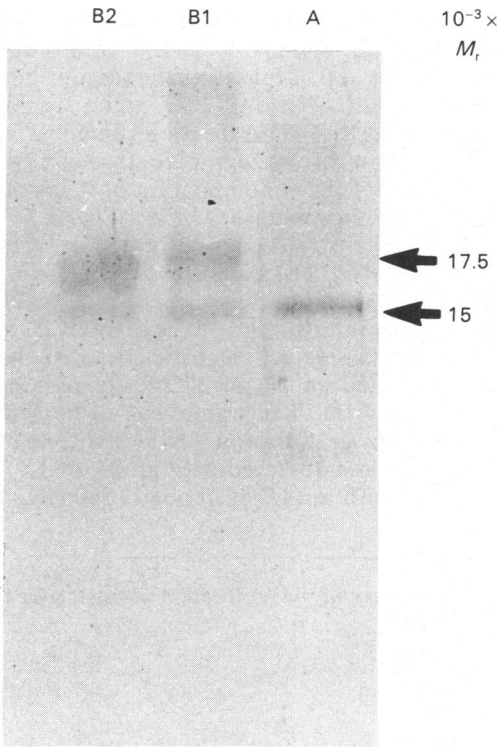

Fig. 4. SDS/polyacrylamide-gel electrophoresis of subunits A and B separated by h.p.l.c.

B1 and B2 refer to the leading and trailing edge of the doublet peak.

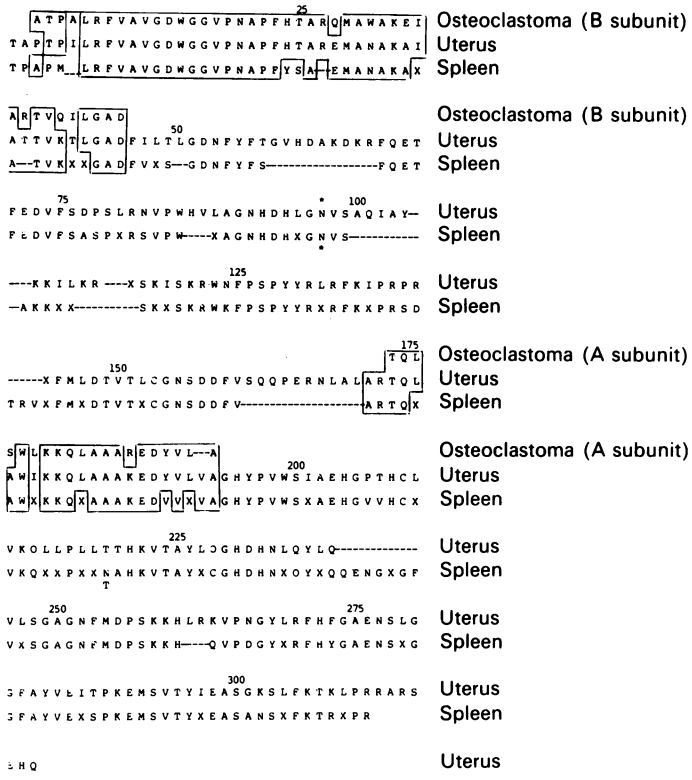

Fig. 5. Comparison of the amino acid sequences of osteoclastoma, bovine spleen and pig uterus tartrate-resistant acid phosphatases

Boxes indicate identical sequences.

(Fig. 7). Methylumbelliferone, at concentrations up to 4 mM, had no effect on the activity of the enzyme (results not shown). Transition-state analogues of phosphate such as molybdate were potent inhibitors of the osteoclastoma enzyme. The inhibition by molybdate was competitive, with a K_i of 0.18 μM (Fig. 8). As discussed by Lau *et al.* [25], these results are consistent with a pseudo Uni/Bi hydrolytic two-step transfer reaction mechanism involving the formation of a covalent phosphoryl-enzyme intermediate. However, fluoride, which has also been suggested to act as a transition-state analogue of phosphate, was a much less potent inhibitor of the enzyme than molybdate. In addition, the inhibition was non-competitive with a K_i of 3.1 mM (Table 3). The slopes and intercepts of the inhibitor plots were determined by least-squares regression analysis.

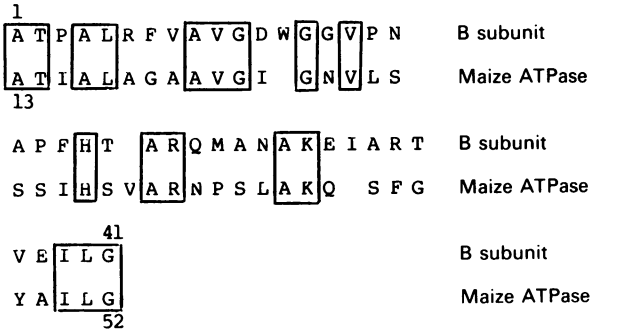

Fig. 6. Comparison of the amino acid sequences of osteoclastoma tartrate-resistant acid phosphatase and the F_o subunit of maize mitochondrial ATPase

Boxes indicate identical sequences.

The majority of tartrate-resistant acid phosphatases are activated by reducing agents (e.g. 2-mercaptoethanol, ascorbic acid, Fe^{2+}) and inhibited by divalent cations with low oxidation potential, suggesting that sulphydryl groups are required for activity. However, the osteoclastoma enzyme was inhibited by reducing agents although it was also inhibited in a non-competitive manner by a variety of divalent metal ions (Table 3). Copper was the most potent inhibitor, with a K_i of 6.8 μM, whereas Fe^{2+} was the least effective inhibitor with a K_i of 800 μM. Zn^{2+} has been shown to be an uncompetitive inhibitor of the enzyme from bovine cortical bone matrix, whereas the inhibition by Fe^{2+} is partially non-competitive [25]. Unlike other tartrate-resistant acid phosphatases, the osteoclastoma enzyme is inhibited by EDTA, suggesting a requirement for a divalent cation(s). The rates of hydrolysis of methylumbelliferyl phosphate, ATP and GTP by the osteoclastoma enzyme are increased, to a small extent, by both Mg^{2+} and Mn^{2+}. A summary of inhibitors of the osteoclastoma enzyme is shown in Table 3.

DISCUSSION

Tartrate-resistant acid phosphatases have been purified from a number of sources including Gaucher spleen [6], bovine spleen [7], spleens of patients with hairy cell leukaemia [13], human, bovine and rat bone [25,28–30], lung [31], and pig uterus [32]. These enzymes share a number of properties, including M_r values of 30000–40000, pH optima of 5–6, and extreme sensitivity to inhibition by molybdate. Although the enzyme has previously been purified from bone [25,28,29], these studies utilized non-human tissue, and moreover the cellular source of these enzymes is not entirely clear. We have purified, to apparent homogeneity, a tartrate-resistant acid phosphatase from human osteoclastomas in which the osteoclast is a major cell type. The specific activity of the purified enzyme from bone is generally about 10-fold lower than that of the spleen enzyme [13,26,27]. The final specific activity of the osteoclastoma enzyme was 387 units·mg^{-1}.

The subunit structure of the osteoclastoma enzyme is similar to that reported for the tartrate-resistant acid phosphatase purified from bovine spleen [27], and the *N*-termini of both subunits show extensive sequence similarities with the *N*-termini of the spleen enzyme subunits [33]. However, the B subunit is considerably smaller than the corresponding subunit of the spleen enzyme, having an M_r of 17500 compared with 24000 for the spleen enzyme subunit. An enzyme purified from rat bones has subunits of similar size to those of the osteoclastoma enzyme [34]. In addition, both osteoclastoma enzyme subunits lack the two *N*-terminal amino acids found in the spleen enzyme subunits. The *N*-terminal sequences of both osteoclastoma and spleen enzymes are similar to the sequence of tartrate-resistant acid phosphatase (also known as uteroferrin) purified from pig uterus. However, this latter enzyme is synthesized as a single polypeptide. The *N*-termini of these enzymes also show about 40% identity with that of

Table 2. Substrate specificity of osteoclastoma tartrate-resistant acid phosphatase

The specific activities K_m and $k_{cat.}$ of acid phosphatase for various phosphorylated substrates were determined by double-reciprocal plot analysis. Liberated phosphate from reaction mixtures containing up to 4.0 mM substrate was determined spectrophotometrically.

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ $s^{-1}\cdot$(mmol of enzyme)$^{-1}$
p-Nitrophenylphosphate	198	1.000	198
4-Methylumbelliferyl phosphate	121	0.284	424
ATP	119	0.265	448
UTP	119	0.714	167
GTP	113	0.260	434
Phenolphthalein diphosphate	109	0.276	394
TTP	107	0.299	358
CTP	93	0.377	248
GDP	86	0.348	246
UDP	79	0.615	129
ADP	73	0.333	220
TDP	69	0.476	146
Phenyl phosphate	62	0.606	102
CDP	58	0.606	96
α-Naphthyl phosphate	53	0.909	59
Phosphoenolpyruvic acid	2	0.339	3
AMP	–	–	–
CMP	–	–	–
GMP	–	–	–
TMP	–	–	–
UMP	–	–	–
Phosvitin	–	–	–
4-Methylumbelliferyl pyrophosphate	–	–	–
α-D-Glucose 1-phosphate	–	–	–
α-D-Glucose 6-phosphate	–	–	–

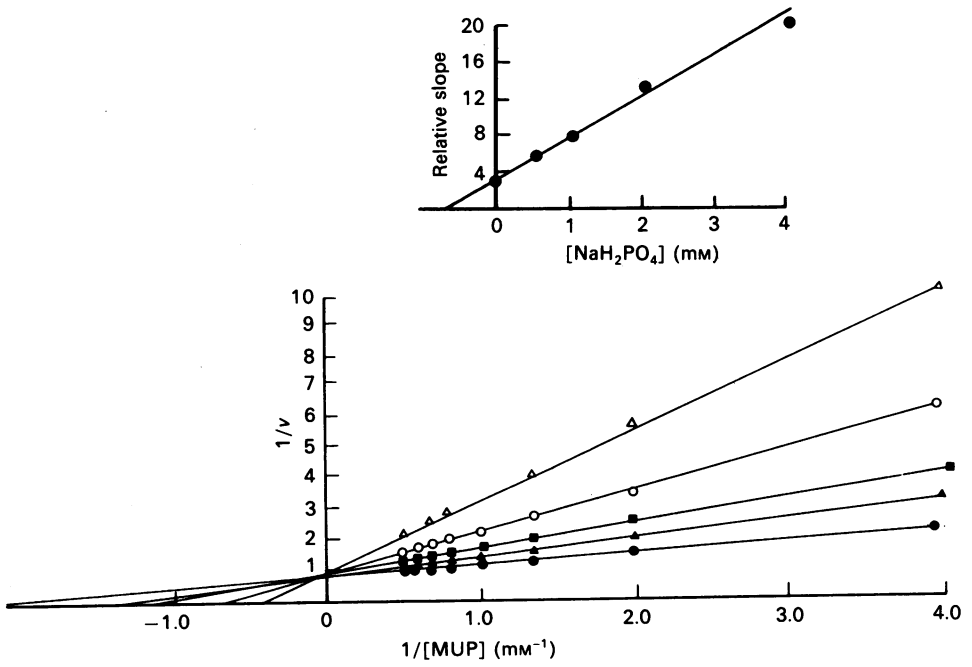

Fig. 7. Product inhibition of tartrate-resistant acid phosphatase by phosphate

A Lineweaver–Burk plot and replot (insert) are given. MUP, 4-methylumbelliferyl phosphate. The effects of no phosphate (●), 0.5 mM- (▲), 1 mM- (■), 2 mM- (○) and 4 mM-phosphate (△) are shown.

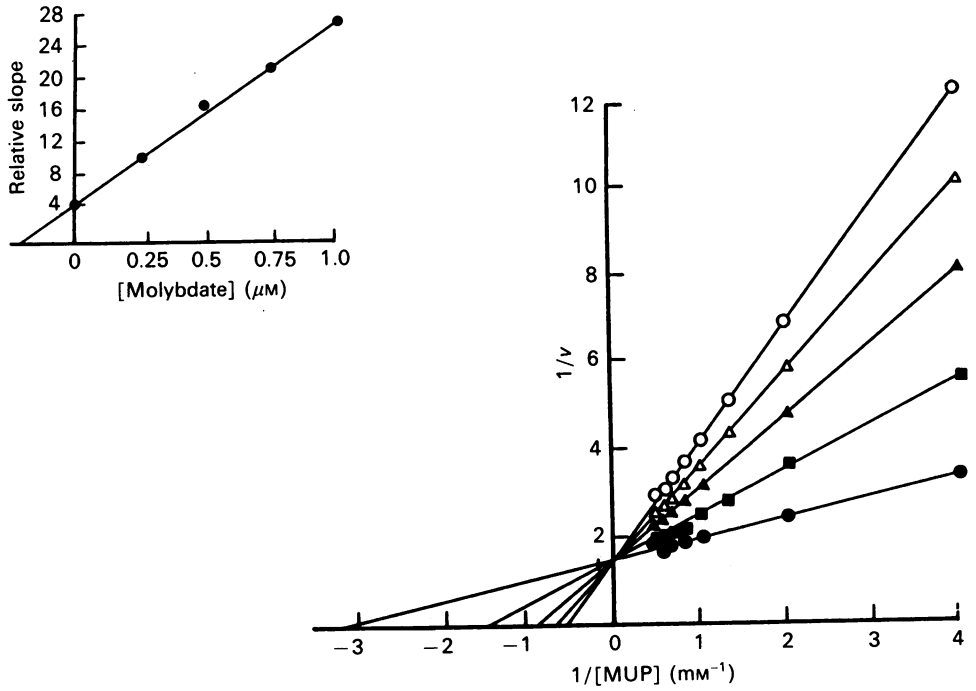

Fig. 8. Kinetics of inhibition of tartrate-resistant acid phosphatase by molybdate

A Lineweaver–Burk plot and replot (insert) are given. MUP, 4-methylumbelliferyl phosphate. The effect of no molybdate (●), 0.25 μM- (■), 0.5 μM- (▲), 0.75 μM- (△) and 1.0 μM-molybdate (○) are shown.

the non-catalytic, lipid-binding subunit of mitochondrial F_0-ATPase which acts as a proton pump. Osteoclasts contain an active proton pump which acidifies the space between the osteoclast and the bone surface, resulting in the breakdown of bone mineral [35]. Clearly, further investigations are required to determine if tartrate-resistant acid phosphatases contribute to proton transport. Tartrate-resistant acid phosphatases do not appear to share any sequence identity with lysosomal acid phosphatase [36].

We also investigated a number of characteristics of the osteoclastoma enzyme in order to determine if this enzyme had substantially different properties from other tartrate-resistant acid phosphatases. Acid phosphatases from both spleen and bone contain iron, and this may be essential for activity [27,29]. The osteoclastoma enzyme

Table 3. Effect of compounds on the activity of acid phosphatase

Several compounds were tested for their ability to inhibit acid phosphatase. These were included in the reaction mixture and the effects determined fluorimetrically. n.d., not determined.

Inhibitor	Concentration (mM)	Inhibition (%)	K_i (mM)	Type of inhibition
Disodium tartrate	100.0	None	–	–
Molybdic acid	0.001	73	0.00018	Competitive
Sodium dihydrogen orthophosphate	0.5	40	0.754	Competitive
Sodium fluoride	1.0	46	3.1	Non-competitive
Sodium chloroaurate	0.005	64	0.00053	Non-competitive
Cupric sulphate	0.005	36	0.0068	Non-competitive
β-Aminophenyl mercuric acetate	0.025	73	0.11	Non-competitive
Zinc chloride	0.15	40	0.066	Non-competitive
Ferrous ammonium sulphate	1.0	60	0.8	Non-competitive
Magnesium chloride	1.0	20% activation	–	–
Manganese chloride	1.0	20% activation	–	–
EDTA	1.0	48	n.d.	n.d.
1,10-Phenanthroline	10.0	None	–	–
β-Mercaptoethanol	200.0	19	n.d.	n.d.
Ascorbic acid	20.0	40	10.213	Non-competitive
Sodium dithionite	0.5	47	0.171	Competitive

contains 4.8 mol of iron per mol of protein. Although the osteoclastoma enzyme was inhibited by EDTA, the iron chelator phenanthroline had no effect on enzyme activity, suggesting either that the iron is inaccessible to chelators, or that it is not required for the activity of tartrate-resistant acid phosphatase. Indeed, the enzyme is inhibited by Fe^{2+} at all concentrations tested. Both Mn^{2+} and Fe^{2+} have been reported to be activators of the enzyme purified from bovine cortical bone matrix [25]. An acid phosphatase containing Mn^{2+} but not Fe^{2+} has been reported to be present in sweet potatoes [37] but this has been disputed [38]. The enzyme purified from whole rat bone has been reported to be activated by Fe^{2+} and to be resistant to EDTA [28,29]. These results suggest that there are differences in the metal requirements of acid phosphatases purified from whole bones and from osteoclastomas.

The role of acid phosphatases *in vivo* is unclear. The osteoclastoma enzyme might play a role in the dissolution of bone mineral. Contradictory reports exist concerning the pyrophosphatase activity of the enzyme purified from whole bone [28,29], whereas the spleen enzyme hydrolysed pyrophosphate relatively efficiently when isolated from the spleens of patients with hairy-cell leukaemia [13] but not when isolated from Gaucher spleen [26]. Possibly one enzyme is derived from B cells and the other from macrophages. We were unable to demonstrate any activity towards pyrophosphate by the osteoclastoma enzyme. Although bone contains phosphoproteins, the osteoclastoma enzyme was similar to those isolated from rat bone and Gaucher spleen in that it failed to dephosphorylate the phosphoprotein phosvitin [26,29]. The tartrate-resistant acid phosphatase purified from bovine cortical bone matrix has been shown to dephosphorylate histones [25]. The significance of the hydrolysis of nucleoside tri- and diphosphates is unclear, unless the enzyme is related to a proton pump. They are usually hydrolysed at 25–100% of the rate of synthetic substrates [13,26,28,29]. The osteoclastoma enzyme hydrolysed these compounds at 30–60% of the rate of hydrolysis of *p*-nitrophenyl phosphate, unlike the enzyme from bovine cortical bone matrix where the rate of hydrolysis is only 7–15% [25].

Studies on the structure, substrate specificity, the effects of inhibitors and activators, and metal ion requirements have demonstrated that there are some similarities and differences between the osteoclastoma tartrate-resistant acid phosphatase and similar enzymes purified from whole bone and other sources. It is known that several cell types, including macrophages, red cells and osteoblastic cells, all of which are present in whole bone, also contain small amounts of tartrate-resistant acid phosphatase [9,31,39], and since whole bone contains only small numbers of osteoclasts compared with these cell types, the source of tartrate-resistant acid phosphatase in some of these studies remains unclear. The source of our enzyme was chosen specifically for its high content of osteoclasts. Since the osteoclast enzyme is secreted during bone resorption, more specific identification of the enzyme may enable improved assays to be developed for osteoclastic function *in vivo* and *in vitro*.

This investigation was supported, in part, by grants from The Cancer Research Campaign and The Medical Research Council.

REFERENCES

1. Lam, K. W., Li, O., Li, C. Y. & Yam, L. T. (1973) Clin. Chem. **19**, 483–489
2. Burstone, M. S. (1958) J. Natl. Cancer Inst. **21**, 523–539
3. Eggert, F. M. (1980) Histochemistry **66**, 319–329
4. Vaes, G. (1968) J. Cell Biol. **39**, 676–684
5. Chambers, T. J., Fuller, K. & Darby, J. A. (1987) J. Cell. Physiol. **132**, 90–97
6. Chen, J., Yam, L. T., Janckila, A. J., Li, C. Y. & Lam, W. K. W. (1979) Clin. Chem. **25**, 719–722
7. Campbell, D. M. & Moss, D. W. (1961) Clin. Chim. Acta **6**, 307–315
8. Stephan, J. J., Silinkova-Malkova, E., Havranek, T., Formankova, J., Zichova, M., Lachmanova, J., Strakova, M., Bronlik, P. & Pacovsky, V. (1983) Clin. Chim. Acta **133**, 189–200
9. Lau, K. H. W., Onishi, T., Wergedal, J. E., Singer, F. R. & Baylink, D. J. (1987) Clin. Chem. **33**, 458–462
10. Yam, L. T., Li, C. Y. & Finkel, H. E. (1972) Arch. Intern. Med. **130**, 248–256
11. Robinson, D. B. & Glew, R. H. (1980) Clin. Chem. **26**, 371–382
12. Helfrich, M. H., Thesingh, C. W., Mieremet, R. H. P. & van Iperen-van Gent, A. S. (1987) Cell Tissue Res. **249**, 125–136
13. Ketcham, C. M., Baumbach, G. A. Bazer, F. W. & Roberts, R. M. (1985) J. Biol. Chem. **260**, 5768–5776
14. Lam, K. W. & Yam, L. T. (1977) Clin. Chem. **23**, 89–94
15. Lam, K. W., Li, C. Y., Yam, L. T. & Desnick, R. J. (1981) Clin. Biochem. **14**, 177–181
16. Chambers, J. P., Peters, S. P., Glew, R. H., Lee, R. E., McCafferty, L. R., Mercer, D. W. & Wenger, D. A. (1978) Metabolism **27**, 801–814
17. Chambers, T. J., Fuller, K., McSheehy, P. M. J. & Pringle, J. A. P. (1985) J. Pathol. **145**, 297–307
18. Horton, M. A., Lewis, D., McNulty, K., Pringle, J. A. S. & Chambers, T. J. (1985) Cancer Res. **45**, 5663–5669
19. Atkinson, A., Gatenby, A. D. & Lowe, A. G. (1973) Biochim. Biophys. Acta **320**, 195–204
20. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leingruhei, W. & Weigel, M. (1972) Science **178**, 871–872
21. Laemmli, U. K. (1970) Nature (London) **277**, 680–685
22. Freidman, M., Krull, L. H. & Cavins, J. F. (1970) J. Biol. Chem. **245**, 3868–3871
23. Dewey, R. E., Schuster, A. M., Levings, C. S. & Timothy, D. H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1015–1019
24. Hayman, A. R., Warburton, M. J. & Chambers, T. J. (1988) Biochem. Soc. Trans. **16**, 1064–1065
25. Lau, K. H., Freeman, T. K., & Baylink, D. J. (1987) J. Biol. Chem. **262**, 1389–1397
26. Robinson, D. B. & Glew, R. H. (1980) J. Biol. Chem. **255**, 5864–5870
27. Davis, J. C., Lin, S. S. & Averill, B. A. (1981) Biochemistry **20**, 4062–4067
28. Anderson, T. R. & Toverud, S. U. (1979) Calcif. Tissue Int. **27**, 219–226
29. Kato, T., Hara, A., Nakayama, T., Sawada, H., Hamatake, M. & Matsumoto, Y. (1986) Comp. Biochem. Physiol. **83B**, 813–817
30. Allen, S. H., Nuttleman, P. R. & Roberts, R. M. (1987) J. Bone Min. Res. **2**, 122
31. Efstradiadis, T. & Moss, D. W. (1985) Enzyme **33**, 34–40
32. Schlosnagle, D. C., Bazer, F. W., Tsibris, J. C. M. & Roberts, R. M. (1974) J. Biol. Chem. **249**, 7574–7579
33. Hunt, D. F., Yates, J. R., Shabanovitz, J., Zhu, N.-Z., Zirino, T., Averill, B. A., Daurat-Larroque, S. T., Shewale, J. G., Roberts, R. M. & Brew, K. (1987) Biochem. Biophys. Res. Commun. **144**, 1154–1160

34. Andersson, G., Ek-Rylander, B. & Hammarstrom, L. (1984) Arch. Biochem. Biophys. **228**, 431–438
35. Baron, R., Neff, L., Louvard, D. & Courtoy, P. J. (1985) J. Cell Biol. **101**, 2210–2222
36. Pohlmann, R., Krentler, C., Schmidt, B., Schroder, W., Lorkowski, G., Calley, J., Mersmann, G., Geier, C., Wahead, A., Gottschalk, S., Grzeschik, K.-H., Hasilik, A. & von Figura, K. (1988) EMBO J. **7**, 2343–2350
37. Sugiura, Y., Kawabe, H. & Tanaka, H. (1980) J. Am. Chem. Soc. **102**, 6581
38. Hefler, S. K. & Averill, B. A. (1987) Biochem. Biophys. Res. Commun. **146**, 1173–1177
39. Wergedal, J. E. & Baylink, D. J. (1969) J. Histochem. Cytochem. **17**, 799–806

Received 28 September 1988/27 February 1989; accepted 3 March 1989