Charged with meaning: the structure and mechanism of phosphoprotein phosphatases

Many phosphatases require two metal ions for catalysis. New structural information on two serine/threonine phosphatases offers insight into how the metals contribute to catalysis. A comparison with the structures of protein tyrosine phosphatases, which do not use metal ions, shows that the only similarity at the active site is that of charge.

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Many biological processes are regulated by a simple chemical event — the cleavage or formation of phosphate esters. In nature, these reactions are catalyzed by two sets of enzymes, phosphatases and kinases. Kinases operate in the synthetic direction (phosphorylation) while phosphatases catalyze the hydrolysis (cleavage) reaction. Over the last decade there has been a growing realization that phosphatases are extremely important in cellular and organismal functions [1]. Here, we focus on recent advances in our understanding of phosphatases, especially phosphoprotein phosphatases, with a view to clarifying some of the mechanistic and structural complexities and ambiguities presented by this diverse group of enzymes. The newly reported structural information on two serine/threonine (Ser/Thr) phosphatases [2,3] is particularly enlightening.

Categories of phosphatases

The substrates for phosphatases range from small phosphorylated metabolites such as glucose-6-phosphate and second messengers (e.g. phosphoinositols) all the way up to large phosphorylated proteins. Over a hundred phosphatases are known, and it is likely that the total number of these enzymes is well over a thousand, or >1 % of the proteins encoded by the human genome. To make sense of this large family of enzymes, a classification scheme is essential (Fig. 1). There are three major groups of phosphatases: (1) nonspecific — these enzymes will catalyze the hydrolysis of almost any phosphate ester; (2) phosphoprotein specific — enzymes that use phosphoproteins or phosphopeptides as preferred substrates; and (3) small-molecule specific — enzymes that hydrolyze one (or a group of structurally similar) substrate(s), for example, phosphoinositol monophosphatase (this enzyme may be the clinical target of lithium ion therapy for depression [4]).

A second useful classification scheme is one according to the enzyme's mechanism of action. Some phosphatases use an active site nucleophile as the initial phosphoryl group acceptor; others transfer it immediately to water (Fig. 2). The first group can be further subdivided according to the phosphoryl group acceptor (cysteine, histidine, or serine). Members of the second group characteristically use a two-metal-ion dyad to bind phosphate esters and catalyze their subsequent hydrolysis. Indeed, this metal ion motif is ubiquitous in phosphoryl group transfer biochemistry, as it is also used by phosphodiesterases (e.g. nucleases [5]) and phosphotriesterases [6]. Two-metal-ion phosphatases are very heterogeneous, however, varying in metal-ion type, protein sequence, structure, ligands, active-site catalytic residues and even mechanism. For example, alkaline phosphatase transfers the phosphoryl group to a serine residue, not to water, and has a third active-site metal (a Mg(II) ion that does not directly contact the phosphate ester) [7]. Zn(II) ions are frequently found in the active site of two-metal-ion phosphatases, but many other divalent metal ions are also common. Mammalian purple acid phosphatase, which contains an unusual binuclear Fe(II)–Fe(III) metal dyad [8], serves as a reminder that the two-metal-ion enzymes have enormous diversity.

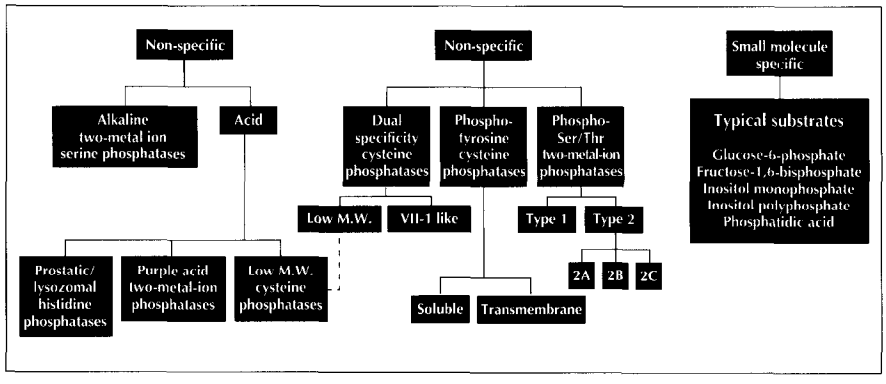

Fig. 1. A hierarchy of phosphatases. Initial classification is based on substrate specificity; secondary classification is based on mechanism. The low MW acid phosphatases and the low MW dual specificity phosphatases seem to be a related family of enzymes.

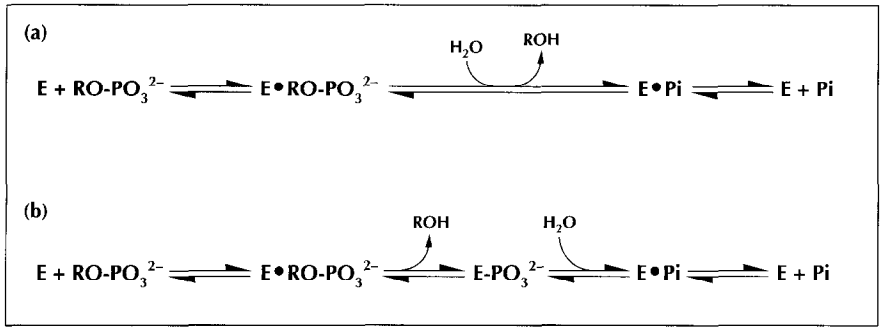

Fig. 2. Typical phosphatase reaction mechanisms. **(a)** Direct transfer to water, normally catalyzed by active-site metal ions. **(b)** Hydrolysis via a phosphoenzyme intermediate. Serine, cysteine, and histidine are nucleophiles commonly used as the initial phosphoryl group acceptor.

Phosphoprotein phosphatases

Both phosphoprotein phosphatases and protein kinases are important in the regulation of the phosphorylation state of proteins. Often, phosphatases simply reverse the effects of kinases, for instance by inactivating a protein that has been activated by phosphorylation. But things are not always so straightforward. Phosphatases can also regulate kinase activity, thus indirectly regulating the phosphorylation state of the substrates of the kinase. Some phosphatases contain SH2 domains which specifically recognize and bind to phosphotyrosine residues, and their activity is therefore modulated by protein tyrosine kinases. Such examples of crosstalk allow for the exquisite fine tuning of signal transduction cascades necessary for regulating cell function.

Phosphoprotein phosphatases are subdivided into the Ser/Thr phosphatases [9], which are probably all two-metal-ion phosphatases, the protein tyrosine phosphatases (PTPases) [10], and the dual-specificity phosphoprotein phosphatases [11–13]. PTPases and dual-specificity phosphoprotein phosphatases are mechanistically related and use an active-site cysteine located in a phosphate binding loop as the phosphoryl group acceptor. True PTPases are highly specific for phosphotyrosine, whereas the dual-specificity enzymes hydrolyze both phosphotyrosine and phosphoserine (the low-molecular-weight dual-specificity phosphatases prefer aromatic phosphate esters and phosphotyrosine substrates, but can still often hydrolyze phosphoserine-containing peptides and proteins [14]). For most phosphatases the nature of the physiologically relevant substrate(s) is unknown. Thus, the fact that a phosphatase is classified as having Ser/Thr phosphatase activity (Fig. 1) does not necessarily imply that this is its sole activity *in vivo*. Indeed, calcineurin, a prototypical Ser/Thr phosphatase, can hydrolyze phosphotyrosine-containing peptides as well as aromatic phosphates such as *p*-nitrophenyl phosphate [15]. Even prostatic acid phosphatase, a so-called nonspecific phosphatase, has phosphoprotein substrates (in particular, those containing phosphotyrosine) with K_M values in the low nanomolar range [16]; perhaps a phosphoprotein is a major target *in vivo*.

Functions of Ser/Thr phosphoprotein phosphatases

The Ser/Thr phosphatases fall into four classes, which account for virtually all cellular Ser/Thr phosphatase activity [9]. Type 1 enzymes (PP-1 enzymes; PP-1 is both an enzyme in the class and the name of the class) specifically dephosphorylate the β subunit of phosphorylase kinase and are inhibited by two low molecular weight proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2). Type 2 enzymes prefer the α subunit of phosphorylase kinase as a substrate and are insensitive to I-1 and I-2. The type 2 enzymes are subdivided according to their divalent metal ion requirements. PP-2A enzymes (like PP-1 enzymes) are stimulated by Mn(II), whereas PP-2B enzymes and PP-2C enzymes use Ca(II) and Mg(II), respectively. PP-1, PP-2A, and PP-2B enzymes have highly homologous catalytic domains, and the differences between their activities are mostly caused by regulatory subunits bound to the catalytic domain. PP-2C enzymes, however, appear to be monomeric, and are structurally unrelated to the other Ser/Thr phosphatases.

Much is known about the biological roles of some of the Ser/Thr phosphatases, particularly those of the type 1 family. Early studies on the regulation of glycogen synthesis and breakdown provided seminal information on the effect of protein phosphorylation/dephosphorylation on the activities of individual enzymes involved in metabolic and catabolic processes [17]. Dephosphorylation by PP-1 reverses the activity of protein kinase A and regulates the phosphorylation state (and therefore the activity) of three of the key enzymes involved in glycogen metabolism: phosphorylase kinase, glycogen synthase and glycogen phosphorylase. The activity of PP-1 is itself tightly regulated by levels of cyclic AMP, and the degree of phosphorylation of the inhibitors I-1 and I-2. These and other inhibitors of PP-1 (such as the polyether, okadaic acid, and the cyclic heptapeptide toxins known as microcystins) have been useful for the study and manipulation of signal transduction cascades. Studies on inhibitors of calcineurin-A (PP-2B), such as the cyclosporins and FK506, have added greatly to our understanding of T-cell activation and the design of new immunosuppressants [18].

Structure and mechanism

Despite a wealth of information about the biological processes involving Ser/Thr phosphatases, chemical and structural information about these enzymes is sparse and little is known about how they catalyze phosphate ester hydrolysis. Most of the mechanistic information on these proteins stems from mutagenesis studies of bacteriophage λ phosphatase, a phosphatase with substantial homology

to the catalytic domain of PP-1, PP-2A and PP-2B enzymes [19]. These studies established the importance of a variety of residues that are conserved in all Ser/Thr phosphatases and suggested potential roles for these residues in both metal-ion binding and catalysis. Many of these conserved residues form the Ser/Thr phosphatase signature motif Gly-Asp-Xaa-His-Gly-$(Xaa)_n$-Gly-Asp-Xaa-Val-Asp-Arg-Gly-$(Xaa)_n$-Arg-Gly-Asn-His-Glu ($n \approx 25$), which is part of a more general signature motif common to a variety of other phosphoesterases [20].

Very recently, crystal structures of both PP-1 [2] and calcineurin-A [3] were obtained. As the high degree of sequence homology between these proteins might suggest, their overall architectures are similar, and they seem to have nearly identical active sites. These structures also firmly establish that Ser/Thr phosphatases bind two metal ions in the active site. This is an important advance because it is often difficult to establish the nature, stoichiometry, location and function of enzyme-bound metal ions; this has certainly been the case for Ser/Thr phosphatases. The localization of the two metal ions in these structures permits assignment of structural and mechanistic roles to conserved residues and sheds light on the mechanism of enzyme catalysis and on the nature of inhibitor binding and regulation. Furthermore, because of the homology between PP-1, PP-2A and PP-2B enzymes, these structures enhance our understanding of a variety of Ser/Thr phosphatases.

Although the exact nature of the active-site metals of many of the Ser/Thr phosphatases remains a subject of some controversy [21], data combined from both biochemical and X-ray diffraction studies suggests that PP-1 uses a Mn(II) dyad for catalytic purposes, whereas calcineurin-A uses a binuclear Zn(II)–Fe(III) cluster. The metal ion binding sites in these enzymes are similar (despite the lack of sequence homology) to that of purple acid phosphatase; all have a central structural unit composed of α-helices and strands of β-sheet in the configuration β–α–β–α–β (Fig. 3). This scaffolding contains the Ser/Thr phosphatase signature motif identified during studies with bacteriophage λ phosphatase. The recent crystal structures of PP-1 and calcineurin-A illustrate distinct structural and mechanistic roles for several residues in this motif and also identify other active-site residues.

The first domain of the Ser/Thr phosphatase signature motif contains two metal-binding ligands, Asp64 and His66 (numbering as in PP-1 [2]), which coordinate to metal ion 1 (M1, see Fig. 3a). M2 is coordinated in part by Asn124 from the third domain of the motif and by His173 and His248. Although the latter two ligands are not included in the signature motif, they are conserved between several Ser/Thr phosphatases. Asp92, found in the second conserved domain, forms a monodentate bridge between the two metals. Two arginine residues, Arg96 and Arg221, are positioned to stabilize phosphate bound in the active site, suggesting that they are

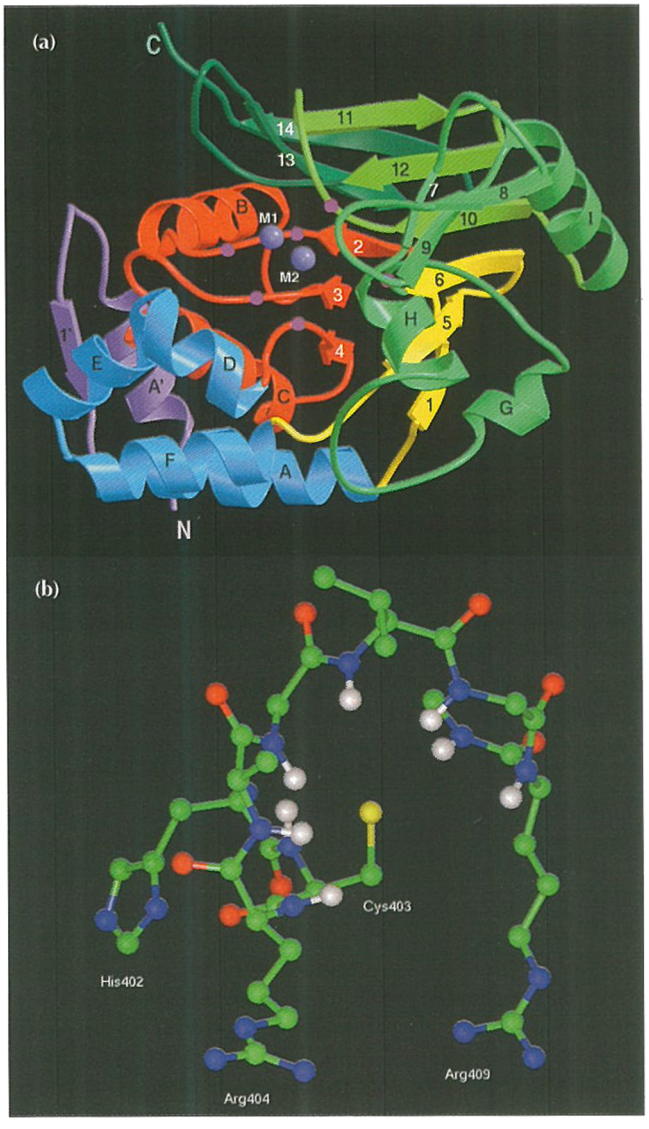

Fig. 3. Active-site structure of two phosphoprotein phosphatases. **(a)** Ribbon diagram of PP-1 showing the β–α–β–α–β metal-binding motif (red), active-site metals (violet spheres), and metal ligands (violet circles) [2]. **(b)** Ball and stick representation of Yop51 phosphate binding loop showing the cysteine–histidine pair as well as two arginines that flank the active site. Backbone amides direct several hydrogen bonding arrays into the active site (for clarity, only amide protons are depicted) [23].

important in substrate binding and perhaps in transition state stabilization. Arg96 is found in the second conserved domain, whereas Arg221, like His173 and His248, is conserved but is not a member of the formal signature motif. Asp95, from the second domain, and His125, from the third domain, round out the conserved residues positioned at the active site. Possible roles for these two amino acids are discussed below.

The Ser/Thr phosphatases presumably use their active site metal ions to bind substrate, activate a nucleophile, and/or stabilize the transition state(s) of phosphate-ester hydrolysis. A plausible mechanism for two-metal-ion catalysis of this reaction is shown in Figure 4. The nature of the active site nucleophile is unknown, although (by

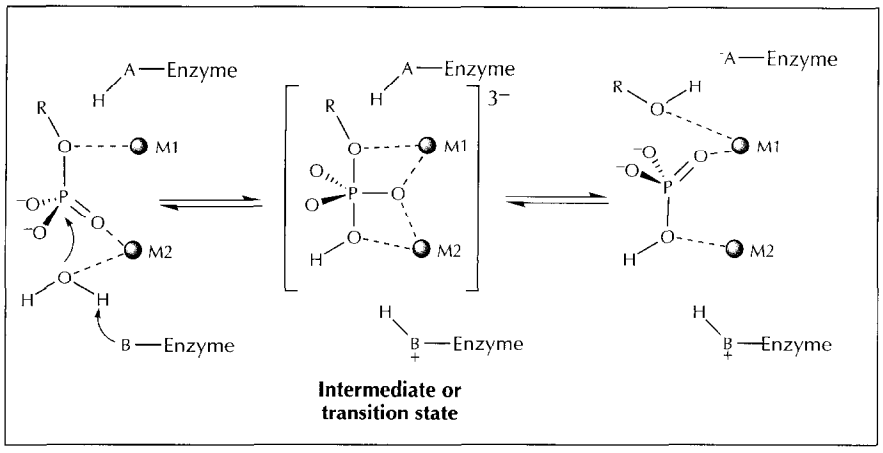

Fig. 4. Two-metal ion catalysis of phosphate ester hydrolysis. The active site metals probably act to bind substrate, stabilize the transition state(s) of the reaction, facilitate leaving group departure, and activate the phosphoryl group acceptor (shown here as H_2O, but possibly an enzyme nucleophile or metal-bound hydroxide). A variety of phosphatases have metal ions at the catalytic active site. An active site base catalyzes the deprotonation of the attacking water molecule, and an active-site acid protonates the leaving group.

analogy with other phosphatases and based on model building) a metal-bound water molecule or hydroxyl group is a reasonable candidate. Alternatively, the conserved His125 (coupled with Asp95) could act as the nucleophile or perhaps as a general acid to promote the departure of the leaving serine or threonine residue.

Despite the noted similarities in structure and in active site composition, PP-1 and calcineurin-A have distinctly different biological roles and regulatory mechanisms. These differences apparently arise from variations in the surface structure of the catalytic domain and from regulatory regions near the carboxyl-termini of these enzymes. The surface topology of PP-1 is characterized by three distinct grooves that radiate from the central, shallow active site: a 'hydrophobic' groove, an 'acidic' groove, and a 'carboxy-terminal' groove (Fig. 5a). These grooves have a role in inhibitor binding and presumably in substrate binding as well. Microcystin inhibits PP-1 by embedding itself in the hydrophobic groove and overlapping the active site. Inhibitor-1 may act in a similar manner, though modeling indicates that its major interactions are with the acidic groove. The third (carboxy-terminal) groove may be involved in the inhibition of PP-1 caused by the phosphorylation of Thr320, located near the carboxyl terminus of the protein.

The topology and architecture of the catalytic domain of calcineurin is similar to that of PP-1, with the striking exception of the regulatory carboxyl-terminus. Calcineurin-A has a five-turn amphipathic helix known as the calcineurin-B binding helix (BBH) linked to the phosphatase domain. The hydrophobic face of this helix fits into a complementary groove in calcineurin-B, leaving the polar face mostly exposed. The immunophilin–immunosuppressant complex FKBP12–FK506 binds at the base of the BBH, contacting both calcineurin-A and calcineurin-B in the process. This brings FKBP12 into close proximity to the hydrophobic groove of calcineurin-A, apparently inhibiting enzymatic activity by hindering the approach of macromolecular substrates. The active site of calcineurin-A, however, remains accessible to solvent and perhaps also to small molecules. As with PP-1, the mode of substrate binding to the catalytic domain has yet to be determined.

Although many additional mechanistic questions about the Ser/Thr phosphatases remain unanswered, these new structural data provide the necessary background for starting to understand the mechanisms of most of the known Ser/Thr phosphatases, as well as a number of other enzymes that share the two-metal-ion phosphatase signature motif.

Protein tyrosine phosphatases

This subgroup is characterized by a conserved catalytic domain of ~250 amino acids, containing the active site signature sequence Pro-Xaa-Ile/Val-Ile/Val-His-Cys-Ser-Ala-Gly-Xaa-Gly-Arg-Ser/Thr-Gly. PTPases can be divided into two classes: (1) transmembrane, receptor-like PTPases, and (2) soluble intracellular PTPases. The transmembrane enzymes (with two exceptions) contain two intracellular catalytic domains. The amino-terminal region (domain I) is thought to be constitutively active, while the carboxy-terminal region (domain II) has little or no enzymatic activity, at least *in vitro*. The amino-acid sequence outside of the catalytic domains varies considerably in both transmembrane and intracellular PTPases. This structural diversity may allow for control of PTPase activity through mechanisms such as ligand binding, cell adhesion and protein compartmentalization [10] and may account for substrate specificity.

There is much evidence indicating that phosphate ester hydrolysis catalyzed by PTPases proceeds through a phosphocysteine intermediate. X-ray structures of PTP1B [22] and Yop51 [23] depict an active-site phosphate binding loop, with a cysteine positioned to attack the phosphate ester. This residue is apparently maintained as the thiolate by a flanking histidine residue (reminiscent of the Cys–His catalytic dyad of cysteine proteases) as well as an extensive hydrogen-bonding network radiating from the active site (Fig. 3b). Treatment of PTPases with ^{32}P labeled substrate results in the incorporation of radioactivity into the enzyme [24,25], whereas mutants in which the active site cysteine is replaced with serine have no catalytic activity and do not incorporate label, although they do retain the ability to bind substrate [25]. For Yop51 the breakdown of the phosphocysteine intermediate is apparently rate-limiting for both small aromatic phosphate

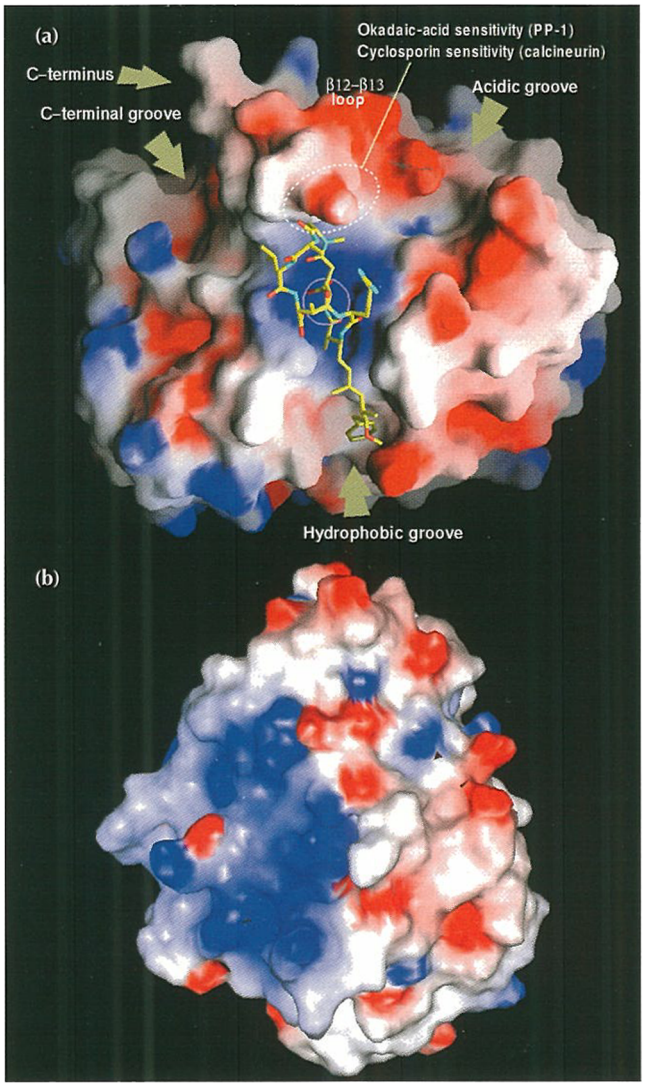

Fig. 5. Electrostatic surface potentials of **(a)** PP-1 (reprinted with permission from [2]) and **(b)** Yop51 [23]. Despite the vast differences in sequence, structure, and catalytic mechanism, these enzymes have surprisingly similar surface charges, suggesting that they have evolved a similar strategy for active site charge distribution — a feature no doubt dictated by the catalytic requirements of phosphate ester hydrolysis.

esters and for phosphopeptide substrates [26]. Site-directed mutagenesis studies have also identified a conserved aspartate residue that apparently acts as an active-site acid [27]. This residue is located on a mobile loop that covers the active site when substrate is bound.

Ser/Thr phosphatases compared to PTPases

Tyrosine kinases and Ser/Thr kinases share a great deal of structural and mechanistic homology. The same cannot be said for PTPases and Ser/Thr phosphatases. Although the two classes of phosphatases carry out very similar reactions, PTPases are not metalloenzymes and seem to have evolved a completely different strategy for catalyzing phosphate-ester hydrolysis from that of Ser/Thr phosphatases. The major structural similarity between these enzymes seems to be the presence of multiple arginine residues positioned at the active site. Ser/Thr phosphatases have two arginine residues located near the surface of the active site, whereas PTPases have three. Positively charged groups in the active site (such as metal ions or arginine residues) seem to be important in the catalytic mechanisms of many phosphatases. The surfaces of both PTPases and PP-1 are largely negative or neutral, with the exception of the active site and surrounding areas, which contain a region of densely packed positive charge (Fig. 5). This is not unexpected, since both proteins need to bind the negatively charged phosphate group and stabilize the negatively charged phosphorane intermediate that forms during nucleophilic attack. The charge presumably also facilitates the deprotonation of the nucleophile (cysteine for PTPases, possibly water for PP-1 enzymes) and charge development on the leaving group. All the steps necessary to catalyze the hydrolysis of a phosphate ester should be promoted by the high degree of positive charge in the active sites of these two types of enzymes. Thus the general approach to phosphoester hydrolysis used by these two families of phosphatases is similar, despite the large differences in the structural and chemical details of catalysis.

Summary

The new structural data obtained for PP1 and calcineurin-A resolve many of the outstanding questions about the catalytic nature of the Ser/Thr phosphatases. These enzymes may now be firmly classed as two-metal ion phosphatases. These data also provide a model for understanding a variety of phosphatases that share the two-metal-ion binding signature motif found in the Ser/Thr phosphatases. These enzymes are clearly important in the control of a variety of cellular functions and in a number of cases are important targets for therapeutic intervention. The structural data obtained for PP1 and calcineurin-A should certainly facilitate efforts to understand, and design inhibitors of, these proteins and related phosphatases.

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