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Purple acid phosphatases from bacteria: similarities to mammalian and plant enzymes

Gerhard Schenk [a,*], Michael L.J. Korsinczky [a,b], David A. Hume [a], Susan Hamilton [a], John DeJersey [a]

[a] *Department of Biochemistry, The University of Queensland, Brisbane 4072, Australia*
[b] *Institute for Molecular Biosciences, The University of Queensland, Brisbane 4072, Australia*

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Abstract

Mammalian and plant purple acid phosphatases have similar active site structures despite low sequence identity (<20%). Although no bacterial enzyme has been purified, a sequence database search revealed that genes that could encode potential purple acid phosphatases may be restricted to a small number of organisms (i.e. myco- and cyanobacteria). Analysis of their deduced amino acid sequences and predicted secondary structures indicates that the cyanobacterial enzyme is similar to both the mammalian and the recently discovered low-molecular-weight plant purple acid phosphatases, while the mycobacterial enzyme is homologous to the fungal and high-molecular-weight plant purple acid phosphatases. Homology models indicate that both bacterial proteins appear to be similar to mammalian purple acid phosphatases in the immediate vicinity of the active site. It is likely that these enzymes act as Fenton-type catalysts in order to prevent damage caused by reactive oxygen species generated by invaded host cells (*M. tuberculosis*) or by the light-harvesting complex (*Synechocystis* sp.).

Keywords: Binuclear metal centre; Cyanobacteria; Fenton-type catalysis; Mycobacteria; Phosphate metabolism; Purple acid phosphatase

1. Introduction

Purple acid phosphatases (PAPs) comprise a family of binuclear metalloenzymes, which catalyse the hydrolysis of a wide range of phosphate esters and anhydrides. The characteristic purple colour is due to a tyrosine→Me(III) charge-transfer transition (Klabunde and Krebs, 1997). Due to their insensitivity towards inhibition by tartrate, PAPs are also known as tartrate-resistant acid phosphatases (TRAPs).

Mammalian PAPs from human, pig, bovine, mouse and rat have been isolated and characterised; all are monomers (~35 kDa) and contain redox-active Fe(III)–Fe(III)/Fe(II) centres. A comparison of their amino acid sequences shows that these proteins are >80% identical (for a review, see Klabunde and Krebs, 1997).

PAPs (high-molecular-weight form) have also been extracted from a number of plant sources. However, only the enzymes from red kidney bean and soybean and two isoforms from sweet potato have been characterised in detail. All are homodimers (subunits ~55 kDa) and reveal extensive sequence homology (Schenk et al., 1999). They display some variability in their metal compositions; the enzymes from red kidney bean (Beck et al., 1986) and soybean (Schenk et al., 1999) and one isoform from sweet potato (Durmus et al., 1999) contain Fe(III)–Zn(II) centres, whereas a separate sweet potato isoform utilizes manganese instead of zinc (Schenk et al., 1999). Sequences encoding a third isoform from sweet potato and two from *Arabidopsis thaliana* have been cloned, but the corresponding proteins have not yet been purified (Schenk et al., 1999; Durmus et al., 1999). Sequence homology between these plant enzymes and mammalian PAPs is restricted to five short motifs containing the seven invariant metal coordi-

Abbreviations: rmsd, root mean square deviation; PAP, purple acid phosphatase; PK, protein kinase; PP, protein phosphatase; PTP, phosphotyrosine phosphatase.

* Corresponding author. Tel.: +61-7-33654286; fax: +61-7-33654699.
E-mail address: schenk@biosci.uq.edu.au (G. Schenk)

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nating amino acids (Klabunde et al., 1995). However, the recent cloning of cDNA sequences from Easter lily, red kidney bean, soybean, sweet potato and *A. thaliana* and the discovery of several homologous ESTs from *A. thaliana*, rice and magnolia strongly suggest a second plant PAP gene family (low-molecular-weight form) with a greater sequence homology to the mammalian PAPs (Schenk et al., 2000).

The only purified PAP from a microorganism is that from *Aspergillus ficuum* (Ullah and Cummins, 1988). Although this protein appears to be monomeric (~85 kDa), its sequence is more closely related to the high-molecular-weight plant PAPs than to the mammalian enzymes. Other *Aspergillus* species [*A. niger* (Mullaney et al., 1995), *A. nidulans* (Sarkar et al., 1996)] contain sequences homologous to *A. ficuum* PAP, but no related sequence is evident in *Saccharomyces cerevisiae*. Furthermore, only a limited number of prokaryotic organisms appear to contain genes encoding potential PAPs; a search of complete and partial genome sequences listed in the TIGR Microbial Database (www.tigr.org/tdb/mdb/mdb.html) indicated their presence only in the cyanobacterium *Synechocystis* sp. as well as in *Mycobacterium tuberculosis* and *M. leprae* (Schenk et al., 2000).

The roles of PAP remain unknown. Mammalian enzymes have been implicated in bone resorption (Hayman et al., 1996) and iron transport (Nuttleman and Roberts, 1990), while the high-molecular-weight plant enzymes may have a function in phosphate acquisition (Duff et al., 1994). It also remains unclear why plants appear to have two distinct types of PAP (high- and low-molecular-weight forms) and why the occurrence of this enzyme may be restricted to only a few microorganisms. A phylogenetic analysis has shown that the cyanobacterial sequence appears to be related to the mammalian and the low-molecular-weight plant PAPs, while the mycobacterial sequences are likely to share a common ancestral gene with the *Aspergillus* and the high-molecular-weight plant enzymes (Schenk et al., 2000). Hence, it is likely that the bacterial sequences encode proteins, which may serve as model systems to study eukaryotic PAPs. In particular, for *Synechocystis*, powerful systems for genetic manipulation are well established (e.g. Poncelet et al., 1998), which will be valuable for the investigation of both mechanistic and physiological aspects of PAPs. The aim of this study was to (1) discuss sequence and structure similarities between prokaryotic and eukaryotic PAPs and (2) speculate on the metabolic role and the limited occurrence of bacterial PAPs.

2. Materials and methods

Amino acid alignments between PAPs from pig and *Synechocystis* and between those from red kidney bean and *M. tuberculosis* were performed using ClustalW software (Thompson et al., 1994). Further refinements were introduced manually based on the predicted secondary structures [determined with the algorithm PEPTIDESTRUCTURE (Jameson and Wolf, 1988)] of the bacterial enzymes. Gaps were inserted into the alignment that minimized the probability of disrupting α-helices and β-sheets.

Based on these alignments and the coordinates of the PAPs from pig (Guddat et al., 1999) and red kidney bean (Sträter et al., 1995), three-dimensional models were constructed for the *Synechocystis* and *M. tuberculosis* proteins, respectively. Initial modelling was carried out using the HOMOLOGY package included in INSIGHT 98 (MSI). The models were refined by applying energy minimization procedures in the program DISCOVER (also part of INSIGHT 98). All buried charged or polar amino acids were, or could potentially, be involved in the formation of either hydrogen bonds or salt bridges. The final models contained no significant internal cavities. The quality of the models was further validated by statistical analysis with the programs PROCHECK (Laskowski et al., 1993), WHAT IF (Vriend, 1990) and PROFILES-3D (MSI). Comparison between the three-dimensional structures of red kidney bean and pig PAP showed the core Cα structures to be similar with a root mean square deviations (rmsd) of 1.4 Å for 204 Cα atoms (Guddat et al., 1999). For red kidney bean and *M. tuberculosis* PAP, the rmsd for the corresponding 193 Cα atoms is 1.7 Å and for 193 Cα atoms from pig and *Synechocystis* PAP the rmsd is 1.8 Å. Additionally, in the three comparisons above, the seven metal-coordinating residues can be superimposed with an rmsd of 0.20, 0.41 and 0.48 Å, respectively. Given the similar arrangements of the metal-coordinating residues in the crystal structure of pig PAP and the model of the *Synechocystis* enzyme and the crystal structure of red kidney bean PAP and the model *M. tuberculosis* PAP, two iron atoms could readily be placed into the structures of the two models. Illustrations in this paper were generated using Molscript version 2.0.1 (Kraulis, 1991).

3. Results

Sequence alignments between the *M. tuberculosis* sequence (*Mtu*) and red kidney bean PAP (*Phaseolus vulgaris*, *Pvu*) and between the *Synechocystis* (*Syn*) and the pig enzymes are shown in Fig. 1. Known PAPs contain signal peptides (27 amino acids for both the red kidney bean and the pig enzymes). Due to the absence of sequence homology in the N-terminal regions, it is diffiult to assign the most likely cleavage sites in the bacterial proteins. We tentatively removed the N-terminal 34 and 37 amino acids for Mtu and Syn, respectively. The sequences are aligned in the regions

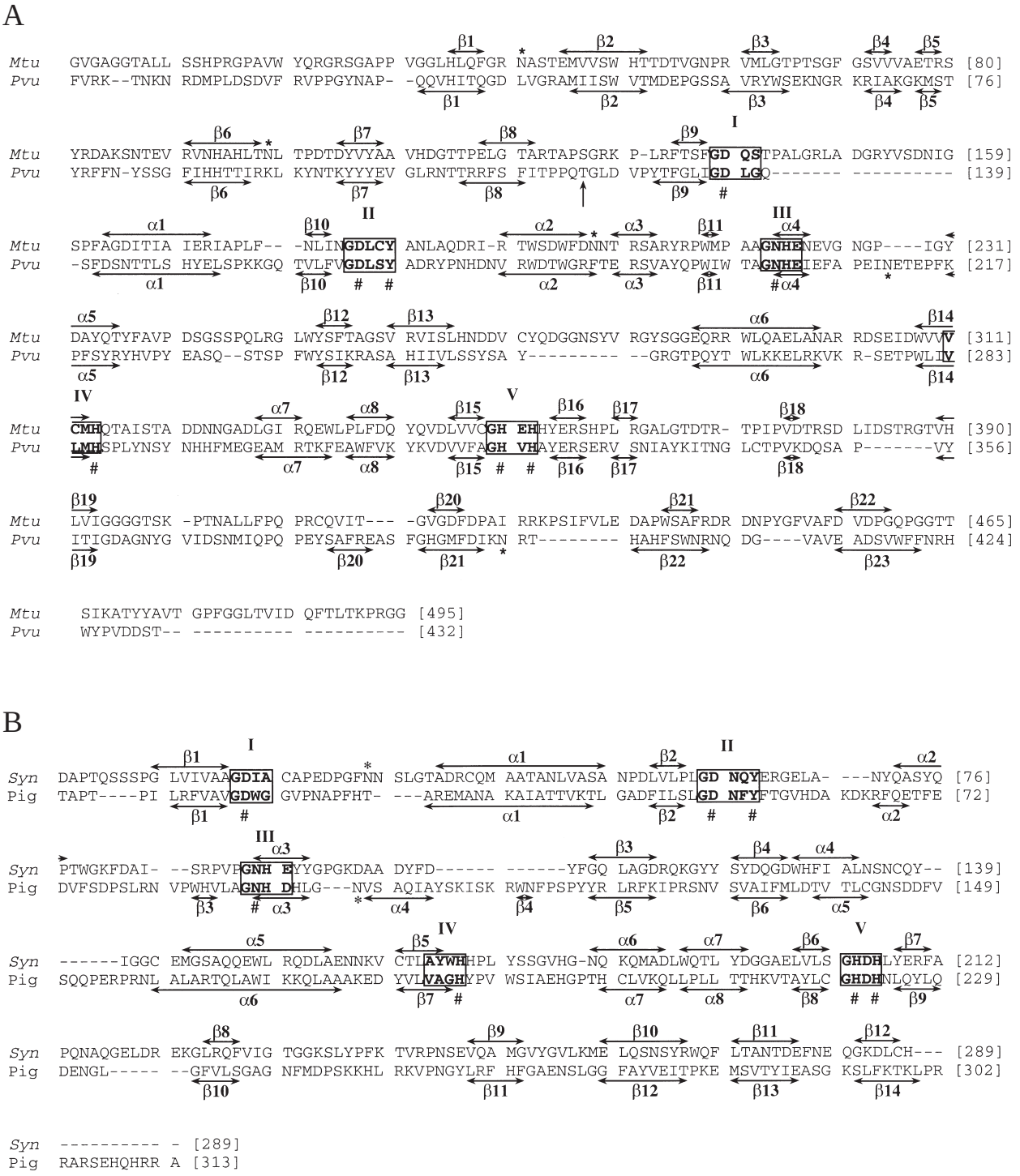

Fig. 1. Sequence alignments between PAPs from red kidney bean (*Pvu*) and *M. tuberculosis* (*Mtu*) (**A**) and from pig and *Synechocystis* (*Syn*) (**B**). Secondary structure elements (β: β-sheets; α: α-helix), as predicted (*Mtu*, *Syn*) or deduced from the structure (*Pvu*, Pig) are indicated by bars and numbered consecutively. Numbers in brackets at the end of each line indicate the number of residues. The five conserved regions (boxed) that contain the seven invariant metal-ligating amino acids (#) are also shown. Signal peptides are not included.

(boxes I–V in Fig. 1) containing the conserved metal ligating residues (#). Homology models for Mtu and Syn were constructed using the alignments in Fig. 1 together with the coordinates for the red kidney bean enzyme (for Mtu) (Sträter et al., 1995) and pig PAP (for Syn) (Guddat et al., 1999). Statistical analysis of the two homology models not only validated their quality but also showed their similarity to plant (Mtu) and mammalian (Syn) PAPs (see Section 2). Similar to red kidney bean PAP, the mycobacterial enzyme consists

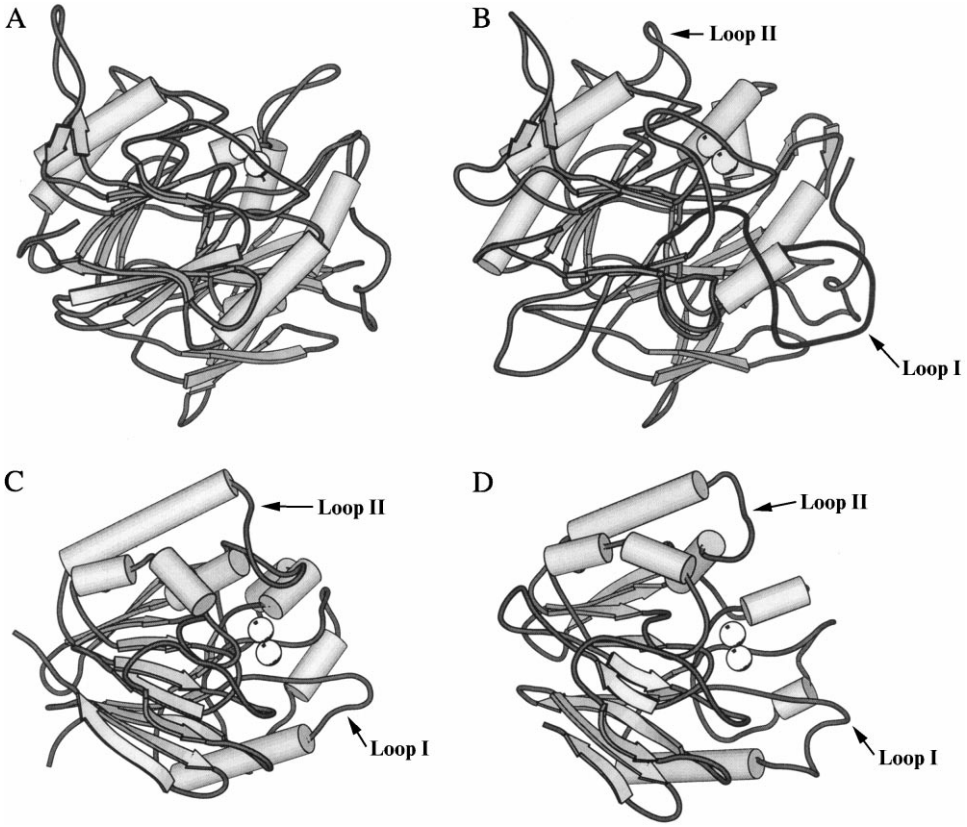

Fig. 2. Ribbon diagrams of the three dimensional structure of PAPs from (**A**) red kidney bean, (**B**) *M. tuberculosis*, (**C**) pig and (**D**) *Synechocystis*. The metals of the binuclear centres are drawn as spheres. Loops I and II are described in the text.

of two domains, an N-terminal domain and a presumably catalytic C-terminal domain. The boundary between these domains is indicated by an arrow in Fig. 1A. As in the pig enzyme, the N-terminal domain is absent from the cyanobacterial PAP. Fig. 2 shows ribbon diagrams of the four structures. Unlike the pig enzyme, red kidney bean PAP is a homodimer whose subunits are linked via a disulphide bridge (Cys345); further contacts between the two monomers mainly occur between α7 and amino acids in the loop from residue 253 to 260 (numbered according to the red kidney bean sequence) (Sträter et al., 1995). The disulphide bridge is not conserved in the mycobacterial enzyme. However, interactions between α5 are possible, indicating that *M. tuberculosis* PAP could be homodimeric.

All known PAPs are glycosylated. Potential glycosylation sites for the myco- and cyanobacterial PAPs are indicated by an asterisk in Fig. 1. The most obvious difference between the published pig and red kidney bean PAP structures in the immediate vicinity of the binuclear centres is the presence of two loops in the pig enzyme (Fig. 2C) (Guddat et al., 1999). Both loops are present in the bacterial proteins (Fig. 2B and D). One of those loops (loop I, comprising residues 18–24 in the pig enzyme, residues 143–160 in Mtu and 22–32 in Syn; Fig. 1) may be involved in substrate binding (Guddat et al., 1999). The other loop (loop II) comprises residues 142–155 in the pig enzyme, residues 272–283 in Mtu and 134–139 in Syn (Fig. 1). The disulphide bridge observed in pig PAP (between Cys142 and Cys200) is absent in both bacterial enzymes.

An interesting feature of PAPs is their pseudo-twofold symmetry around the binuclear metal centre, which is located on the twofold axis surrounded by two βαβαβ units ('Motifs' A and B) (Guddat et al., 1999). A similar topology is observed in the models of *M. tuberculosis* and *Synechocystis* PAP. In the mycobacterial (and red kidney bean) protein, 'motif' A comprises β-sheets 9–11 connected by α-helices 1 and 2 (residues 134–214) and 'motif' B extends from β-sheet 13 to 15 with α-helices 6 and 7 connecting these sheets (residues 261–349). In the cyanobacterial PAP, the third β-sheet in 'motif' A is absent. The two 'motifs' extend from residues 10 to 74 ('motif' A) and residues 122 to 202 ('motif' B), respectively. Most of the metal coordinating residues lie in loops adjacent to the C-terminal end of β-sheets in 'motifs' A and B (Fig. 1).

4. Discussion

A database search has revealed only three genes (one cyanobacterial and two mycobacterial sequences) that

encode potential PAPs. An extensive search of various other bacterial databases did not reveal any further PAP-encoding sequences (Schenk et al., 2000). Here, we have shown that despite low sequence similarities, the *M. tuberculosis* and *Synechocystis* sequences align and have a similar predicted secondary structure to that of PAPs from red kidney bean and pig, respectively (Fig. 1). Energy-minimized homology models of the putative bacterial PAPs (Fig. 2) form a first approximation to their true three-dimensional structures and further support a structural similarity with red kidney bean and pig PAP. Since all seven amino acids involved in metal binding are totally conserved and can be correctly positioned in the three-dimensional models after energy minimization, it is very likely that the bacterial enzymes also fold in a manner similar to known PAPs.

The chromophoric site in PAPs characterised to date is occupied by Fe(III). All mammalian enzymes also have a redox-active Fe(III)/Fe(II) in the divalent site. The studied plant PAPs contain Zn or Mn (Beck et al., 1986; Durmus et al., 1999; Schenk et al., 1999). Hence, the redox potential of these enzymes will differ significantly from that of mammalian PAPs. The precise biological role(s) of PAPs is not yet known, although a functional overlap seems likely. For example, in addition to their phosphatase activity, the mammalian enzymes may also function as iron carriers (Nuttleman and Roberts, 1990) and/or generators of reactive oxygen species (Sibille et al., 1987). In contrast, the plant enzymes have been implicated in phosphate acquisition (Duff et al., 1994). Since both bacterial proteins appear to be more similar to the pig enzyme than to red kidney bean PAP in the immediate vicinity of the active site (Fig. 2), we speculate that the two bacterial PAPs may contain Fe–Fe centres with properties similar to those of the mammalian enzymes.

The role of the non-catalytic N-terminal domain in the mycobacterial (and red kidney bean) enzyme is obscure except that two out of three potential glycosylation sites in Mtu are located in this domain (Fig. 1A). Glycosylation amongst bacterial proteins is much less frequent than amongst eukaryotic proteins but several potential antigens in *M. tuberculosis* have been shown to be glycosylated (e.g. Herrmann et al., 1996). Before PAP can be considered as a potential virulence factor, it has to be shown that its function assists the course of the disease. Acid phosphatases have been shown to be of crucial importance for the survival of *Leishmania donovani* (Remaley et al., 1985), *Legionella micdadei* (Saha et al., 1985) and *Francisella tularensis* (Reilly et al., 1996) within their hosts' phagocytic cells due to their ability to inhibit the respiratory burst of their hosts. Although PAPs have no apparent sequence similarity to the acid phosphatases from *L. donovani*, *L. micdadei* and *F. tularensis*, all seem to have broad substrate specificities and are resistant to inhibition by tartrate. *M. tuberculosis* resides in the highly oxidative environment of human macrophages; both reactive oxygen and reactive nitrogen intermediates are antimycobacterial effector molecules (Riley, 1995). A recombinant peroxynitritase was recently shown to prevent oxidative damage to the bacterium by removing reactive oxygen and nitrogen species (Wengenack et al., 1999). By analogy, PAPs can remove reactive oxygen intermediates in a Fenton-type reaction (Sibille et al., 1987). Hence, in *M. tuberculosis*, a PAP enzyme could assist pathogen survival by (1) reducing the respiratory burst of its host and (2) removing potentially lethal free radicals. In order to understand better the role of PAP in *M. tuberculosis*, it is now necessary to purify the enzyme, determine its cellular location and characterise its physical properties.

It is generally accepted that all prokaryotic organisms may have contained 'eukaryote-like' protein kinases (PKs) and phosphatases (PPs) (Shi et al., 1998). *Synechocystis* contains the largest number of different ORFs encoding PKs and PPs, and it has been hypothesized that this may reflect this organism's ability to survive in extreme environments (Shi et al., 1998). Mammalian PAPs can act as phosphotyrosine phosphatases (PTPs), raising the possibility for a role of this enzyme in signal transduction (Nash et al., 1993). Since PTP activity might be retained in the related cyanobacterial PAP, a similar role for this enzyme cannot be precluded. Additionally, PAP may also act in *Synechocystis* as a defence against free radicals, in particular in association with the light-harvesting complex, which generates a great number of potentially damaging active oxygen species.

Despite their apparent resemblance to mammalian PAPs in the vicinity of the active site, it cannot be ruled out that myco- and cyanobacterial PAPs also have a role similar to that of the plant enzymes, i.e. phosphate uptake (Duff et al., 1994). If this is the case, the two bacterial PAPs form interesting targets for bioengineers attempting to improve biological phosphorus removal in waste water plants. Although it is not yet known which bacteria are important in phosphate accumulation, *Actinobacteria* and members of the β-2 subclass of the *Proteobacteria* are likely candidates (Bond et al., 1999). Hence, overexpression of *M. tuberculosis* or *Synechocystis* PAP in any of these bacteria may increase their ability to rid waste water of phosphorus.

The evolutionary history of PAP seems complex, in particular in view of the similarity between mycobacterial, fungal and high-molecular-weight plant enzymes on one side and cyanobacterial, mammalian and the recently discovered low-molecular-weight plant PAPs (Schenk et al., 2000) on the other side. It has been speculated that the catalytic domains may have evolved through the combination of mononuclear centres (Guddat et al., 1999). The origin of the non-

catalytic N-terminal domain present in some PAPs remains obscure and does not preclude a convergent type of evolution. While the phylogeny of PAP remains unknown, the presence of this enzyme in a limited number of microorganisms is an indication of its role in specialised metabolic pathways. Hence, it is important to advance our knowledge of the functions of bacterial PAPs since this will also lead to a better understanding of the physiological roles of the mammalian enzymes.

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