

**TARTRATE-RESISTANT
ACID PHOSPHATASE:
THREE-DIMENSIONAL
STRUCTURE AND
STRUCTURE-BASED
FUNCTIONAL STUDIES**

Studies on the enzyme using recombinant protein produced by baculovirus expression vector system in insect cells

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Research Center for Molecular Endocrinology,
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Biocenter Oulu,
University of Oulu

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Oulu, Finland

2002

Abstract

Osteoporosis is a disease characterized by abnormalities in the amount and architectural arrangement of bone tissue, which leads to impaired skeletal strength and increased susceptibility to fractures. Type 5 tartrate-resistant acid phosphatase (TRACP, AcP5) has been suggested to participate directly in bone resorption.

In this study, baculovirus expression vector system in insect cells was used to gain large amounts of recombinant type 5 acid phosphatase for structure determination, structure-based functional studies and production of monoclonal antibodies. Active and inactive forms of the enzyme were separated from each other by cation-exchange chromatography, and characterized. The enzyme was crystallized and the three-dimensional structure was determined. Based on the three-dimensional structure of the active site five different enzyme variants were constructed, produced in insect cells, and purified. The wild type enzyme and the mutated forms were characterized, and their kinetic parameters were determined. The importance of amino acids that were expected to be essential for the acid phosphatase activity was confirmed. The acid phosphatase activity and reactive oxygen species generating activity of this dual enzyme proved to exploit different amino acids in their reaction mechanisms.

Further studies are needed to clarify the physiological substrates of TRACP *in vivo*. The findings of this study could form a base for construction of inhibitors for TRACP that could be useful therapeutic agents for osteoporosis and related bone disorders.

Keywords: recombinant proteins, tartrate-resistant acid phosphatase, bone resorption, three-dimensional structure, monoclonal antibodies, site-directed mutagenesis, reactive oxygen species

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Oulu, June 2002

Helena Kaija

"Life what is it but a dream"

Verses in italics: Lewis Carroll, Through the Looking Glass and Alice in the Wonderland

Abbreviations

AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AcP	acid phosphatase
BEVS	baculovirus expression vector system
bp	base pair(s)
EPR	electron paramagnetic resonance
IEF	isoelectric focusing
K_m	Michaelis-Menten constant
k_{cat}	turnover number
k_{cat}/K_m	catalytic efficiency
kDa	kilodalton
Mab	monoclonal antibody
MHC	major histocompatibility complex
M-CSF	macrophage colony-stimulating factor
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κ B	transcription factor NF- κ B
NMR	nuclear magnetic resonance
P_i	inorganic orthophosphate
PAP	prostatic acid phosphatase
<i>p</i> NPP	<i>para</i> -nitrophenyl phosphate
PPase	protein serine/threonine phosphatase
PTPase	protein tyrosine phosphatase
PTH	parathyroid hormone
PU.1	transcriptor factor PU.1
RANK	receptor activator of NF- κ B
RANKL	receptor activator of NF- κ B ligand
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>Sf</i>	<i>Spodoptera frugiperda</i>
TNF	tumor necrosis factor
TRACP	tartrate-resistant acid phosphatase

TRAP	tartrate-resistant acid phosphatase
TR-AP	tartrate-resistant acid phosphatase
UV	ultra violet
V_{max}	maximal reaction velocity
Å	Ångström

List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Halleen J, Kaija H, Stepan J, Vihko P & Väänänen HK (1998). Studies on the protein tyrosine phosphatase activity of tartrate-resistant acid phosphatase. *Arch Biochem Biophys* 352:97-102.
- II Kaija H, Jia J, Lindqvist Y, Andersson G & Vihko P (1999). Tartrate-resistant bone acid phosphatase: Large-scale production and purification of the recombinant enzyme, characterization, and crystallization. *J Bone Miner Res* 14:424-430.
- III Lindqvist Y, Johansson E, Kaija H, Vihko P & Schneider G (1999). Three-dimensional structure of a mammalian purple acid phosphatase at 2.2 Å resolution with a μ -(Hydr)oxo bridged di-iron center. *J Mol Biol* 291:135-147.
- IV Kaija H, Alatalo S, Halleen J, Lindqvist Y, Schneider G, Väänänen HK & Vihko P (2002). Phosphatase and oxygen radical generating activities of mammalian purple acid phosphatase are functionally independent. *Biochem Biophys Res Commun* 292:128-132.

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1 Introduction

Mammalian tartrate-resistant acid phosphatase (TRACP), also known as type 5 acid phosphatase (AcP5, E.C. 3.1.3.2.), is a basic glycoprotein whose expression under physiological conditions is restricted to osteoclasts and activated macrophages. Together with similar enzymes isolated from animals, plants and fungi, it belongs to the group of purple acid phosphatases. The physiological functions of purple acid phosphatases in biological systems are not yet known, and no natural substrates have been unambiguously established. Mice deficient in TRACP develop mild osteopetrosis (Hayman *et al.* 1996), and overexpression of TRACP leads to accelerated bone turnover (Angel *et al.* 2000), suggesting that TRACP has an important role in bone resorption.

The catalytic site of mammalian TRACP contains a binuclear iron center, where one of the irons is redox-active, while the other is stabilized to ferric state (Antanaitis & Aisen 1983). The binuclear iron center of purple acid phosphatases has been studied extensively using various spectroscopic techniques. The enzyme may exist in two forms: an inactive purple form, where the redox-active iron is in the ferric state, and an active pink form, where it is reduced to the ferrous state (Antanaitis & Aisen 1983). The active enzyme is also capable of generating reactive oxygen species through Fenton's reaction, where the ferrous ion reacts with hydrogen peroxide to produce highly destructive hydroxyl radicals (Garret *et al.* 1990).

Red kidney bean acid phosphatase was the first purple acid phosphatase whose three-dimensional structure was determined (Sträter *et al.* 1995). Our group has long experience of studying prostatic acid phosphatase (PAP), an enzyme that is sensitive to inhibition by tartrate. We have carried out studies on PAP's enzymatic properties and described its structure both on gene and protein levels (Vihko *et al.* 1988, Roiko *et al.* 1990, Vihko *et al.* 1993, Schneider *et al.* 1993, Lindqvist *et al.* 1993, Lindqvist *et al.* 1994, Porvari *et al.* 1994, Virkkunen *et al.* 1994). The aim of this study was to determine the three-dimensional structure of a mammalian tartrate-resistant acid phosphatase and in particular its active site, to characterize amino acids predicted to be important for the acid phosphatase activity, to study the substrate specificity of the enzyme, and to produce monoclonal antibodies against TRACP. Knowledge of the active site structure is valuable for the design of enzyme inhibitors that could be useful as therapeutic agents for conditions with accelerated bone resorption.

2 Review of the literature

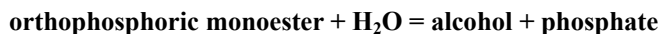
2.1 Phosphatases

Phosphate ester bond functions as an extremely important linkage within the living cell. It participates in storage and transfer of the genetic information, carries chemical energy and regulates the activity of enzymes and signaling molecules in the cell. Enzymes capable of acting on ester bonds (Enzyme Commission classification number EC 3.1) and catalyzing the cleavage of phosphate esters (EC 3.1.3) constitute the subclass of phosphohydrolases, i.e. phosphatases.

Phosphatases can be further classified according to several frameworks (Vincent *et al.* 1992). They can be divided into groups based on their substrate type. Non-specific phosphatases catalyze the hydrolysis of almost any phosphate ester, whereas protein phosphatases prefer phosphoproteins or phosphopeptides as substrates. Non-specific phosphatases can be divided into alkaline and acid phosphatases based on their optimal pH for catalysis. Non-specific alkaline and acid phosphatases recycle phosphate in metabolic reactions (Lohse *et al.* 1995).

Acid phosphatases (EC 3.1.3.2) form a group of four isoenzymes with differences originating at the structural level of the gene: prostatic, lysosomal, erythrocytic and macrophagic acid phosphatases. Acid phosphatases can also be distinguished based on their molecular weight into low molecular weight acid phosphatases and high molecular weight acid phosphatases, or on the basis of their resistance to inhibition by tartrate into tartrate-sensitive and tartrate-resistant acid phosphatases.

Reaction catalyzed by acid phosphatases:



Protein phosphatases comprise two groups based upon their substrate specificity. Protein-tyrosine phosphatases (PTPases, EC 3.1.3.48) prefer to remove phosphate from tyrosine residue and serine/threonine protein phosphatases (PPases, EC 3.1.3.16) from serine or threonine residue. The PTPase group includes dual-specificity phosphatases,

which are capable of hydrolyzing phosphorylated tyrosine, serine and threonine. Protein phosphatases regulate signal-transduction pathways (Lohse *et al.* 1995, Neel & Tonks 1997, Zhang 1998). Approximately 30% of intracellular proteins are subject to reversible protein phosphorylation (Barford *et al.* 1998).

One obvious difference among phosphatases is the presence or absence of metal ion cofactors.

2.2 Purple acid phosphatases

Purple acid phosphatases can easily be distinguished from other acid phosphatases by their purple color in solution. The color is due to the presence of a binuclear iron center (Davis & Averill 1982) and arises from tyrosine-to-iron charge transfer transition (Gaber *et al.* 1979, Davis & Averill 1982). The Committee of Scientific Advisors of the International Osteoporosis Foundation has endorsed the name type 5 acid phosphatase (AcP5) for mammalian purple acid phosphatases. The name is based on the relative electrophoretic motility of acid phosphatases, when these enzymes are being sorted electrophoretically under non-denaturing conditions. Type 5 acid phosphatases migrate fastest (Li *et al.* 1970). Purple acid phosphatases or type 5 acid phosphatases are not yet listed in Enzyme Nomenclature.

Type 5 acid phosphatases are resistant to inhibition by tartrate, and are therefore called also tartrate-resistant acid phosphatases (TRACP, other abbreviations used: TRAP, TR-AP). Resistance to inhibition by L-tartrate distinguishes type 5 acid phosphatases from acid phosphatases of lysosomal (von Figura & Weber 1978) or prostatic (Vihko *et al.* 1978) origin. Terms related to the enzyme's origin, osteoclastic acid phosphatase and macrophagic acid phosphatase, are also used. The abbreviation PAP is frequently used as reference to type 5 acid phosphatases referring to the name purple acid phosphatases. However, PAP is commonly known as the abbreviation for prostatic acid phosphatase, and it is therefore misleading.

In the present work, the name tartrate-resistant acid phosphatase (TRACP) is used for type 5 acid phosphatases and the name purple acid phosphatases for corresponding non-mammalian enzymes.

2.3 TRACP

2.3.1 Expression and cell localization

TRACP is expressed by bone-resorbing osteoclasts and activated macrophages (Minkin 1982, Efstradiadis & Moss 1985, Bevilacqua *et al.* 1991). It was first purified from

bovine spleen in 1954 (Sundararajan & Sarma 1954). In 1970 it was identified in hairy cells of patients with leukemic reticuloendotheliosis (hairy cell leukemia) (Li *et al.* 1970), and in 1973 a similar enzyme was isolated from porcine uterine fluid (Chen *et al.* 1973). In 1973 it was discovered to contain iron (Campbell & Zerner 1973), but the presence of two iron atoms per molecule of enzyme was not confirmed until 1978 (Campbell *et al.* 1978). It has since been isolated from various mammalian tissues including human (Allen *et al.* 1989), bovine (Lau *et al.* 1987a), and rat (Andersson *et al.* 1984) bone, human (Ketcham *et al.* 1985) and rat (Hara *et al.* 1984) spleen, human lungs (Efstratiadis & Moss 1985) and placenta (Ketcham *et al.* 1989).

TRACP mRNA expression has been detected in neonatal rats in skeletal tissues and with lower levels also in spleen, thymus, liver, skin, kidney, lung, heart, and brain (Ek-Rylander *et al.* 1991a) and in adult rats in cells of central and peripheral nervous system (Lång *et al.* 2001). Using murine tissues TRACP expression and activity has been demonstrated to be localized in tissues that possess cells originating from the osteoclast/macrophage lineage (Hayman *et al.* 2000a,b). The expression of TRACP is increased in some pathological conditions: in Gaucher's disease (Robinson & Glew 1980, Lam *et al.* 1981), which is a lysosomal storage disease, in leukemic reticuloendotheliosis (Yam 1974, Lam *et al.* 1976, 1981, Ketcham *et al.* 1985), in HIV-induced encephalopathy (Schindelmeiser *et al.* 1989) and in osteoporosis and metabolic bone diseases (Delmas 1992, Cheung *et al.* 1995, Halleen *et al.* 1996).

Purple acid phosphatases have been described from plants: red kidney bean (Klabunde *et al.* 1994), sweet potato (Durmus *et al.* 1999), *Arabidopsis thaliana* (del Pozo *et al.* 1999), and soybean (LeBansky *et al.* 1992, Hegeman & Grabau 2001), microbial organisms and fungi (Schenk *et al.* 2000a,b). Mammalian and plant enzymes are enzymatically and spectroscopically very similar, despite the fact that instead of two iron atoms plant enzymes contain one iron and one zinc or manganese atom (Merks and Averill 1999, Schenk *et al.* 1999, 2001), and differ by their amino acid composition and molecular weight (Klabunde *et al.* 1995, Schenk *et al.* 2000b). Sequence homology is low and the only totally conserved residues are the seven invariant metal coordinating amino acids (Klabunde *et al.* 1995).

Histochemical, cytochemical and immunocytochemical studies reveal that TRACPs are localized in lysosomes and lysosome-like organelles or intracellular transcytotic vesicles in osteoclasts, and in antigen presentation route in activated macrophages (Katayama *et al.* 1972, Lam *et al.* 1976, Andersson *et al.* 1986, Clark *et al.* 1989, Schindelmeiser *et al.* 1987, Reinholt *et al.* 1990a, Halleen *et al.* 1999a, Hayman *et al.* 2000b, Räisänen *et al.* 2001). No obvious hydrophobic membrane-spanning domain has been identified.

The major secretory product of the porcine uterus under the influence of progesterin is a tartrate-resistant acid phosphatase called uteroferrin. Uteroferrin and other TRACPs are products of the same gene and share high sequence identity (Ling & Roberts 1993). Uteroferrin differs from intracellular TRACPs in the fact that it is secreted by pig uterine glandular epithelium cells and transported to fetal blood circulation (Buhi *et al.* 1982, Baumbach *et al.* 1986).

2.3.2 TRACP gene

Tartrate-resistant acid phosphatases constitute a group of related metalloenzymes. One single gene encodes mammalian TRACPs (Ling & Roberts 1993). The TRACP gene has been localized to human chromosome 19 (Lord *et al.* 1990, Leach *et al.* 1994) and to mouse chromosome 9 (Grimes *et al.* 1993), being a member of syntenic genes localized to these human and mouse chromosomes.

The genes encoding pig (Simmen *et al.* 1989, Vallet & Fahrenkrug 2000), rat (Ek-Rylander *et al.* 1991a), human (Cassady *et al.* 1993, Reddy *et al.* 1995a, Fleckenstein *et al.* 1996), and mouse (Cassady *et al.* 1993, Reddy *et al.* 1993) TRACP have been cloned and characterized. The sequences are highly conserved. Human and porcine enzymes have 85% homology (Ketcham *et al.* 1989) and porcine and bovine enzymes 90% homology (Hunt *et al.* 1987). Rat bone TRACP is 94% identical with human placental enzyme (Ek-Rylander *et al.* 1991a) and 89% identical with uteroferrin (Ek-Rylander *et al.* 1991a). The overall sequence similarity of rat osteoclastic acid phosphatase to rat lysosomal acid phosphatase is only 41%, and to prostatic acid phosphatase 44% (Himeno *et al.* 1989, Roiko *et al.* 1990, Ek-Rylander *et al.* 1991a). Studies of human and murine TRACP genes have revealed that both of these genes contain five exons with the initiation code lying in exon 2, exon 1 remaining noncoding (Fleckenstein & Drexler 1997). Exon/intron organization of the 5'-flanking region of murine, human and porcine uteroferrin gene is similar, but they differ in respect to the size of the intron (Reddy *et al.* 1995a, Vallet & Fahrenkrug 2000). Figure 1 indicates box diagrams comparing sizes of the exons for porcine uteroferrin, human TRACP and mouse TRACP gene.

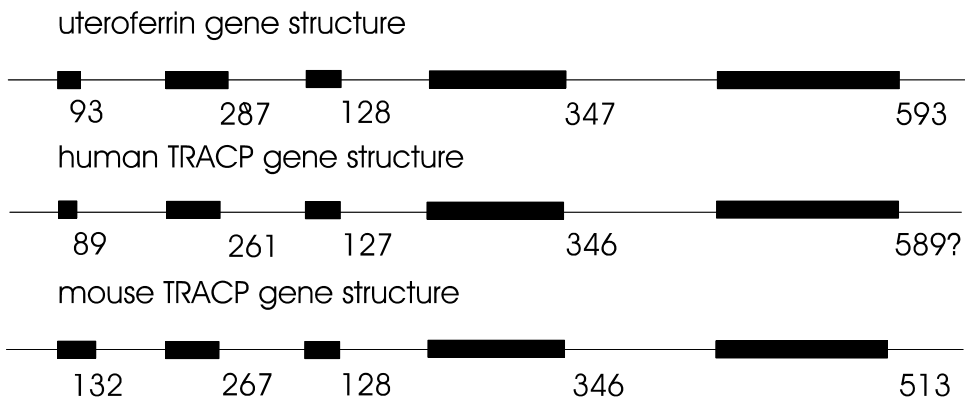


Fig. 1. Comparing sizes of the exons for porcine uteroferrin gene, human TRACP gene and mouse TRACP gene (Figure modified from Vallet & Fahrenkrug 2000).

Studies on the 5'-flanking region of the TRACP gene have indicated that transcriptional regulation of this enzyme is rather complex (Lamp & Drexler 2000) and not yet fully described. Two different promoters have been identified locating in human gene within the sequence from -1903 bp to -1bp (Reddy *et al.* 1995a,b). A putative repressor for one of the promoters and a putative enhancer for both promoters have been described (Reddy *et al.* 1995b). Iron and hemin have been found to regulate one of these promoters transcriptionally (Alcantara *et al.* 1994, Reddy *et al.* 1995b, 1996, 1998, Fleckenstein *et al.* 2000). The most important region for iron control lies between sequences -1846 bp and -1240 bp in mouse gene (Reddy *et al.* 1996). TRACP gene expression is enhanced by iron and inhibited by hemin.

Estrogen has been shown to prevent bone loss by reducing the mRNA levels of osteoclastic bone resorbing enzymes TRACP and carbonic anhydrase II (Zheng *et al.* 1995). Porcine uteroferrin gene contains progesterone and estrogen response elements (Simmen *et al.* 1989). They have, however, not been found in other TRACPs, and it has therefore been suggested that cells other than osteoclasts mediate estrogen effects in the bone environment (Collier *et al.* 1998).

TRACP is translated as a single polypeptide (Hayman *et al.* 1991). Ketcham and his co-workers have reported an open reading frame of 969 bp corresponding to a protein of 323 amino acids for human placental TRACP (Ketcham *et al.* 1989). According to Lord and his co-workers, the open reading frame of human macrophage sequence is 975 bp encoding 325 amino acids (Lord *et al.* 1990). Both groups have reported a putative signal sequence of 19 amino acids. Some of the isolated enzymes are single polypeptides while others are isolated as two-subunit proteins. It has been suggested that TRACPs are synthesized as latent proenzymes and activated by cleavage into subunits (Orlando *et al.* 1993, Ljusberg *et al.* 1999).

2.3.3 Structure of the active site

A binuclear metal center is a common feature for proteins in the class of metallohydrolases. These proteins have, however, many different kinds of structures, functional groups and chemical mechanisms to accelerate hydrolysis reactions (reviewed by Wilcox 1996, Sträter *et al.* 1996, Kimura 2000). Sequence alignments have revealed a signature motif DXH-(X)_{≈25}-GDXXD-(X)_{≈25}-GNHD/E that is common for a wide variety of phosphoesterases cleaving phosphoesterbonds in many different substrates. It has been identified in tartrate-resistant/purple acid phosphatases, Serine/Threonine phosphatases, diadenosine tetraphosphatases, exonucleases, 5'-nucleosidases and sphingomyelin phosphodiesterases. (Koonin 1994, Zhuo *et al.* 1994, Lohse *et al.* 1995, Aravind & Koonin 1998, Oddie *et al.* 2000, Knöfel & Sträter 2001). All those metallophosphatases which contain the first sequence motif seem also to possess a second common motif GH-(X)_{≈50}-GHXH (Klabunde *et al.* 1996). Sequences of proteins belonging to these groups contain five conserved blocks of residues that surround the seven invariant metal binding residues (Lohse *et al.* 1995). Alignment of some TRACP-like sequences from different sources in table 1 (Oddie *et al.* 2000).

Table 1. Conserved blocks of sequences in animal, plant, and microbial tartrate-resistant/purple acid phosphatases.

	Source	1	2	3	4	5	Length	Accession number				
Animal	Human	GDWG	34	GDNFY	34	GNDH	87	VAGH	33	GHDH	325	P13686
	Mouse	GDWG	34	GDNFY	34	GNDH	89	VAGH	33	GHDH	327	Q05117
	Rat	GDWG	34	GDNFY	34	GNDH	89	VAGH	33	GHDH	327	P29288
	Pig	GDWG	34	GDNFY	34	GNDH	89	VAGH	33	GHDH	338	P09889
Plants	Sweet potato	GDIG	25	GDLSY	32	GNHE	78	VLVH	35	GHVH	473	AF200825
	Soybean	GDLG	25	GDLSY	32	GNHE	78	VLMH	35	GHVH	464	AF236109
	Red kidney bean	GDLG	25	GDLSY	32	GNHE	78	VLMH	35	GHVH	459	Q38924
	<i>Aspergillus</i>	NDMG	25	GDLSY	84	GBHE	151	VMSH	33	GHIH	614	U18554
Micro-organisms	<i>sp.</i>											
	<i>Mycobacteria</i>	GDQS	40	GDLCY	31	GNHE	89	VCMH	35	GHEH	529	Z77724

Table 1 is modified from Oddie et al. 2000. Five conserved blocks of residues surround the seven metal-binding residues (in bold). Numbers of residues between blocks and total length are indicated.

All isolated tartrate-resistant acid phosphatases have similar physical and enzymatic properties. They are all monomeric iron-containing glycoproteins with M_r 30-40 kDa. TRACPs have basic isoelectric point (Vincent & Averill 1990a, Ek-Rylander *et al.* 1991b) and optimal enzymatic activity at acidic pH. (Vincent & Averill 1990a).

Many research groups have studied tartrate-resistant/purple acid phosphatases and their binuclear metal center using various spectroscopic methods (reviewed by Doi *et al.* 1988). The distinctive purple color of TRACP was identified by resonance Raman spectra to be due to tyrosine phenolate-to-metal charge transfer transition (Gaber *et al.* 1979, Antanaitis *et al.* 1982). Final evidence for a spin-coupled binuclear iron center at the active site of TRACP was gained from EPR (electro paramagnetic resonance) spectra and magnetic susceptibility data (Davis & Averill 1982, Antanaitis *et al.* 1983). Each molecule of TRACP binds two iron atoms in a binuclear cluster (Campbell *et al.* 1978, Davis & Averill 1982). The presence of two types of iron, a chromophoric tyrosine coordinated species that remains ferric and a colorless species that cycles between ferric and ferrous states depending on the reduction-oxidation state has been demonstrated (Antanaitis & Aisen 1983).

The enzyme can exist in two different forms: an enzymatically active pink form where the binuclear iron unit is in the ferrous-ferric state, and enzymatically inactive purple diferric state (Davis & Averill 1982). Mild reductive agents can change the inactive form into active form (Davis & Averill 1982). The reduction does not disrupt the tyrosine-iron coordination (Gaber *et al.* 1979, Antanaitis *et al.* 1982, Lambert *et al.* 1997). The diferric-state is EPR silent (Antanaitis *et al.* 1983, Day *et al.* 1988) and ferrous-ferric state elicits an intense EPR signal, distinctive to this class of enzymes (Davis & Averill 1982, Antanaitis *et al.* 1983, Day *et al.* 1988). Resonance Raman spectra show vibration, which suggests that the strong antiferromagnetic coupling between the irons results from an μ -oxo-bridge (Sjöberg *et al.* 1982).

The visible spectrum of TRACP was first published in 1960 (Glomset & Porath 1960) and after that in numerous studies (Campbell & Zerner 1973, Schlosnagle *et al.* 1974, Keough *et al.* 1980, Davis *et al.* 1981, Antanaitis & Aisen 1983, Antanaitis *et al.* 1983, Vincent *et al.* 1991a, and references therein). The purple form of the enzyme has absorption maximum between 550-570 nm and a near-UV shoulder between 315-320 nm. The pink form has absorption maximum between 505-510 nm and a near-UV shoulder at 310 nm. Preservation of the near-UV shoulder at the wavelength that is distinct to disulfide bond suggests that such potential bonds remain unaffected by reduction (Doi *et al.* 1988).

2.3.4 Three-dimensional structure

The three-dimensional structure of kidney bean purple acid phosphatase was determined in 1995 by Sträter and his co-workers at 2.9 Å resolution (Sträter *et al.* 1995). Kidney bean purple acid phosphatase is a metalloenzyme with Fe(III)-Zn(II) dinuclear active site. Kidney bean enzyme is a homodimeric protein with M_r 110 kDa containing two domains in each subunit. It has the shape of a twisted heart and overall dimensions 40 Å by 60 Å by 75 Å. The active site is at the bottom of the pocket formed by the monomers. The function of the smaller NH₂-terminal domain of each subunit is unclear. The COOH-terminal domain contains the active site. The larger domain forms an α/β -type structure. It contains five α helices and two mixed β sheets with 6 and 7 β strands. The β sheets form a sandwich-like structure. The α helices connect the parallel strands in the β sheets. The two sandwich-like structures are connected with one α helix and two β strands. The iron is coordinated by Tyr167, His325 and Asp135, and the zinc is coordinated by His286, His 323 and Asn201. The metal ions are bridged by Asp164. (Sträter *et al.* 1995).

The primary structure of mammalian enzymes from different sources is conserved with over 80% homology. Except for the blocks of sequence homology in the metal coordinating region there is low amino acid similarity and very low sequence homology between mammalian and plant enzymes. NMR studies of uteroferrin and bovine spleen enzymes reveal the ligation of one tyrosine and one histidine to the Fe(III) and the ligation of one histidine to the Fe(II) (Wang *et al.* 1992, 1996). These residues are also conserved in kidney bean enzyme (Sträter *et al.* 1995). When the zinc ion of kidney bean enzyme is replaced with Fe(II), the enzyme's spectroscopic and kinetic properties show close similarity to mammalian enzymes (Battistuzzi *et al.* 1997).

The active site of mammalian TRACPs could be expected to be similar to that of kidney bean purple acid phosphatase and an average secondary structure prediction suggested that mammalian TRACPs belong to the $\beta\alpha\beta\alpha\beta$ -type structures, as does the kidney bean enzyme (Sträter *et al.* 1995, Denu *et al.* 1996). In 1999 three independent structures of mammalian tartrate-resistant acid phosphatase were reported almost simultaneously: pig TRACP at 1.65 Å resolution (Guddat *et al.* 1999), the rat bone enzyme first at 2.7 Å resolution (Uppenberg *et al.* 1999) and a month later at 2.2 Å resolution in this study.

2.3.5 Substrate specificity, inhibitors and activators

TRACPs prefer as substrates phosphoric esters of aromatic alcohols such as *p*NPP and phosphotyrosine, and compounds containing a phosphoanhydride linkage such as nucleotide di- and triphosphates (Hara *et al.* 1984, Ketcham *et al.* 1985, Anderson & Toverud 1986, Kato *et al.* 1986, Lau *et al.* 1987a, Hayman *et al.* 1989, Allen *et al.* 1989, Andersson *et al.* 1989, Janckila *et al.* 1992a, Cheung *et al.* 1995). Phosphoric esters of aliphatic alcohols, such as nucleotide monophosphates, phosphoserine and phosphothreonine are not hydrolyzed (Anderson & Toverud 1986, Lau *et al.* 1987a, Andersson *et al.* 1989, Cheung *et al.* 1995).

Acid phosphatase activity of TRACP is inhibited by phosphate, molybdate, zinc, copper, fluoride, vanadate, arsenate, tungstate, and ascorbate (Robinson & Glew 1980, Keough *et al.* 1982, Antanaitis & Aisen 1985, Ketcham *et al.* 1985, Anderson & Toverud 1986, Hayman *et al.* 1989, Allen *et al.* 1989, Crans *et al.* 1992, Janckila *et al.* 1992a, Cheung *et al.* 1995, Andersson & Ek-Rylander 1995, Halleen *et al.* 1996). Oxidation by e.g. hydrogen peroxide causes inhibition of the enzyme activity (Ketcham *et al.* 1985, Anderson & Toverud 1986, Hayman *et al.* 1989, Janckila *et al.* 1992a, Halleen *et al.* 1996, Beck *et al.* 1999). Reaction product phosphate and its analogs vanadate, arsenate and molybdate cause competitive inhibition (Keough *et al.* 1982, Antanaitis & Aisen 1985, Janckila *et al.* 1992a). Fluoride, tungstate, copper and zinc cause noncompetitive inhibition (Hayman *et al.* 1989, Crans *et al.* 1992, Halleen *et al.* 1996).

The enzyme is activated by mild reducing agents, such as β -mercaptoethanol (Ketcham *et al.* 1985, Hayman *et al.* 1989, Janckila *et al.* 1992a, Halleen *et al.* 1996). Strong reducing agents, such as dithionite, lead to removal of iron and thus cause inhibition of the activity (Keough *et al.* 1980, Ketcham *et al.* 1985, Hayman *et al.* 1989). Ascorbate together with iron activates TRACP (Hayman *et al.* 1989, Halleen *et al.* 1996). Manganese and magnesium are reported to have an activating effect on TRACP (Hayman *et al.* 1989).

2.3.6 Proposed mechanism of hydrolysis of phosphate esters by TRACP

Phosphoryl-transfer reactions have been intensively studied because of their primary significance in biological events. Hydrolysis of phosphate esters proceeds either through dissociative or associative mechanism (Vincent *et al.* 1992). The main question is whether or not there is a covalent phosphoenzyme intermediate in the reaction. (Reviewed by Sträter *et al.* 1996). A study on bovine tartrate-resistant acid phosphatase mechanism reported evidence for a phosphoenzyme intermediate (Vincent *et al.* 1991b). More recent studies have, however, not found such intermediate (Wynne *et al.* 1995).

Based on spectroscopic studies and on the crystal structure of plant purple acid phosphatase (Sträter *et al.* 1995), the following mechanism of hydrolysis shown in Figure 2 has been suggested by Klabunde and his co-workers (Klabunde *et al.* 1996). The phosphate group of the substrate binds to the divalent metal ion, which together with the histidine ligands preorientates the phosphate group and enhances the electrophilicity of the phosphorus center. After that a nucleophilic attack occurs by the hydroxide ligand bound to the Fe^{3+} ion, and the configuration at the phosphorus center is inverted through a pentacoordinate transition-state in which the leaving group and the attacking nucleophile are in apical position. In addition to the metal ions the protonated side chains of histidine residues reduce the energy of the pentacoordinate transition-state through the formation of hydrogen bonds. An additional histidine may facilitate by a small movement the protonation of the leaving alcohol group.

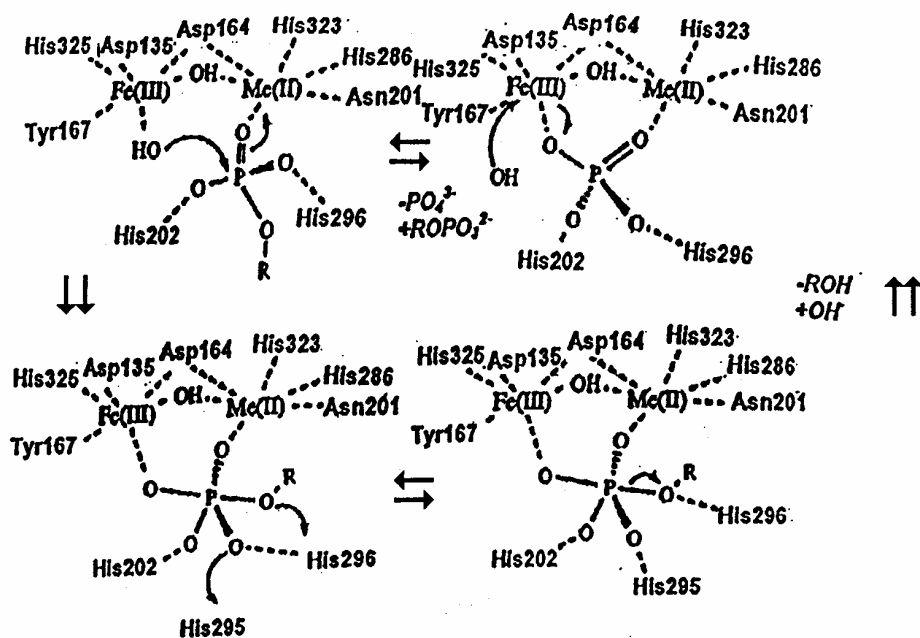


Fig. 2. Proposed mechanism of hydrolysis of phosphate group by TRACP (Figure modified from Klabunde *et al.* 1996).

Tartrate-resistant/purple acid phosphatases have been studied in order to clarify the role of metal ions in the catalytic mechanisms of metalloenzymes. Three possible mechanisms have been proposed, presented in Figure 3 as reviewed by Kimura (Kimura 2000). In mechanism a), the dianionic phosphate monoester substrate binds to Me^{2+} ($\text{Me} = \text{Fe}$ or Zn) and after that phosphate is attacked by the Fe^{3+} -OH. In mechanism b), phosphate ester forms a bridging between the Fe^{3+} and Me^{2+} ions. The bridged phosphate ester is attacked by the μ -OH bridge, which acts as a nucleophile. In mechanism c), the inert Fe^{3+} -OH acts as a general base and deprotonates a second water molecule, which then attacks the Me^{2+} bound phosphate ester.

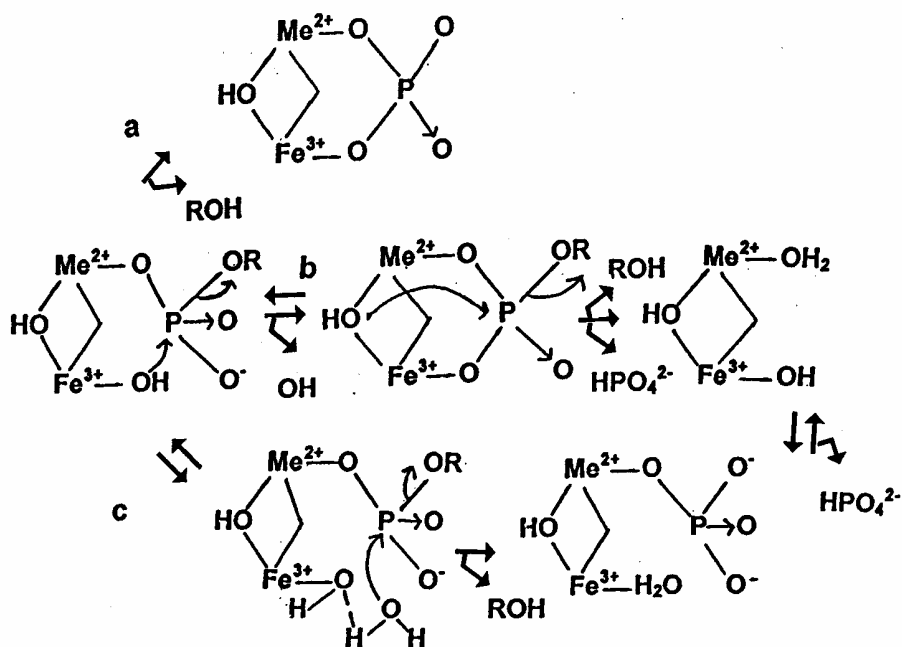


Fig. 3. Role of metal ions in the catalytic mechanism of metalloenzymes (Figure modified from Kimura *et al.* 2000).

2.3.7 Mechanism of ROS generation by TRACP

In osteoclasts and macrophages, superoxide is generated by the action of NADPH-oxidase on molecular oxygen (Garret *et al.* 1990, Steinbeck *et al.* 1992, 1994, Key *et al.* 1994, Silverton 1994, Silverton *et al.* 1995, Hall *et al.* 1995, Darden *et al.* 1996). TRACPs, like other metalloproteins containing a redox-active iron, are able to catalyze the generation of reactive oxygen species from superoxide by the mechanism known as Haber-Weiss-Fenton chemistry (Sibille *et al.* 1987, Hayman & Cox 1994, Halleen *et al.* 1999a, Räsänen *et al.* 2001). Reactive oxygen species (ROS) include hydroxyl radicals, hydrogen peroxide, and singlet oxygen. Superoxide itself may not be directly injurious. Highly reactive hydroxyl radicals formed through Fenton's reaction can break peptide bonds and cause disruption of RNA, DNA and protein structures (Greenwald *et al.* 1989,

Ries *et al.* 1992, Boldt 1999). Figure 4 describes reactions important for the ROS generation by TRACP.

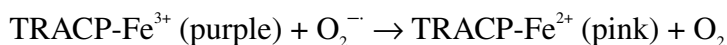
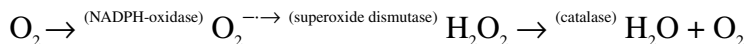


Fig. 4. Reactions important for ROS generation.

2.3.8 Proteolytic cleavage and carbohydrate content

Some TRACPs appear as one single polypeptide chain after purification, while others are cleaved into two fragments by proteolytic cleavage (Ketcham *et al.* 1985, Hayman *et al.* 1991, Halleen *et al.* 1996). TRACP purified from bone tissue exists primarily as two smaller fragments (Hayman *et al.* 1989, Halleen *et al.* 1996, Ek-Rylander *et al.* 1997, Ljusberg *et al.* 1999). The size of the smaller fragment varies between M_r 15-16 kDa and that of the larger fragment between M_r 18-23 kDa. Purified recombinant bone-derived enzyme exists as one polypeptide chain form (Hayman & Cox 1994, Ek-Rylander *et al.* 1997). A small portion of the recombinant enzyme appears after disulfide reduction as two smaller fragments with M_r 16 kDa and 20 kDa (Ek-Rylander *et al.* 1997). The recombinant enzymes can be turned into two-fragment form by cleavage with serine proteinase trypsin or chymotrypsin or cystein proteinase papain (Orlando *et al.* 1993, Ljusberg *et al.* 1999).

The cleavage occurs within an exposed loop in the residue 155-161 region (Ljusberg *et al.* 1999). This highly antigenic loop is a general feature of the structures of TRACPs (Orlando *et al.* 1993, Ljusberg *et al.* 1999). Cleavage of the protein into fragments enhances the acid phosphatase activity (Ljusberg *et al.* 1999). Activation is accompanied by a shift in pH optimum to more basic values (Ljusberg *et al.* 1999, Funhoff *et al.* 2001a). Proteolytic cleavage changes also the EPR spectrum into one distinct to two-fragment TRACPs (Funhoff *et al.* 2001b). The reason for the cleavage of the enzyme into smaller fragments is not clear. It has been suggested that the enzyme is synthesized as a latent proenzyme which is activated by proteolytic cleavage (Ljusberg *et al.* 1999).

The carbohydrate content of tartrate-resistant acid phosphatases has been most thoroughly studied using porcine uteroferrin (Chen *et al.* 1973, Saunders *et al.* 1985, Roberts *et al.* 1986, Baumbach *et al.* 1991). The uteroferrin polypeptide chain has two potential N-glycosylation sites, Asn97 and Asn128 (Baumbach *et al.* 1991). Both of these glycosylation sites are conserved in human (Ketcham *et al.* 1989, Lord *et al.* 1990) and rat (Ek-Rylander *et al.* 1997) enzymes. Both sites are located in the N-terminal larger fragment, residues Asn97 and Asn128 in human placental TRACP (Ketcham *et al.* 1989) and Asn118 and Asn149 in rat bone TRACP (Ek-Rylander *et al.* 1997). Bovine spleen enzyme has been reported to have only one attachment site for N-linked oligosaccharides (Ketcham *et al.* 1989). In uteroferrin only one of the sites, Asn 97 seems to carry carbohydrate chain (Ketcham *et al.* 1989, Baumbach *et al.* 1991). There is significant charge heterogeneity among TRACPs, and this might reflect varying degrees of glycosylation (Lau *et al.* 1987a). Human bone TRACP has been demonstrated to contain only high-mannose type carbohydrates (Halleen *et al.* 1996). The small molecular mass difference in bone-derived TRACP and recombinant osteoclastic TRACP seems not to be due to differences in N-glycosylation, since the difference in motility is still present after N-glycase treatment (Ek-Rylander *et al.* 1997).

2.3.9 Serum TRACP as a marker of bone resorption

Type 5 acid phosphatases present in serum have been named 5a and 5b (Stepan *et al.* 1989). They differ in pH optimum, which is 5.0 for 5a and 5.5-6.0 for 5b (Alatalo *et al.* 2000), and in carbohydrate content, 5a containing sialic acid while 5b does not (Janckila *et al.* 1992a,b, Halleen *et al.* 2000). Isoform 5b is derived from osteoclasts and therefore useful as a marker for osteoclast activity (Alatalo *et al.* 2000, Janckila *et al.* 2001a,b). The source of isoform 5a is so far unestablished.

TRACP expression and activity is associated with bone resorption (Lam *et al.* 1982, Anderson & Toverud 1986, Lau *et al.* 1987a, Zaidi *et al.* 1989, Kraenzlin *et al.* 1990, Moss 1992, Rico & Villa 1993, Chamberlain *et al.* 1995, Moss *et al.* 1995, Halleen *et al.* 1996, 2000, 2001, Hannon *et al.* 1998, Nakasato *et al.* 1999, Seregini *et al.* 2001). Increased bone resorption can be found in physiological bone remodeling and growth in children (Lam *et al.* 1978) and in various pathological osteolytic processes. Elevated TRACP levels have been measured in the serum of patients with osteoporosis (Rico and Villa 1993, Chamberlain *et al.* 1995, Cheung *et al.* 1995, Halleen *et al.* 1996), bone metastases in cancer (Lam *et al.* 1982, Lau *et al.* 1987b, Wada *et al.* 1999), hyperparathyroid bone disease (Cheung *et al.* 1995, Lau *et al.* 1987b, Kraenzlin *et al.* 1990) and Paget's disease (Lau *et al.* 1987b, Kraenzlin *et al.* 1990, Kehely & Moss 1992, Rico & Villa 1993).

TRACP has been known for decades to be a marker for hairy cell leukemia (Hoyer *et al.* 1997, Lamp & Drexler 2000). Increasing proof of its participation in bone metabolism has made it a promising marker for osteoporosis. Instability of the enzyme activity causes a problem in assays based on measuring the activity of the enzyme. In serum TRACP has also been demonstrated to form complexes with α_2 -macroglobulin (Brehme *et al.* 1999).

Specimens with high bilirubin content can cause nonspecificity of the analytical reaction (Alvarez *et al.* 1999). Several immunological tests for TRACP have been developed for research purposes and also for clinical use (Lam *et al.* 1982, Echetebe *et al.* 1987, Kraenzlin *et al.* 1990, Chamberlain *et al.* 1995, Cheung *et al.* 1995, Yaziji *et al.* 1995, Janckila *et al.* 1996, 1997, Halleen *et al.* 1996, 1998, 1999b).

2.4 Cells containing TRACP

2.4.1 Osteoclasts and bone resorption

The skeleton is a complex organ system that is under a constant state of flux. It serves mechanical, protective and metabolic functions, most importantly calcium homeostasis (Mundy & Guise 1999). The cellular process of bone activity is referred to as bone remodeling, the balance between bone formation and bone resorption. There is an interdependency of bone resorbing osteoclastic and bone forming osteoblastic activities. Osteoclasts are recruited to a particular site on the bone surface, and when their task is completed, they signal the osteoblasts to attend the same site (Edelson & Kleerekoper 1996). Osteoblasts have been shown to produce collagen that activates osteoclasts (Holliday *et al.* 1997).

Imbalance in the interrelationship between osteoblasts and osteoclasts can lead to osteoporosis. Osteoporosis is characterized by decreased bone mass and increased susceptibility to fracture in an aging individual or as a consequence of metabolic bone disease of unknown (Paget's disease) or known (hyperparathyroid bone disease) etiology. (Edelson & Kleerekoper 1996). Sufficient dietary calcium intake and serum vitamin D levels have beneficial effects on bone volume (Mezquita-Raya *et al.* 2001). Both bone mass and density is influenced by genetic factors as well (Brown *et al.* 2001). It has recently been appreciated that, as well as cell proliferation and activity, bone cell apoptosis may be a significant determinant of bone shape and size (Boyce *et al.* 1999, Plotkin *et al.* 1999, Manolagas 2000, Weinstein & Manolagas 2000, da Paz *et al.* 2001).

Osteoclasts are multinucleated bone-resorbing cells that originate from precursors of the monocyte-macrophage lineage of hemopoietic stem cells in the bone marrow (Walker 1975, Quinn *et al.* 1996, 1998). The precursors are released as monocytes to the bloodstream and collect at sites of bone resorption (Scheven *et al.* 1986). Osteoclasts are formed by fusion of post-mitotic monocytes by influence of bone-derived stromal cell elements (Quinn *et al.* 1996).

The role of stromal cells in the control of osteoclast differentiation and activity seems to be important but not yet fully understood (Matayoshi *et al.* 1996, Yasuda *et al.* 1998, Udagawa *et al.* 1990, Suda *et al.* 1999, Migliaccio *et al.* 2000, Kondo *et al.* 2001). Osteotropic factors such as parathyroid hormone (PTH), interleukin 11 (IL-11) and 1,25-dihydroxyvitamin D₃ enhance osteoclast formation (Suda *et al.* 1992, Takeda *et al.* 1999) and activity (Martin & Udagawa 1998). The mechanism of function of these regulators

can be both direct and indirect (Martin & Ugadawa 1998, Oursler *et al.* 1991, Oursler *et al.* 1993). Target cells for these factors are most likely osteoblasts/stromal cells, in which they induce expression of elements that control osteoclast formation and differentiation (Yasuda *et al.* 1998, Suda *et al.* 1999). These elements probably include NF- κ B, TNF, RANK, RANKL (Yasuda *et al.* 1998, Abu-Amer *et al.* 1998, Blair *et al.* 2000, Shevde 2000, Zhang *et al.* 2001, Mancino *et al.* 2001, Hofbauer & Heyfelder 2001), hemopoietic transcription factor PU.1 (Tondravi *et al.* 1997) and M-CSF (Takeshita *et al.* 2000, Toyosaki-Maeda *et al.* 2001, Mancino *et al.* 2001, Nishino 2001). Studies of the sequence of events during differentiation have revealed that TRACP and proteinase cathepsin K activity appear simultaneously at 3-5 days, whereas major increases in expression of proteinases and acid secretion occurs later, at 10-14 days (Blair *et al.* 2000).

Osteoclasts are characteristically TRACP positive (Minkin 1982, Price *et al.* 1995) and have the ability to form resorption pits (Howship's lacunae) on bone surface (Suda *et al.* 1992, Teitelbaum 2000). Resorbing osteoclasts reveal four functionally and structurally different membrane domains: sealing zone (clear zone), ruffled border, basolateral membrane and functional secretory domain (Salo *et al.* 1997). Figure 5 shows an illustration of a bone resorbing osteoclast.

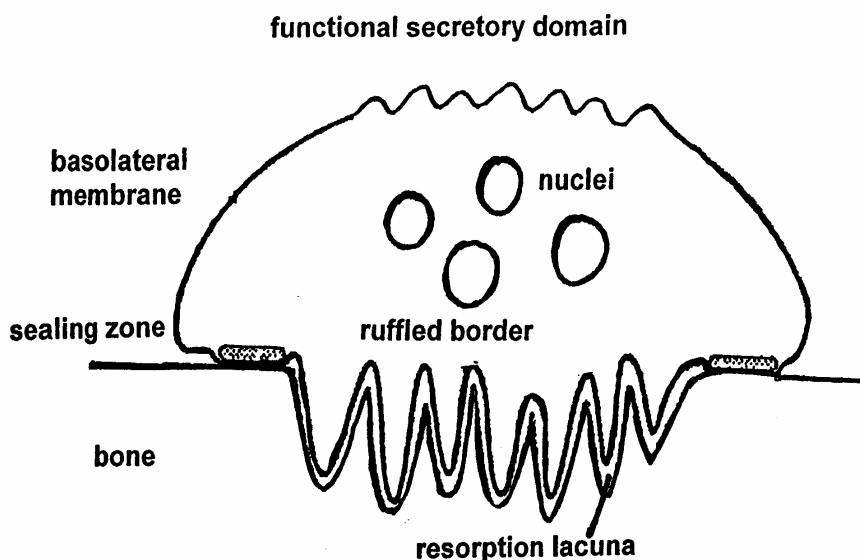


Fig. 5. Schematic illustration of a bone resorbing osteoclast (Figure modified from Väänänen & Horton 1995).

During the resorption osteoclasts are tightly sealed to the surface of bone through sealing zone membrane domain (Silver *et al.* 1988, Väänänen & Horton 1995). Osteoclasts attach via cell-surface receptors to the bone (Flores *et al.* 1992, Duong & Rodan 1999, Ihara *et al.* 2001). The $\alpha_v\beta_3$ integrin is the major receptor on the osteoclast that can interact with ligands such as osteopontin and vitronectin in the bone matrix (Medhora *et al.* 1993, Mimura *et al.* 1994, Ruoslahti 1996). Osteopontin and vitronectin contain an RGD amino acid sequence that interacts with integrins, and their attachment can be inhibited by RGD-containing peptides (Flores *et al.* 1992). It has been demonstrated that besides osteoblasts also osteoclasts are able to synthesize osteopontin, thus being able to facilitate their own attachment to the bone surface (Merry *et al.* 1993, Dodds *et al.* 1995) and initiation of the resorption process (Arai *et al.* 1993).

Osteoclasts dissolve bone minerals and degrade organic matrix by secretion of acid and proteases through ruffled border (Miller 1985, Blair *et al.* 1986, 1989, Silver *et al.* 1988, Baron *et al.* 1986, Moonga *et al.* 1990, Väänänen *et al.* 1990, Inoue *et al.* 1999, Reinholt *et al.* 1999). Targeted disruption of the proton pump has been shown to cause osteopetrosis (Li *et al.* 1999). Inhibition of the acidification affects osteoclast differentiation (Laitala-Leinonen *et al.* 1999) and activity (Lees *et al.* 2001).

The degraded bone material is endocytosed via ruffled border membrane and transported through the osteoclast in membranous vesicles to the functional secretory domain (Isaki & Hanaoka 1995, Salo *et al.* 1997, Nesbit & Horton 1997). TRACP has been found in these transcytotic vesicles, suggesting that TRACP participates in the fragmentation of endocytosed material (Halleen *et al.* 1999a).

Sex steroids regulate bone mineral density both on women and men (Greendale *et al.* 1997, Riggs 2000, Paglia *et al.* 2001). Estrogen has an inhibitory effect on osteoclast function (Oursler *et al.* 1993, 1994, Gruber *et al.* 2001), possibly by promoting osteoclast apoptosis (Hughes *et al.* 1996, Boyce *et al.* 1999), or by modulating osteoclast differentiation (Huang *et al.* 1998, Qu *et al.* 1999). The anabolic effects of high-dose estrogen are caused by increased osteoblast activity (Bord *et al.* 2001). Osteoclasts have been shown to possess functional androgen receptors (Pederson *et al.* 1999), and androgen is therefore a potential regulator of bone resorption activity of osteoclasts (Pederson *et al.* 1999). The powerful inhibitory effect of calcitonin on osteoclast activity is mediated by calcitonin receptors on osteoclasts (Cornish *et al.* 2001).

Oxygen-derived free radicals have been suggested to be involved in the events in formation, differentiation and activation of osteoclasts (Garret *et al.* 1990, Hall *et al.* 1995).

2.4.2 Macrophages and immune defence

Macrophages are mononuclear cells capable of endocytosing material destined to degradation. This material may be either cells/cell components or injurious and infectious foreign agents and micro-organisms. Macrophages together with polymorphonuclear leukocytes are responsible for non-specific tissue defense by phagocytosing and thus for removing foreign material and tissue debris caused by tissue damage. Macrophages are

also involved in the highly specific immune defense system. Macrophages act as antigen presenting cells by taking up foreign material and breaking it down into fragments, which can be recognized by T-cells, leading to the activation of the immune response. (Burkitt *et al.* 1993). Macrophages originate from the same lineage of progenitor cells in the bone marrow as osteoclasts. Activated macrophages are known to possess elevated levels of TRACP. It has been suggested that TRACP has an important role in immune defense, and an analogous route to osteoclast transcytosis might function in macrophages in the antigen presentation (Hayman *et al.* 2000b, Räsänen *et al.* 2001).

2.5 Biological function of TRACP

The physiological function(s) of TRACP are not known. Diverse functions for mammalian TRACPs have been proposed, including acid phosphatase activity, reactive oxygen species generating activity and iron transport. These activities may have different roles in different cells and cell compartments.

Targeted disruption of TRACP gene results in mild osteopetrosis in mice (Hayman *et al.* 1996) whereas overexpression of TRACP has been shown to cause increased rate of bone turnover leading to osteoporosis (Angel *et al.* 2000). These findings suggest an important role for TRACP in bone metabolism. It has been reported that TRACP is able to dephosphorylate two bone-matrix proteins, osteopontin and bone sialoprotein, thus regulating the attachment of osteoclasts to the bone surface (Ek-Rylander *et al.* 1994, 1997). These studies refer to the role of acid phosphatase activity of the enzyme in the resorption lacuna, which has an acidic pH. In bone resorbing osteoclasts, TRACP has been found in vesicles transporting bone matrix degradation products through the cell (Clark *et al.* 1989, Salo *et al.* 1997, Halleen *et al.* 1999a). In osteoclasts the physiological function of TRACP has therefore been hypothesized to be the destruction of endocytosed matrix degradation products by highly destructive reactive oxygen species (Halleen *et al.* 1999a).

Mice with disrupted TRACP gene have disordered inflammatory responses and reduced population of phagocytosing macrophages (Bune *et al.* 2001). This would imply an important role for TRACP in the defense mechanisms of macrophages. The observation that TRACP is found in bone marrow derived dendritic cells and macrophages that function as antigen-presenting cells suggests that it participates in processing of macromolecular antigens (Hayman *et al.* 2001). This hypothesis is supported by co-localization of TRACP with MHC II molecules and phagocytosed bacteria in the same late endosomal/lysosomal vesicles (Räsänen *et al.* 2001). Phagocytosing cells containing TRACP activity have been found associated with aged and deformed erythrocytes in bovine spleen (Schindelmeiser *et al.* 1987). TRACP may participate in degradation of cell membrane phosphoproteins in phagocytosed red blood cells (Schindelmeiser *et al.* 1987) and in iron metabolism (Antanaitis & Aisen 1983).

The major function of porcine uteroferrin appears to be iron transport from the mother to the developing fetus (Buhi *et al.* 1982, Roberts *et al.* 1986).

2.6 Recombinant protein production

Recombinant protein production technology provides means for obtaining large quantities of protein for function and structure analysis, drug design, diagnostics, therapy, and vaccines. Mammalian protein production is no longer restricted to the cells that generate the protein naturally. The baculovirus expression vector system (BEVS) in insect cells was first used by Smith and his coworkers in 1983 (Smith *et al.* 1983). This method has since become widely used in the production of complicated recombinant proteins, giving biologically active products on a short time scale with high yields (Summers & Smith 1987, Vihko *et al.* 1993). The baculovirus-based system is an eukaryotic expression system and it is therefore able to produce overexpressed recombinant proteins with proper folding, disulfide bond formation and oligomerization. This system is also capable of performing many post-translational modifications including O-linked and N-linked glycosylation, phosphorylation, acylation, amidation, carboxymethylation and cleavage of certain proteins to their active forms. (Luckow 1991).

Baculoviruses are double-stranded DNA viruses that infect different species of insects as their natural hosts. Baculovirus are not known to infect vertebrate hosts (Carbonell *et al.* 1985, Carbonell & Miller 1987), thus providing a safe method for protein production. The baculovirus DNA used in most of the baculovirus expression vector systems is the *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA.

The most frequently used insect cells that are susceptible to AcNPV infections are cell lines *Sf9* and *Sf21*. Both of these lines are originally established from ovarian tissues of *Spodoptera frugiperda* larvae. These cell lines may be grown in suspension and can therefore be used in bioreactor. Transfer vector containing the foreign DNA fragment is cotransfected with linearized AcNPV virus DNA into the insect cells. This allows recombination between homologous sites, transferring the foreign gene from the vector to the AcNPV DNA. The desired recombinant protein can be produced, harvested and purified.

A schematic description of large-scale production of recombinant proteins with *Sf9* or *Sf21* insect cells in 30-liter bioreactor is presented in Figure 6 (Picture kindly provided by Riitta Kurkela & Katja Porvari.)

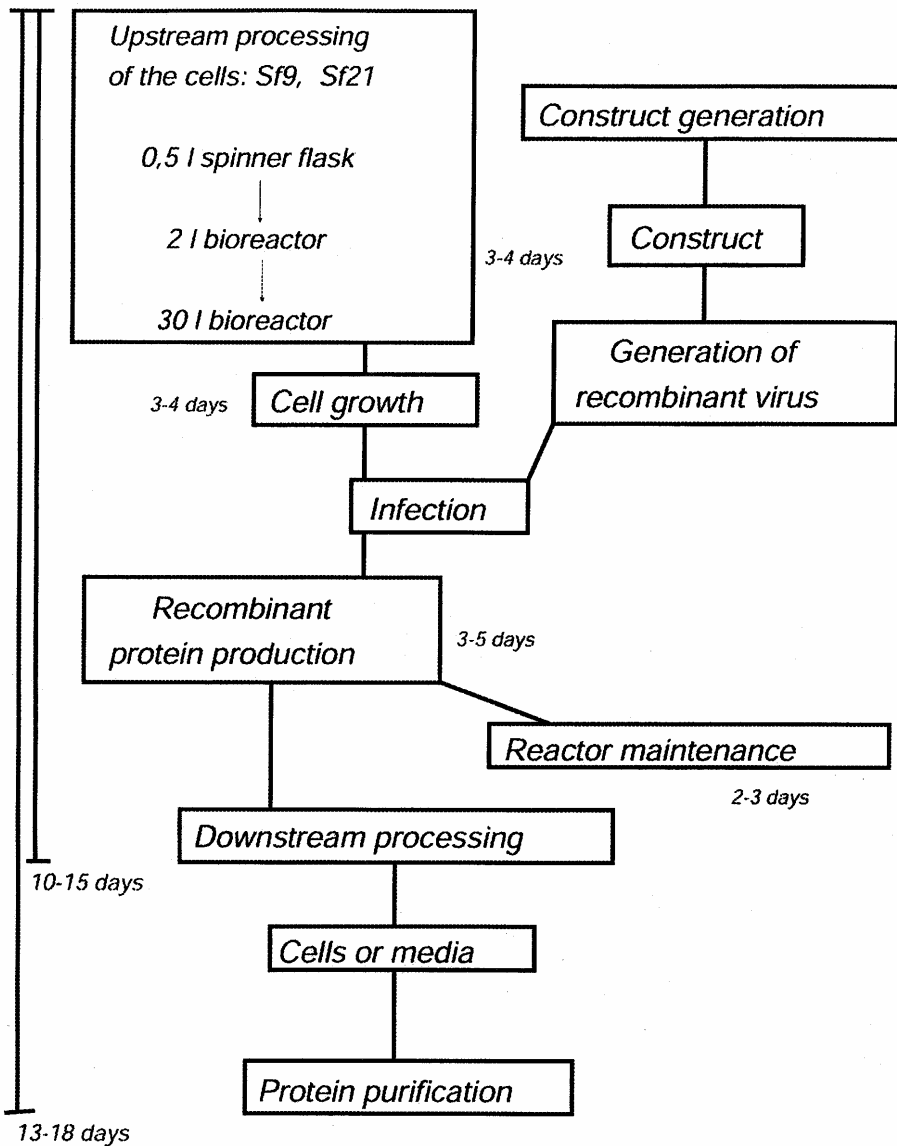


Fig. 6. Recombinant protein production by BEVS.

3 Outlines of the present study

The present work was aimed at clarifying the substrate specificity and function of tartrate-resistant acid phosphatase based on the three-dimensional structure. The knowledge of the active site structure would give valuable information for the design of TRACP inhibitors, which could be useful therapeutic agents for osteoporosis and related bone disorders.

Specific aims of the study were:

- to produce and purify sufficient amounts of recombinant tartrate-resistant acid phosphatase, to crystallize the protein and determine its three-dimensional structure
- to characterize amino acids predicted to be important for the acid phosphatase activity of the enzyme
- to produce monoclonal antibodies

4 Materials and methods

Detailed description of the materials and methods is to be found in the original papers I-IV.

4.1 Production and purification of the recombinant enzyme (II)

Transfer vector pVL1392 containing the rat bone TRACP DNA fragment was cotransfected with linearized *Autographa californica* nuclear polyhedrosis virus DNA into *Spodoptera frugiperda* Sf9 cells (Vihko *et al.* 1993). The construct was prepared in Dr Anderssons laboratory at Huddinge University Hospital, Sweden (Ek-Rylander *et al.* 1997). Five days postinfection the medium was collected and concentrated, and the enzyme was purified using cation exchange and hydrophobic interaction chromatography steps, including Streamline SP, S-Sepharose 26/10, and Phenyl Superose HR 5/5 columns. The purity of the enzyme was evaluated by SDS-PAGE runs under nonreduced and reduced conditions (Wyckoff *et al.* 1977). Protein concentrations were measured according to the Bradford method (Bradford 1976). Proper cleavage of the recombinant protein was confirmed by N-terminal sequencing (Kalkkinen & Tilgmann 1988).

4.2 Characterization of the recombinant enzyme (I, II)

4.2.1 Acid phosphatase activity

Acid phosphatase activities were determined by monitoring the formation of pNP from pNPP. Activity measurements were done at +37°C, pH 4.9 or 5.8 (Ek-Rylander *et al.* 1997) in the presence of L(+) tartrate. Induced activity measurements were done after

reduction by 1 mM ascorbic acid and 0.1 mM FeCl₃ (Ek-Rylander *et al.* 1997). One unit of enzyme releases one μ mol of *p*NP per minute per mg enzyme at +37 °C.

4.2.2 Separation of the active and inactive form and stability measurements

The different forms of purified TRACP were separated by cation exchange chromatography step using Mono-S column PC 1.6/5 connected to a SMART micropurification system (Pharmacia, Sweden).

To determine whether the reduction of the enzyme is associated with color change, the reduction and incubation at +37°C were performed using 600 μ g of purified rat bone TRACP in a total volume of 100 μ l. Absorption spectra were determined using a Shimadzu UV 160-A spectrophotometer (Kyoto, Japan).

4.3 Crystallization (II), structure determination (III) and site directed mutagenesis (IV)

Crystallization and structure determination was done at the Department of Medical Biochemistry & Biophysics, Karolinska Institutet, Stockholm, Sweden. The methods used are described in detail in papers II and III.

Site-directed mutagenesis was achieved by using QuikChange™ Site-directed mutagenesis Kit (Stratagene, TX, USA). This method allows site-directed mutagenesis using double stranded DNA templates (Bremer *et al.* 1996). The mutation products were sequenced using ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, USA) to verify the mutation and the integrity of the rest of the DNA.

4.3.1 Substrate specificity

Substrate specificity was studied by measuring phosphatase activity by the method published by Black & Jones (Black & Jones 1983). *p*NP, β -glycerophosphate, *o*-phospho-L-serine and *o*-phospho-L-tyrosine were used as substrates. One unit of enzyme releases one μ mol of P_i per minute per mg enzyme at +37 °C.

4.3.2 Kinetic parameters for phosphatase activity

In the kinetic measurements of phosphatase activity *p*NPP was used as the substrate. The steady state constants for *p*NPP were derived from the analysis of rate versus substrate concentration. K_m and V_{max} values were calculated using the GraphPadPrism software. For pH profiles of the enzymes, the activity measurements were performed at pH values 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5.

4.3.3 Radical oxygen species generating activity

The formation of ROS was determined by monitoring the formation of malondialdehyde acetal from degradation products of deoxyribose (Halliwell *et al.* 1987). ROS generation activity studies were performed by Dr Jussi Halleen and MSc Sari Alatalo.

4.4 Monoclonal antibodies (I)

Monoclonal antibodies were raised using purified recombinant TRACP as antigen (Höyhty *et al.* 1987). Mabs were produced at Diabor Ltd (Oulu, Finland).

5 Results

5.1 Production and purification of recombinant TRACP (II)

The baculovirus expression vector system was used to overproduce TRACP in insect cells. The amount of TRACP activity in the culture medium reached the maximum level 5-6 days after the infection. The medium was harvested at day 5, when only a few viable cells could be detected, and the contents of the cells had been released into the culture medium. The recombinant protein was purified from the medium to homogeneity by two-step cation exchange chromatography followed by a hydrophobic interaction chromatography step, resulting in 0.5-1.0 mg of pure TRACP/l of harvested medium. N-terminal sequencing confirmed that the recombinant protein was properly cleaved.

The mutant proteins were produced and purified the same way as the wild-type enzyme.

5.2 Characterization of wild-type TRACP and monoclonal antibodies (I, II)

The purified protein was analyzed by SDS-PAGE. Under nonreducing conditions, a 34 kDa protein was identified. Isoelectric focusing showed the isoelectric point of recombinant TRACP to be at ~9, corresponding to the calculated pI of rat bone TRACP (Ek-Rylander 1991a).

The specific acid phosphatase activity of the purified wild-type recombinant protein varied in different purification batches. The average value for specific activity after the final purification step was ~ 120 U, 1 unit corresponding to 1 μ mol of *p*-nitrophenol formed/minute/mg enzyme at +37°C. When the purified protein was mildly reduced, the activities measured were 2- to 6-fold higher. Incubation at +37°C activated the protein to the same level as attained by the addition of β -mercaptoethanol. Prolonged incubation at

+37°C dropped the activity near the original value. At this stage the enzyme could not be activated by β -mercaptoethanol. The activation and inhibition of TRACP were associated with color changes. The non-reduced purple form had its absorption maximum at 540 nm, the reduced pink form at 505 nm, and the further reduced yellowish form at 450 nm.

The specific activity of the enzyme toward phosphotyrosine was comparable to the activity with *p*-nitrophenyl phosphate. Phosphoserine and β -glycerophosphate were not hydrolyzed by the enzyme.

Mono-S cation exchange chromatography of the purified wild-type recombinant TRACP resulted in separation of the purple and pink forms of the protein. The less active purple form was significantly more abundant and it eluted before the pink form from the column. The specific activity of the purple form was 54.2 U/mg and of the pink form 349.2 U/mg.

Monoclonal antibodies were raised in mice. Three Mab-producing clones - U1, Z1, and V1 - were selected. U1 detected TRACP in Western blotting, while Z1 and V1 were able to bind the active enzyme. Monoclonal antibody Z1 was used in developing a new bone-specific immunoassay for rat TRACP 5b activity (manuscript in press).

5.3 Crystallization and structure determination (III)

In the final conditions for crystallization, the reservoir solution contained 0.1 M HEPES, pH 7.5, 2.24 M ammonium sulphate, 10 % polyethylene glycol 400, and 0.05 M zinc sulphate. The formation of crystals took two months to two years. The crystals formed were small with a typical diameter of 30 μ m. Removal of glycosyl units did not speed the process. The crystals were orthorhombic, space group $P2_12_12_1$, with cell dimensions $a = 57.2$ Å, $b = 69.5$ Å, and $c = 87.2$ Å. The data collection on the few available crystals succeeded with some difficulties at the second attempt. Structure determination with molecular replacement proceeded without major obstacles.

The three-dimensional structure of rat bone TRACP was determined at 2.2 Å resolution, Figure 7. The mammalian enzyme is a monomeric enzyme comprising one domain. The core of the enzyme was formed by two seven-stranded β -sheets which packed together, making up a β -sandwich. The β -sandwich was surrounded by three solvent-exposed α -helices on both sides. The di-iron center was located on the other edge of the β -sandwich. Two N-acetylglucosamine (NAG) molecules linked to the side chain of Asn118 were found in well-defined electron density. One zinc and one sulphate ion were found in the active site and one further zinc and sulphate ion were involved in crystal contacts. One of the iron ions was ligated to the side chains of Asp35, Tyr76, and His244 and the other iron ion was ligated to Asn112, His207, and His242. The latter iron formed a coordination bond to one of the oxygen atoms of a bound sulphate ion. This oxygen atom formed also a hydrogen bond to the side chain of Asn112. Another oxygen of the sulphate ion interacted with the side chain of His113 and the third oxygen coordinated the bound Zn ion. The metal ions were bridged through the coordination to one of the carboxylate oxygen atoms of Asp73. The distance of the metal ions was 3.5 Å. The electron density map indicated that a μ -(hydr)oxo bridge is found in the metal center.

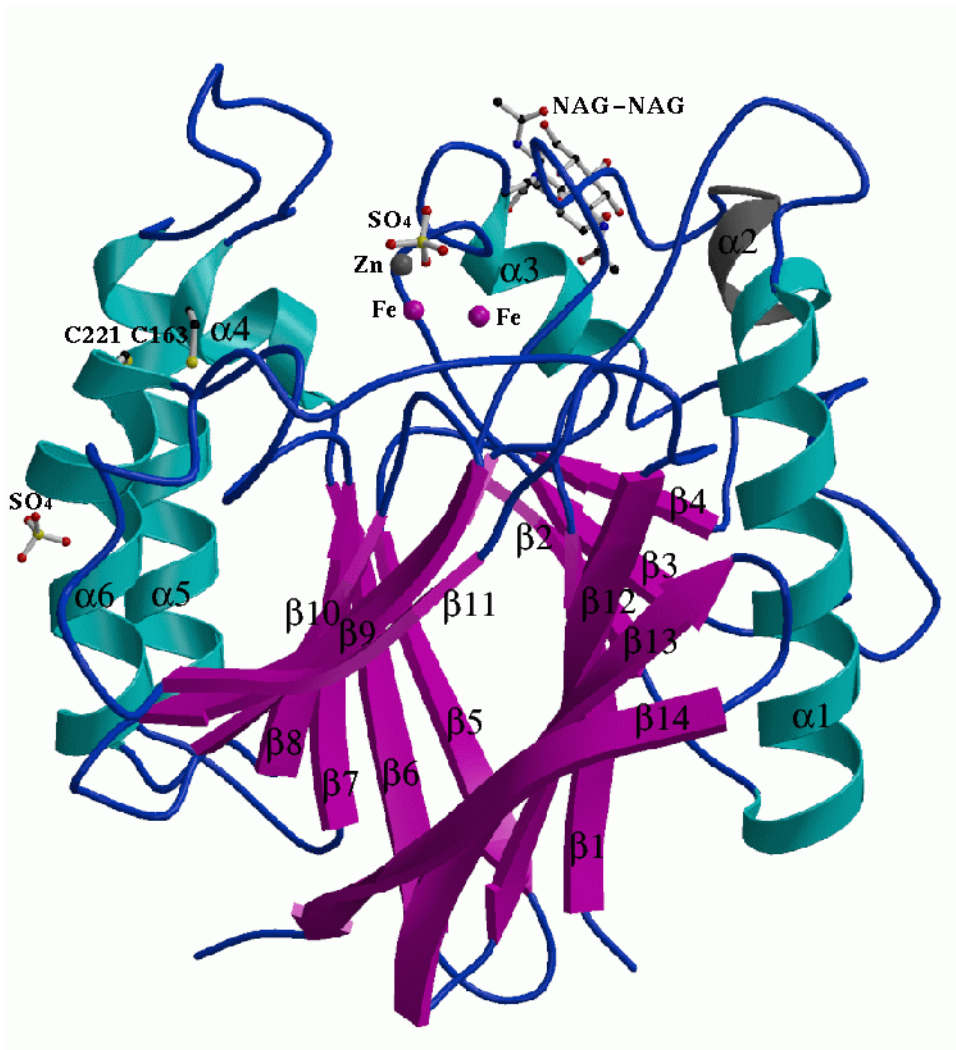


Fig. 7. Three-dimensional structure of mammalian TRACP.

5.4 Site-directed mutagenesis and amino acids important for acid phosphatase activity (IV)

The substrate specificity of TRACP was not affected by the mutations. The K_m values for *p*NPP were of the same order of magnitude for all mutants, with the largest decrease (about five-fold) occurring in the His113Gln and His216Ala mutants compared to the wild-type enzyme. Significant decreases in the k_{cat} values were seen for the mutants at the positions His113 and His216, where substitutions led to a drop in rate by up to two orders of magnitude. In terms of catalytic efficiency, the mutations at position 113 were most deleterious, with a drop in k_{cat}/K_m by up to 75-fold (His113Ala).

The k_{cat}/K_m versus pH profiles of wild-type TRACP and the Asp267Ala mutant were very similar, and the catalytic efficiency of this mutant was not significantly impaired over the whole pH range measured. The His216Ala mutant showed a shift in pH dependency and recovered to reach similar k_{cat}/K_m values as wild-type TRACP at very low pH values. Substitutions at the position His113 led to a mutant enzyme with very low catalytic efficiency over the whole pH range.

The rate versus pH curves for the AcP and ROS-generating activities of wild-type recombinant rat TRACP were different from each other, with the pH optimum for phosphoester hydrolysis close to pH 4.5 and the pH optimum for ROS-generating activity at pH 6.5. In order to determine further whether there are any differences between the AcP and the ROS-generating activities of recombinant rat TRACP, we studied if the mutants at the positions His113 and His216, which were almost inactive as acid phosphatase, were able to generate ROS. We found out that the ROS-generating capacity was only slightly impaired in the four mutants. For both histidine residues, the mutation to alanine inactivated the ROS-generating activity more than the mutation to glutamine.

The absorption maximum of the wild-type enzyme in the visible region of the spectrum was at 514 nm. The Asp267Ala mutant was slightly more oxidized, as indicated by the shift in the absorption maximum to 534 nm. The absorption maximum of all the other variants was at 507 nm – 514 nm.

6 Discussion

6.1 Baculovirus expression vector system

Application of the baculovirus expression vector system (BEVS) provides an easy access to large quantities of recombinant protein, which is biologically active and in most cases properly processed post-translationally. This kind of recombinant protein can be used for both enzymatical and structural characterization. Bone tissue is a particularly challenging material to be used in protein purification. Gaining sufficient amounts of bone tissue may be difficult or cause ethical problems, and the yields are usually not very high (Allen *et al.* 1989, Halleen *et al.* 1996). We were able to purify recombinant rat TRACP 0.5-1.0 mg to homogeneity per each liter of culture medium. In the production we used either 0.5-liter spinner flasks or produced the protein as a batch culture in a 30-liter bioreactor. Large-scale production and purification systems provided us with adequate quantities of protein for crystallization experiments (Vihko *et al.* 1993, Kurkela *et al.* 1995). Purification yields of up to 8 mg/liter culture medium for TRACP have been reported (Hayman & Cox 1994, Ek-Rylander *et al.* 1997). The baculovirus expression vector system is becoming an increasingly popular method for recombinant protein production due to its ability to produce functional proteins. Accelerated protein expression rate in cells may lead to a situation where glycosylation and amidation fail to "keep pace" with the protein production (Luckow 1991). In the case of glycoproteins this may result in lower glycosylation of the recombinant protein compared to the native form and to heterogeneity of the protein produced. This is considered as the only weak point of BEVS. In structure-based studies heterogeneity of the protein may cause difficulties in the crystallization process.

6.2 Characterization of TRACP

Recombinant rat tartrate-resistant acid phosphatase was produced as a mixture of the active, mixed valenced and the inactive, diferric forms of the protein. The specific activity of the purified preparation varied from one purification batch to another, depending on the relative amount of both forms, the diferric form being, however, more dominating in each batch. Large amounts of the protein allowed us to purify and separate these forms in sufficient amounts for their characterization. These forms should not be mixed up with the two differentially glycosylated forms of tartrate-resistant acid phosphatases 5a and 5b (Stepan *et al.* 1989) existing in serum and originating from different sources.

The inactive, diferric purple form could be transformed by reductive agents to the active, mixed valenced pink form and further to the extensively reduced yellowish form. The purple form was also spontaneously activated to the pink form upon incubation in +37°C. TRACP isolated from blood had significantly lower acid phosphatase activity compared to the bone-derived protein. These results led us to the hypothesis of a redox regulation of osteoclastic TRACP activity: TRACP is synthesized as an inactive oxidized form and then reduced to its functionally active form. Once it has completed its biological function in the cell, it is secreted into the circulation and inactivated by further reduction. This was further supported by our observation that the yellowish form brought about by reduction could no longer be activated. The yellowish form could thus represent the form described earlier inactivated by loss of its iron ions (Doi *et al.* 1988). In the blood circulation the enzyme would be destroyed and its iron content could be recycled. TRACP would thus have one possible function in iron metabolism.

In our studies, and shown also by other groups, proteolytic cleavage of the enzyme to its two-subunit form activates the enzyme. Ljusberg and her co-workers have suggested that the cysteine proteinase family might have a regulatory role in TRACP activation by converting the newly synthesized single polypeptide chain to its enzymatically active form (Ljusberg *et al.* 1999). This form is also more redox-sensitive (Ljusberg *et al.* 1999). According to their studies the cleavage shifts the pH optimum of the acid phosphatase activity to more basic values (Ljusberg *et al.* 1999, Funhoff *et al.* 2001b). This is consistent with our observation of the recombinant protein's more acidic pH optimum compared to the protein isolated from tissues. This group has postulated that the proteolytic cleavage results in the loss of a three-residue segment, thus changing interactions between residues and leading to an increase in Lewis acidity of the divalent metal with a concomitant increase in the electrophilicity of the enzyme-bound substrate (Funhoff *et al.* 2001a,b).

6.3 Monoclonal antibodies

Recombinant protein production using BEVS is a suitable method to gain antigen for raising monoclonal antibodies. Two of our antibodies raised in mouse using purified recombinant TRACP were able to bind the active rat recombinant enzyme, and one of the antibodies recognized the denatured form of the protein and could be used in Western blotting. None of the antibodies produced by us recognized native or denatured human bone derived TRACP (unpublished observation). Janckila and his co-workers studied the species specificity of monoclonal antibodies to human TRACP, and obtained both a species-specific and nonspecific antibody, demonstrating that in spite of the highly conserved sequences of mammalian TRACPs species-specific determinants exist and should be recognized when using experimental animals (Janckila *et al.* 1998). Several urinary and serum markers for studying human skeletal disorders have been developed, but use of biochemical markers in animal studies is less examined. Monoclonal antibodies produced in this study were used to develop a specific immunoassay that can be used to monitor the effects of different drugs *in vivo* in an experimental model, where osteoporosis has been induced by orchidectomy or ovariectomy in rats (manuscript in press).

6.4 Three-dimensional structures and reaction mechanism

Crystallization of the enzyme required thousands of experiments and took from several months up to two years. The crystals were very small with a typical diameter of 30 μ m. Heterogeneity of the glycosylation of the recombinant protein was suspected to cause the delay in crystal formation. The reason for this may also have been a mere technical detail of incompletely sealed crystallization wells allowing evaporation of the liquid. The crystals did, however, diffract well, and the data of the few available crystals were collected and processed. The structure was determined using the coordinates of the only structure of purple acid phosphatases existing so far, a plant enzyme isolated from red kidney bean.

Plant purple acid phosphatase is a dimer and has an additional domain of unknown function at the N-terminus which was not included in the model used in molecular replacement. The sequence identity between kidney bean and rat enzyme is only 18%. The three-dimensional structures of kidney bean enzyme and mammalian TRACP in this study and in two of the almost simultaneously published studies are nevertheless very similar (Sträter *et al.* 1995, Guddat *et al.* 1999, Uppenberg *et al.* 1999). Especially the core of the enzyme subunit is well preserved, while many of the loops differ in size and conformation. Figure 8 presents superposition of the structure of mammalian TRACP and purple acid phosphatase from red kidney bean.

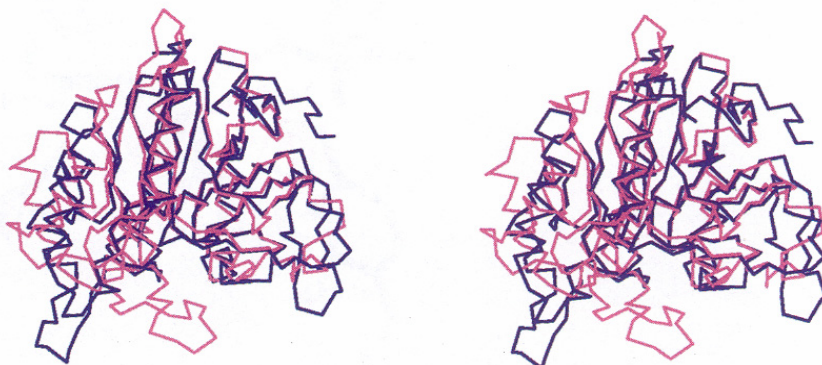


Fig. 8. Superposition of TRACP (pink) and red kidney bean (purple) purple acid phosphatase.

The loop folding over the active site of mammalian TRACP does not exist in the plant enzyme. This loop is also missing in the structure of rat bone enzyme by Uppenberg and his co-workers, presumably due to proteolytic cleavage (Uppenberg *et al.* 1999). The electron density map confirmed the presence of a μ -(hydr)oxo bridge in the metal center in both our study and in the study by Guddat and his coworkers (Guddat *et al.* 1999), while this could not be localized either in plant enzyme or in the rat enzyme structure by Uppenberg and co-workers (Uppenberg *et al.* 1999). The reason for this is probably the low resolution of these structures (kidney bean 2.9Å, rat 2.7Å).

Based on the data in this study and previous spectroscopic studies of tartrate-resistant/purple acid phosphatases we have proposed the following reaction mechanism, which is closely consistent with the mechanism proposed for red kidney bean enzyme (Klabunde *et al.* 1996). In this mechanism the substrate binds to the enzyme with a coordination to the Fe(II). This places the hydroxyl ion in the ligand sphere of the Fe(III) ion in a position suitable for in-line attack on the phosphorus ion. Because the attack is delivered from the side opposite the alcohol group of the substrate, it will result in inversion of the configuration of the phosphorus ion. The inversion of the configuration argues against the formation of a covalent phosphorylated intermediate.

6.5 Classification of TRACP

As suggested by earlier secondary structure predictions, the mammalian TRACP proved to possess a $\beta\alpha\beta\alpha\beta$ -motif, which incorporates it to the large group of metallohydrolases with positioning of two metal ions by a central $\beta\alpha\beta\alpha\beta$ -scaffold. In some cases the metal appears to be essential either for the structure or for the enzymatic mechanism, and in other cases the role of the metal ions is more obscure (Zhuo *et al.* 1994, Nordlund & Eklund 1995). Serine/Threonine protein phosphatases (PPases) belong to this same group of phosphoesterases. PPases and mammalian tartrate-resistant acid phosphatases share fragments of amino acid sequences of significant homology. Among the conserved residues are those important in metal-binding, i.e. tyrosine, histidine, glutamic acid, and aspartic acid (Vincent & Averill 1990b). The active site structures are similar, but they do have some differences (Egloff *et al.* 1995, Kissinger *et al.* 1995, Knöfel & Sträter 2001, reviewed by Wilcox 1996, Barford *et al.* 1998, Kimura 2000). Metallohydrolases have in common that they hydrolyze phosphoproteins phosphorylated at their serine or threonine residues and the artificial substrate *p*-nitrophenyl phosphate (Vincent & Averill 1990b). We and others have reported that TRACP does not hydrolyze free or peptide-bound serine or threonine. TRACP has, however, been reported to hydrolyze protein-bound serine and threonine such as in osteopontin (Ek-Rylander *et al.* 1994, 1997), which is consistent with this classification. Specific phosphorylation of osteopontin has furthermore been shown to be important for its interactions with osteoclasts (Katayama *et al.* 1998).

Protein tyrosine phosphatases, PTPases, form another group of protein phosphatases. TRACP has been shown to hydrolyze phosphotyrosine, and we have suggested that TRACP might function as a PTPase *in vivo*. In spite of substrate specificity, TRACP has not turned out to have similarities with PTPases on structural level. Enzymes belonging to the group of protein tyrosine phosphatases require no metal ions or cofactors (Zhang 1997). PTPases do not share much sequence similarity, but contain an active site motif of one cysteine and one arginine residue, CX₅R. This motif is missing in the structures of TRACPs (Fauman & Saper 1996). Acid phosphatases tend to show rather broad substrate specificity in general. Prostatic acid phosphatase (PAP) hydrolyzes phosphotyrosine *in vitro* (Nguyen *et al.* 1990, Porvari *et al.* 1994) without having structural motifs distinct to PTPases (Schneider *et al.* 1993). PAP has a readily accessible active site, and this has been speculated to give rise to the broad range of substrates hydrolyzed by the enzyme (Schneider *et al.* 1993). In mammalian TRACPs, a long insertion in the loop between β_6 and α_5 folds over the active site, resulting in a less open substrate-binding pocket compared to the plant enzyme (Figure 8) with similar substrate specificity. Fragments of conserved amino acid sequences existing in purple acid phosphatases and PPases are not to be found in PTPases (Zhang 1997). Ljusberg and her co-workers have argued TRACP's function as a PTPase *in vivo* with its cellular localization (Ljusberg *et al.* 1999). TRACP has been shown to be localized in lysosome-like vesicular compartments, whereas tyrosine phosphorylation occurs in cytosomal matrix of cells. The substrate specificity and physiological substrate of TRACP in biological conditions needs further investigation.

6.6 Site-directed mutagenesis

Our results from analyses of mutated forms of TRACP were consistent with most of the conclusions that were made by us and the other groups based on the crystallographic studies (Sträter *et al.* 1995, Guddat *et al.* 1999, Uppenberg *et al.* 1999). The active site structure revealed hydrogen bond interactions of the conserved residues His113 and His216 with oxygen atoms of bound phosphate. Site-directed mutagenesis confirmed these residues to be important in phosphoester hydrolysis. Based on the active site structure we proposed that Asp267, which is also conserved in plant enzyme, acts as a general acid that protonates the leaving group. Our suggestion was argued for by the fact that this residue is not present in PPases and it is too far from the substrate's phosphate group (8Å) to be able to participate directly in the reaction (Knöfel & Sträter 2001). Our mutagenesis studies did indeed reveal that the role of Asp267 is less likely than we had suggested.

The di-iron center is essential for both phosphate hydrolysis and reactive oxygen species generation of TRACP, and the same active site is thus employed in both mechanisms. For site-directed mutagenesis we chose those amino acid residues we expected to be important for the acid phosphatase activity. Mutation of these residues did not impair ROS generating activity as it did acid phosphatase activity, which led us to suggest that these activities are independent of each other. The fact that these two activities have different pH optima supports their independent function and possibly different cell compartment localization. One possibility would also have been to mutate one or more of the metal ion ligand binding residues, and to study its consequences on both of the activities (Zhuo 1994). The three-dimensional structure of an acid phosphatase-inactive form might be useful in clarifying the issue of TRACP's substrate specificity and the identification of natural substrate(s).

7 Conclusions

Here we have used the baculovirus expression vector system to produce recombinant rat tartrate-resistant acid phosphatase for characterization and structural analysis and as method to produce antigen for antibody production. The three-dimensional structure of the active site determined was used to predict the amino acids important for acid phosphatase activity. The role of these amino acids was studied using site-directed mutagenesis. Conserved amino acid residues His113 and His216 proved to have a critical role in acid phosphatase activity, while radical oxygen species generating activity seems to employ other so far unidentified residues. The substrate specificity of the enzyme was not altered by the mutations and kinetic parameters for the wild type enzyme and mutated forms were determined. The three-dimensional structure provided evidence for the μ -(hydr)oxo bridged di-iron center. Hypothesis of the redox regulation of osteoclastic TRACP activity was proposed as well as of the mechanism for phosphate hydrolysis. The di-iron metal center with conserved amino acid residues and the $\beta\alpha\beta\alpha\beta$ core-motif link type 5 tartrate-resistant acid phosphatase to the diverse group of metallohydrolases with different functions and substrate specificities. This study gives information for further investigation on the physiological substrate(s) of the enzyme and for construction of specific inhibitors as well as for studying the role of the enzymes dual activity *in vivo*.

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