Binuclear Metallohydrolases

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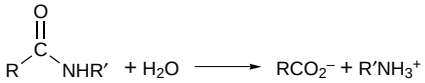
Introduction

Metabolic and signaling biochemical pathways have numerous steps that involve the hydrolytic cleavage of peptide or phosphate ester bonds. Although both types of bonds are thermodynamically unstable to hydrolysis, there are significant kinetic barriers to these reactions. Consequently, peptidases that catalyze the hydrolysis of peptide bonds of proteins (eq 1) and phosphatases and nucleases that



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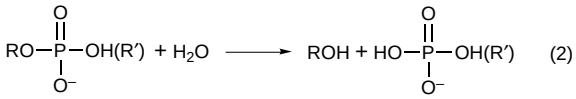
catalyze the hydrolysis of phosphate ester bonds of phosphorylated amino acids and saccharides, nucleotides, DNA, or RNA (eq 2) have evolved to rapidly



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Dean Wilcox was born and raised in Columbus, OH, and received his undergraduate education at a number of institutions, eventually obtaining his B.S. and M.S. degrees in chemistry from the University of California at Riverside in 1978 and 1979, respectively. He then pursued graduate work with Edward Solomon at MIT and Stanford, receiving his Ph.D. in chemistry from MIT in 1984. Thereupon he joined the faculty at Dartmouth College where he is now Professor of Chemistry. His research interests include urease and other binuclear metallohydrolases, the coordination chemistry of Cys- and His-rich peptides including metallothionein and zinc fingers, the biological chemistry of nitric oxide, and EPR spectral characterization of inorganic complexes. Over the years he has found that many good things come in a set of two, including his sons Ethan and Oren, shown here with their father.

and selectively cleave these bonds under physiological conditions. Since these enzymes are involved in processing crucial cellular constituents and play roles in signal transduction, they are ubiquitous and essential for living organisms.

Many of these enzymes have been studied in considerable detail and have been shown to use a variety of structures, functional groups, and chemical mechanisms to accelerate hydrolysis reactions.[1−3] Four different types of peptidases are known: one type, exemplified by chymotrypsin, uses a Ser nucleophile to attack the carbonyl carbon, resulting in an acyl-enzyme intermediate that is subsequently attacked by water; a second type, exemplified by papain, uses a Cys nucleophile in much the same way as the Ser nucleophile; a third type, exemplified by pepsin, uses two carboxylate functional groups in general acid−base catalysis with no enzyme-bound intermediates; the fourth type, exemplified by carboxypeptidase, uses the Lewis acidity of metal ions, typically Zn(II), to increase the electrophilicity of the carbonyl carbon and/or increase the nucleophilicity of water by stabilizing hydroxide in close proximity to the substrate. Similarly, enzymes that catalyze the hydrolysis of phosphate ester bonds use the functional groups of amino acids and/or the Lewis acidity of metal ions. One of the best characterized examples is ribonuclease, which hydrolyzes RNA

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with a concerted general acid−base mechanism using two His residues, and another well-studied example is staphylococcal nuclease, which uses the electrostatic properties of a protein-bound Ca(II) ion and general-base catalysis to hydrolyze DNA. Clearly other factors, including electrostatic stabilization of charge in transition states and intermediates, stabilization of leaving groups, increased effective concentrations, and orientation effects, can also be used by these enzymes to provide lower activation energies and thereby accelerate hydrolysis rates.

Metallohydrolases with peptidase or phosphoesterase activity have been of considerable interest to bioinorganic chemists, and most of the well-studied examples, such as carboxypeptidase[4] and thermolysin,[5] contain a single metal ion that is involved in the enzymatic reaction. However, certain hydrolytic enzymes use two metal ions, typically held in close proximity with bridging ligand(s), and the number of these binuclear metallohydrolases is growing rapidly, as indicated by reports last year of X-ray crystal structures of seven members of this class. Currently known examples include enzymes that catalyze hydrolysis of the C−N bond of urea and guanidine (Arg), the N-terminal peptide bond of proteins, and the P−O bond of alkyl and aryl phosphate monoesters, pyrophosphate, phosphate diesters including DNA and RNA, and phosphate triesters.

This review will provide a current summary of the properties of binuclear metallohydrolases and present some comparisons among members of this emerging class of enzymes,[6] which includes both metalloenzymes that co-purify with their essential metal ions and so-called metal-activated enzymes that need to be reconstituted with metal ions to restore activity after purification. For completeness, metallohydrolases that include a third (and fourth) metal ion, in addition to a well-defined binuclear metal ion site, are included.[7,8] Although the treatment of this class of enzymes is uneven, with considerably more detail for urease and purple acid phosphatases, each case generally includes (a) an overview of the biochemical reaction and the properties of the protein, (b) evidence for the binuclear metal ion site, (c) structural and chemical properties of the native and inhibitor-bound active site and variant enzymes obtained from site-specific mutagenesis, (d) a correlation with enzyme kinetic data, and (e) current model(s) for the enzymatic reaction mechanism, focusing on the role of the metal ions.

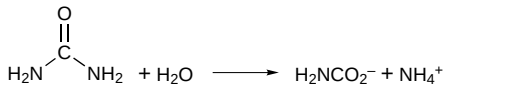
Certain assumptions and recurring concepts need to be mentioned initially. For peptidases, it is widely accepted that hydrolysis involves an associative mechanism with nucleophile (solvent or protein residue) attack on the carbonyl carbon and an intermediate with sp^3 carbon hybridization, which will be referred to as the tetrahedral intermediate. For phosphatases, it is generally accepted that enzyme-catalyzed hydrolysis also involves an associative mechanism with nucleophile attack on the phosphorus opposite the leaving group (so-called in-line attack), resulting in a trigonal-bipyramidal phosphorus intermediate with the entering and leaving groups in the axial positions and leading to inversion of the phosphorus stereochemistry.[9] Coordination of water to metal ions lowers its pK_a significantly, depending on the metal ion, its oxidation state, other ligands, and the local dielectric. Thus, depending on the pH, a di- or tripositive metal ion may have water, hydroxide or even oxide, in the case of bridging coordination, as a ligand. Since the protonation state of a metal-bound water may not be known, it will be identified generally as water (hydroxide). Since similar uncertainty pertains to the protonation state of metal-bound phosphate and the identity of the predominant phosphate species in solution depends on pH and solvent properties, distinction between $H_2PO_4^{1-}$, HPO_4^{2-}, and PO_4^{3-} generally will not be made. Finally, magnetic interaction between open-shell transition metal ions is quantified by the sign and magnitude of the isotropic exchange coupling, $2J$, based on the spin Hamiltonian, $H = -2JS_1S_2$.

The author has attempted to include all hydrolases that have a confirmed binuclear metal ion site and to provide at least a basic description of pertinent properties of each known example. The extent of coverage, however, generally reflects the interests and efforts of bioinorganic chemists. Recent references through mid-1996 and key earlier references are included, but other work on these enzymes can be found as citations in these references. An apology is extended to those whose contributions to our understanding of one or more of these enzymes is not mentioned or referenced explicitly. Finally, the rapidly growing area of structural and mechanistic models for binuclear metallohydrolases has not been included in this review.

Amidohydrolases, Amidinohydrolases, and Peptide Hydrolases

Urease (Urea Amidohydrolase; EC 3.5.1.5)

Certain plants, fungi, and bacteria have a metabolic requirement for the hydrolysis of urea and have an enzyme, urease,[10−13] that hydrolyzes urea to ammonium carbamate (eq 3) with a $\sim 10^{14}$ rate



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enhancement over the uncatalyzed decomposition of urea at pH 7 and 25 °C. This enzyme has two important historical roles. In 1925, urease from jack bean (*Canavalia ensiformis)* became the first enzyme to be crystallized and was shown to retain catalytic activity in the crystalline state.[14] Sumner and others used this result to argue successfully that enzymes were in fact proteins. Some 50 years later, urease from jack bean also became the first enzyme shown to contain and require nickel.[15] Zerner and coworkers also determined the catalytic equivalent weight and showed that the protein contained two nickel ions per protein subunit.[16] More recently, studies of the magnetic and spectroscopic properties of the urease Ni(II) ions demonstrated that they are in close proximity in the native and, particularly, in the thiolate-inhibited enzyme,[17−20] thus establishing urease as a binuclear metallohydrolase. The recent X-ray crystal structure of urease from *Klebsiella aerogenes*[21] has now provided a detailed structure of this binuclear Ni(II) site.

Although the biochemical properties (protein structure, enzyme kinetics, etc.) of jack bean urease, which

consists of a homohexamer of 90.4 kDa[22] subunits, were investigated initially by Sumner,[23] and later by Kistiakowsky[24−27] and others, Zerner and co-workers studied the enzyme extensively subsequent to their discovery that it contained nickel. This included identification of other substrates and inhibitors,[28−31] residues involved in catalysis,[32] and spectral properties of the Ni(II) ions.[33,34] More recently, the biochemical properties of the urease from *K. aerogenes* have been studied by Hausinger and co-workers.[35−39] Although this bacterial urease has a different protein architecture, consisting of three different subunits (60.3 kDa α, 11.7 kDa β, 11.1 kDa γ) in an $(\alpha\beta\gamma)_3$ structure with each α subunit containing two nickel ions, it has >50% sequence homology with jack bean urease.[40]

The properties of the nickel ions in native and inhibited forms of urease have been studied with a number of physical and spectroscopic methods. Although it is difficult to obtain visible absorption spectra of native jack bean urease because of light scattering by the large hexameric protein, ligand field bands similar to those of high-spin 6- or 5-coordinate Ni(II) are observed.[17,34] Nickel EXAFS measurements suggested five or six nitrogen or oxygen donor ligands and the absence of sulfur donor ligands (Cys or Met).[18,41]

The urease Ni(II) ions are not EPR-detectable, so magnetic measurements have been used to characterize their ground-state properties. Magnetic susceptibility data on native jack bean urease showed initially that both Ni(II) ions are paramagnetic with an average magnetic moment of $3.0 \pm 0.1\ \mu_B$. Fitting of these data suggested that there was a weak antiferromagnetic exchange coupling ($2J = -13\ cm^{-1}$) between the Ni(II) ions but also a heterogeneity of the Ni(II) magnetic properties, with ~20% of the Ni(II) lacking exchange interaction in the native enzyme.[17] The evidence for magnetic coupling in the native enzyme was disputed in a saturation magnetization study of *K. aerogenes* and jack bean urease, which reported best-fit parameters that did not include exchange interaction but that did require a percentage of low-spin Ni(II) that varied with pH.[42] However, a recent saturation magentization study of native jack bean urease, which includes correlations with spectroscopic data on the enzyme, has confirmed that best fits of the data require antiferromagnetic exchange coupling and a pH-dependent percentage of active sites with no magnetic interaction between the Ni(II) ions.[43]

Thiolate-to-Ni(II) charge transfer (CT) transitions are observed in the near-UV absorption,[34] CD,[44,45] and MCD[19] spectra of urease upon addition of thiolate competitive inhibitors, indicating reversible thiolate coordination to the Ni(II) ions. Thiolate binding also causes a dramatic reduction of the urease paramagnetism but does not significantly alter the Ni(II) ligand field spectrum[17] or the shape of the nickel X-ray absorption edge,[18] both of which are diagnostic for changes in nickel oxidation state, spin state, and coordination.[46] Thus, good evidence was obtained for increased antiferromagnetic exchange interaction between the Ni(II) ions upon thiolate inhibitor binding, further supporting a binuclear Ni(II) site and suggesting a bridging coordination of the thiolate.

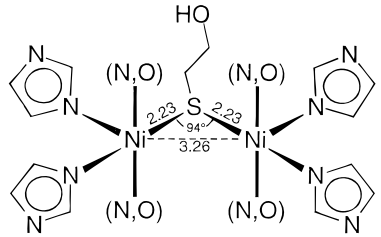


Figure 1. Proposed structure of the binuclear Ni(II) site of urease inhibited by 2-mercaptoethanol. (Reprinted with permission from ref 20. Copyright 1994 American Chemical Society.)

This was confirmed by variable-temperature MCD measurements, which could be fit with an antiferromagnetic exchange interaction of $2J = -80 \pm 10\ cm^{-1}$.[19] Finally, EXAFS data on the thiolate-inhibited form of the enzyme provided metric data on the Ni−Ni separation (3.26 Å), bridging thiolate coordination (Ni−S−Ni angle = 94°), and number of His ligands per Ni(II), as summarized in Figure 1.[20]

Two caveats about the spectroscopic and magnetic characterization of urease need to be mentioned. First, because of only recent success in preparing urease derivatives with a single Ni(II)[47] or with different metal ions,[48,49] only the average of the magnetic and EXAFS properties and the sum of the spectroscopic properties of the urease nickel ions have been reported. Even in the case of the *K. aerogenes* Hα134A variant urease that contains a single Ni(II) ion, its coordination is significantly perturbed from that in the native enzyme.[47] Second, enough physical and spectroscopic data have now been collected on both jack bean and *K. aerogenes* urease to suggest that there are no major differences between the properties of the plant and bacterial urease Ni(II) ions and thus their active sites. This is supported further by the generally high sequence homology of urease from numerous species,[13] and specifically by the homology of the Ni(II) ligand residues.[48]

Although urease was the first enzyme to be crystallized, it was 70 years later, and 20 years after the discovery of its nickel ions, that an X-ray crystal structure of the enzyme was reported. The structure of the *K. aerogenes* urease at 2.2 Å resolution by Karplus and co-workers provided the long-awaited three-dimensional picture of the binuclear Ni(II) active site (Figure 2) and its protein environment.[21] Many features of the urease Ni(II) coordination that had been proposed from spectroscopic and magnetic data were confirmed by the X-ray structure, including two His ligands for each Ni(II) ion and a bridging ligand that can provide a pathway for magnetic interaction. However, the identity of this endogenous bridging ligand, the carbamate derivative of Lysα217, was surprising, although there had been a suggestion of this from the CO_2-dependence for in vitro assembly of *K. aerogenes* urease from its gene products,[50] and there is a precedent in the case of Mg(II)-dependent carbamylation of Lys201 in ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco).[51] The two Ni(II) ions are 3.5 Å apart in native urease. One is 5-coordinate with Nϵ of Hisα134, Nϵ of Hisα136, Aspα360, the bridging carbamylated Lysα217, and a water (hydroxide) as ligands in a geometry distorted between trigonal bipyramidal and square pyramidal, while the other is 3-coordinate, with Nδ

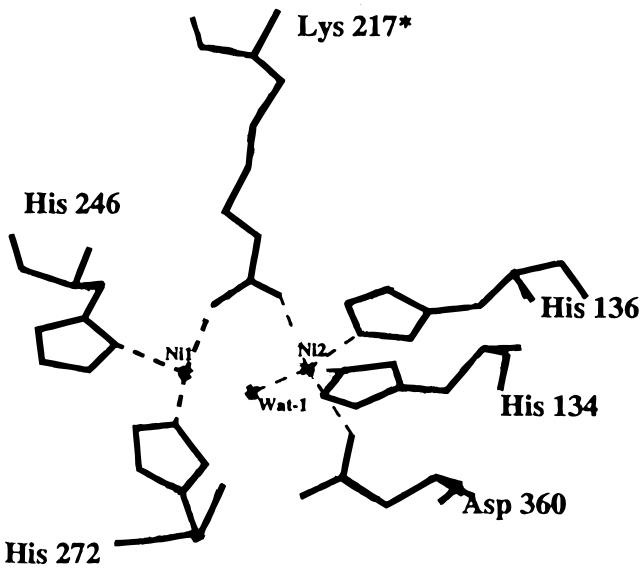


Figure 2. Crystallographically determined structure of the *K. aerogenes* urease binuclear Ni(II) site. (Adapted with permission from ref 21. Copyright 1995 American Association for the Advancement of Science.)

of Hisα246, Nϵ of Hisα272, and the bridging carbamylated Lysα217 as ligands. Partial crystallographic occupancy of the water (hydroxide) ligand of the 5-coordinate Ni(II) in a bridging position was noted, suggesting a heterogeneity of the Ni(II) coordination that may relate to the reported heterogenetity of the urease magnetic properties.[17] In addition, reversible loss of activity of the crystallized enzyme is a concern but may not relate to perturbations at the binuclear Ni(II) site.[52] Clearly an unexpected feature of this structure is the low coordination number of one of the Ni(II) ions, which appears to be either $\sim T_d$ or trigonal pyramidal, depending on whether the water (hydroxide) is bridging or not.

A mechanism for the hydrolysis of urea at a binuclear Ni(II) site was proposed by Zerner[30] over 15 years ago and consists of substrate (urea and a few substituted ureas and $RC(O)NH_2$ compounds) binding to one Ni(II), followed by nucleophilic attack by hydroxide that is bound to the other Ni(II) (Figure 3). General acid−base contributions to the mechanism by other active site residues are also included. This mechanism can be correlated with the pH dependence of V_{max} (or k_{cat}) and V_{max}/K_M, which indicate kinetically important pK_a's of the E·S complex and the native enzyme, respectively. Both jack bean[30] and *K. aerogenes*[35] urease show maximal activity at pH 7.5−8. The k_{cat} vs pH data for jack bean urease, however, do not show simple bell-shaped behavior, but pK_a's of 3, 6.25, and 9 can be obtained from a fit of the data.[30] The k_{cat}/K_M vs pH data, however, show only a lower pK_a of 4.3.[43] Typical bell-shaped behavior is reported for the V_{max}/K_M vs pH data of *K. aerogenes* urease, from which pK_a's of 6.55 and 8.85 are obtained.[35] The upper kinetic pK_a (>8.5) has been associated with a Cys, which originally was thought to serve as a general acid.[30] However, site-specific mutagenesis has been used to show that the kinetically important Cysα319 of *K. aerogenes* is not essential for urea hydrolysis.[39] This Cys is located near the binuclear Ni(II) site, suggesting that chemical modification or substitution

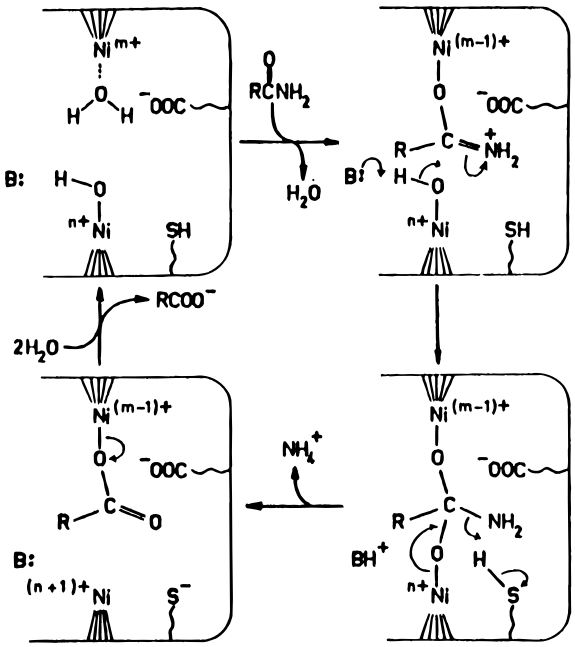


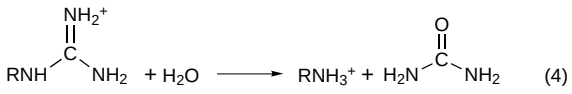
Figure 3. Proposed mechanism for the hydrolysis of urea at the urease binuclear Ni(II) site. (Reprinted with permission from ref 30. Copyright 1980 National Research Council of Canada.)

of this residue results in altered steric interactions at the active site.[21] The kinetic pK_a just below 7 has been associated with a His participating as a general base, and this is consistent with kinetic data for the *K. aerogenes* mutant with Ala replacing Hisα320[53] and its position near the Ni(II) ions.[21,54] Missing from the interpretation of these data, however, is the pK_a of a Ni(II)-bound water ligand, which should be found in the pH range of the enzyme kinetic data, particularly if bridging water (hydroxide) is involved. The pH dependence of the magnetic properties[42,43] and the ligand field spectra[43] of urease suggest a change in the Ni(II) coordination as the pH is lowered, which may be due to protonation of a solvent ligand and associated with the decrease in activity at low pH. The pH dependence of the spectroscopic and kinetic properties of the binuclear Mn(II) derivative of *K. aerogenes* urease, which has 2% of the activity of the native enzyme,[49] may help address this question.

The structural and kinetic properties of *K. aerogenes* urease strongly suggest different mechanistic roles for the two metal ions. The 3- or 4-coordinate Ni(II) is likely involved in binding urea, and this is supported by the dramatic increase in K_M for the mutant with Ala replacing Hisα219, which is located near this metal ion and appears to play a role in substrate binding. The 5-coordinate Ni(II) likely provides an activated solvent nucleophile, and this is supported by the dramatic decrease in k_{cat} for the mutant with Ala replacing Hisα320, which is involved indirectly in hydrogen bonding to the solvent ligand of this Ni(II) ion. However, while the structure of the urease binuclear Ni(II) site supports this model, which retains much of the original Zerner mechanism except for the identity of a general acid, it can also accommodate other mechanisms (e.g., substrate binding to both metal ions) for the hydrolysis of urea at a binuclear Ni(II) site.

Arginase (L-Arginine Amidinohydrolase; EC 3.5.3.1)

The terminal step in the urea cycle of ureolytic organisms is the hydrolysis of the guanidine moiety of L-arginine to give urea and L-ornithine. This reaction (eq 4) is catalyzed by the enzyme arginase,



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which is abundant in the liver[55] but is also found in other tissue where it presumably provides L-ornithine for L-proline and polyamine biosynthesis. Several arginases are isolated with manganese bound to the protein, and enzyme activity generally increases upon incubation with Mn(II)[56] or with a variety of other dipositive metal ions (Co(II), Ni(II), Fe(II), VO^{+2}, Cd-(II)),[57] suggesting that some of the intrinsic Mn(II) is not bound tightly. Yeast arginase has a tightly bound Zn(II), for which a structural role has been proposed.[58] The well-studied arginase from rat liver consists of a homotrimer of 35 kDa subunits,[59] is typically isolated with three tightly bound Mn per trimer, and attains maximal activity with a stoichiometry of six Mn per trimer.[60]

Evidence that arginase is a binuclear metallohydrolase has been provided by EPR spectroscopy. Although the low-temperature EPR spectrum of rat liver arginase is complex (Figure 4), the observation of ^{55}Mn ($I = {}^5/_2$) hyperfine splitting of 45 G,[60] which is approximately half of that expected for magnetically isolated Mn(II), is diagnostic for exchange-coupled Mn(II) ions.[61] This predicts an extensive spin manifold consisting of $S = 0$, 1, 2, 3, 4, and 5 spin states, which is consistent with the complexity of the observed EPR spectrum. Recently the arginase EPR spectral properties were studied in more detail, and the temperature dependence of the intensity of the EPR spectrum of borate-inhibited arginase was correlated with Boltzmann population of the quintet ($S = 2$) state of an antiferromagnetically coupled Mn-(II)−Mn(II) dimer with an isotropic exchange splitting of $2J = -4$ cm^{-1}.[62]

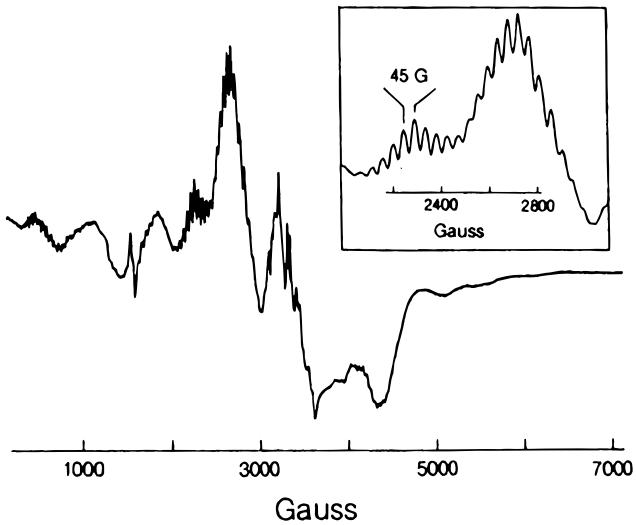


Figure 4. X-band EPR spectrum of Mn(II)-activated arginase at 20 K. (Reprinted with permission from ref 60. Copyright 1992 American Chemical Society.)

No detailed structural information about the arginase protein or its binuclear Mn(II) site is currently available, but site-specific mutagenesis has been used to show that His101 and His126 are required for tight binding of the Mn(II) ions[63] and therefore appear to be ligands for the required metal ions. Histidine-141 reacts with diethyl pyrocarbonate, resulting in loss of activity, but the H141N variant arginase obtained from site-specific mutagenesis has shown that this residue is not essential for catalytic activity; it is likely to be located near the active site and play an ancillary mechanistic role.

The mechanism of arginine hydrolysis by the binuclear Mn(II) site in arginase is still poorly understood, although a recent study of alternative substrates and inhibitors of the enzyme, focusing on the most potent inhibitor borate ($K_i = 1$ mM), has provided some new insight.[64] While the borate anion might mimic a tetrahedral transition state, its non-competitive inhibition and evidence for a ternary L-ornithine−borate−enzyme complex suggests a different mode of interaction with the enzyme. In conjunction with EPR results, which show that borate perturbs the zero-field splitting and thus the coordination of the Mn(II) ion(s),[62] it has been suggested that inhibition results from a reaction with or displacement of a Mn(II)-bound hydroxide.[64] Since Mn(II)-bound water has p$K_a \sim 10.6$,[65] a bridging water (hydroxide) coordination, or the participation of a protein base (possibly His141), may be needed to activate a water nucleophile for hydrolysis of the guanidine C−N bond of L-arginine in the arginase active site. In contrast, creatine amidinohydrolase uses an entirely different mechanism employing general acid−base catalysis to hydrolyze the substituted guanidine of creatine.[66]

Aminopeptidases (α-Aminoacyl-Peptide Hydrolases)

N-Terminal exopeptidases (aminopeptidases)[67] are widely distributed in both eucaryotes and procaryotes, where they play roles in protein degradation and amino acid metabolism, as well as various regulatory roles. In particular, methionine aminopeptidase, which removes the N-terminal Met of nacent polypeptides, plays a key role in protein modification for transport and degradation. Some of the more extensively studied aminopeptidases are the cytosolic leucine aminopeptidases (EC 3.4.11.1) from bovine lens (blLAP)[68] and porcine kidney (pkLAP),[69] the aminopeptidase (EC 3.4.11.10) from the marine bacterium *Aeromonas proteolytica*,[70] and methionine aminopeptidase (EC 3.4.11.18) from *Escherichia coli*.[71]

Many, but not all, aminopeptidases require metal ions. Each 54 kDa subunit of the homohexameric blLAP binds two Zn(II) ions, which are required for activity and which have different kinetics of exchange,[72] suggesting different coordination. The very similar pkLAP and the monomeric 32 kDa *A. proteolytica* aminopeptidase also bind two Zn(II) ions per subunit, but only one is required for catalytic activity. Extensive metal substitution studies suggested that the less easily exchanged Zn(II) (only Co(II) will substitute) plays a catalytic role, while the more easily substituted Zn(II) plays a regulatory or activating role, with different metal ions (Mn(II), Mg-

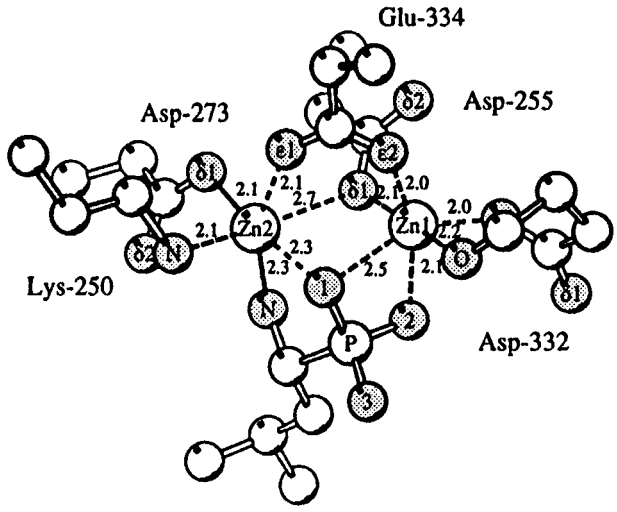


Figure 5. Crystallographically determined structure of the L-leucinephosphonate complex with the bovine lens leucine aminopeptidase binuclear Zn(II) site. (Reprinted with permission from ref 82. Copyright 1995 American Chemical Society.)

(II), Co(II)) providing either depressed or enhanced activity relative to Zn(II). Although the native metal ions of methionine aminopeptidases are not known, the highest specific activity is found with Co(II).[73]

For blLAP[74] and *A. proteolytica* aminopeptidase,[75] the identity of each metal ion affects both K_M and k_{cat}, suggesting that N-terminal exopeptidase activity occurs at an active site containing two Zn(II) ions. This was confirmed[76] and structurally elucidated[77] by X-ray crystallography of native blLAP, which showed that one Zn(II) had both a carboxyl oxygen and the carbonyl oxygen of Asp332, Asp255, and Glu334 as ligands; the latter two carboxylates bridge the 2.9 Å separation to the other Zn(II) ion, which also has Asp273 and the primary amine of Lys250 as ligands. Subsequently the crystal structure of the Zn(II)Mg(II) derivative indicated that the former Zn(II) is more readily exchangable,[78] and a low-temperature 1.6 Å resolution structure of native blLAP has now identified a bridging solvent (hydroxide) ligand, making each Zn(II) pentacoordinate.[79] A recent 1.8 Å resolution X-ray crystal structure of the *A. proteolytica* aminopeptidase shows a somewhat larger 3.5 Å separation between the Zn(II) ions, each of which has a His ligand and a bridging solvent (hydroxide), as well as a bridging Asp.[80] Finally, the recent 2.4 Å resolution X-ray crystal structure of the monomeric 29.3 kDa methionine aminopeptidase from *E. coli* shows a 2.9 Å separation between two 5-coordinate Co(II) ions with two bridging carboxylates, three other protein ligands, and apparently two solvent ligands.[73]

A number of potent aminopeptidase inhibitors with structures that mimic the expected tetrahedral intermediate of peptide hydrolysis are known, including bestatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine), amastatin ((2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L-aspartate), and L-leucinephosphonate (LeuP). Recent X-ray crystal structures of blLAP with these three inhibitors and with L-leucinal have provided important insight about substrate binding and activation by the two metal ions and other active site residues. First, binding of all three inhibitors leads to an increase in the coordination number of each Zn(II) to 6 and to an increase in the Zn−Zn separation to 3.1, 3.3, 3.4, and 3.2 Å for bestatin,[77] amastatin,[81] LeuP,[82] and leucinal,[79] respectively. Figure 5 shows the structure of the LeuP-inhibited blLAP active site, where the inhibitor provides three ligands to the Zn(II) ions, including a bridging phosphate oxygen.

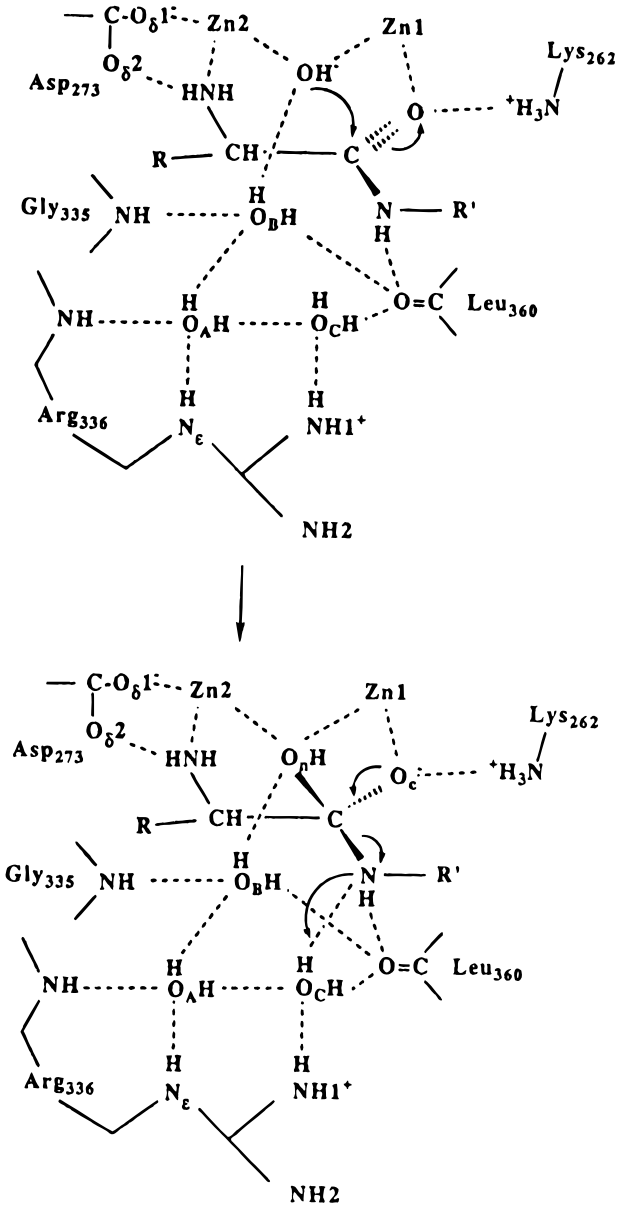


Figure 6. Proposed mechanism for the hydrolysis of an N-terminal amino acid at the bovine lens leucine aminopeptidase binuclear Zn(II) site. (Reprinted with permission from ref 82. Copyright 1995 American Chemical Society.)

A mechanism for peptide hydrolysis by blLAP has been proposed, based on (a) interaction of the Zn(II) ions and other residues (Lys262, Arg 336, Leu360) with inhibitor heteroatoms that are equivalent to the two oxygens and nitrogen of the expected tetrahedral intermediate, (b) identification of a metal-bound solvent nucleophile (hydroxide) bridging the two Zn(II) ions, and (c) the structure of the blLAP complex with the hydrate gem-diol of leucinal, where one hydroxyl bridges the two Zn(II) ions and the other binds to one Zn(II).[79] This model is shown in Figure 6 and suggests the following: (a) coordination of the terminal amine to one Zn(II) and Asp273, (b) coor-

dination of the carbonyl oxygen to one of the Zn(II) ions and Lys262, (c) nucleophilic attack on the sissile carbon by a hydroxide that is bridging the two Zn(II) ions, and (d) stabilization of the tetrahedral intermediate by multiple coordination to the two Zn(II) ions, including bridging coordination that is seen in all four crystallographically characterized complexes. Both Zn(II) ions of blLAP appear to interact with substrate and play a role in solvent activation, although a bridging hydroxide is not as nucleophilic as a terminal hydroxide.

A bridging hydroxide is common to the binuclear Zn(II) sites of blLAP and *A. proteolytica* aminopeptidase. However, *A. proteolytica* aminopeptidase lacks certain residues believed to be mechanistically important for blLAP and has catalytic activity with a single Zn(II), suggesting different and possibly flexible contributions of the metal ion(s) to peptide bond hydrolysis. Finally, additional spectroscopic, kinetic, and structural data on methionine aminopeptidases are required to determine the mechanistic role(s) of the two Co(II) ions in peptide bond hydrolysis.

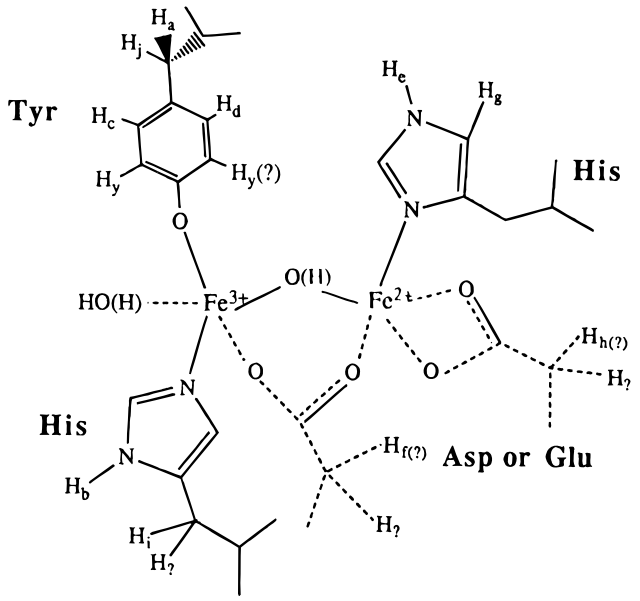


Figure 7. Proposed structure of the binuclear iron site of mammalian purple acid phosphatases. (Reprinted with permission from ref 106. Copyright 1992 American Chemical Society.)

Phosphohydrolases

Purple Acid Phosphatases (Orthophosphoric-monoester Phosphohydrolase (Acid Optimum); EC 3.1.3.2)

Enzymes that catalyze phosphate ester hydrolysis under acidic conditions (optimum pH of 4.9−6.0) resist inhibition by tartrate, contain two irons (or one iron and another dipositive metal ion), and exhibit a characteristic purple color ($\lambda_{max} \sim 550$ nm) in their inactive oxidized form and a pink color ($\lambda_{max} \sim 510$ nm) in their active reduced form are collectively known as purple acid phosphatases (PAP).[83−86] These homologous ~35 kDa proteins were originally isolated from porcine uterine fluid[87] and bovine spleen[88] but have now been found in a variety of animal tissue. In addition, similar acid phosphatases reported to contain two irons, one iron and one zinc, or manganese have been purified from kidney bean,[89] sweet potato,[90,91] and other plants, and similar enzymes are also found in certain bacteria.[83,86] Although the physiological role of these glycoproteins is not known, their localization in lysosomes of osteoclasts and macrophages[92] and their broad substrate specificity support an intracellular monophosphatase function in animals.[84,86] However, the high content of the PAP known as uteroferrin in porcine uterine fluid and its ability to transfer iron to transferrin[93] suggest a role in iron metabolism. Finally, there is some evidence for reactions of these enzymes with oxygen that may have some physiological relevance.[92,94]

Evidence that PAP contains a binuclear metal ion site was obtained initially by magnetic susceptibility and EPR measurements on the bovine spleen enzyme, which showed antiferromagnetic spin coupling in both the oxidized Fe(III)Fe(III) ($S = 0$ ground state) and reduced Fe(III)Fe(II) ($S = {}^1/_2$ ground state) forms.[95] In particular, a rhombic $g \sim 1.7$ EPR signal for the reduced protein is characteristic of antiferromagnetically coupled mixed-valent Fe(III)Fe(II) sites in proteins. Antiferromagnetic coupling of the two irons in reduced and oxidized uteroferrin was subsequently shown by magnetic, EPR, and Mössbauer measurements.[96,97]

Both reduced and oxidized PAP have been studied by numerous physical and spectroscopic methods to characterize the ligands and coordination of the two irons, the Fe−Fe separation and electronic interaction, and the similarities between enzymes from different sources. This has led to a model for the binuclear iron site (Figure 7), the assignment of the salient features of which are summarized here. Since an obvious property of these proteins is their characteristic color, comparison to other iron proteins and model complexes suggested that the intense visible absorption band was a phenolate-to-iron CT transition. This was confirmed by the assignment of phenolate vibrational modes in the resonance Raman spectrum,[98] and comparison of resonance Raman data on the reduced and oxidized enzyme showed that Tyr was a ligand of the redox-inactive Fe(III).[99] Mössbauer measurements identified two distinct low-symmetry 6-coordinate high-spin Fe(III) ions in the oxidized form and a mixed-valence site, consisting of high-spin Fe(III) and high-spin Fe(II), upon reduction to the active form.[97,99] The electronic structure of the Fe(III)Fe(II) PAP site has now been characterized in considerable detail in a recent Mössbauer study.[100] Magnetic susceptibility, EPR, and NMR measurements have indicated a strong antiferromagnetic interaction in the oxidized enzyme ($-2J > 80$ cm^{-1})[95,96,99,101,102] but a weaker antiferromagnetic coupling in the reduced enzyme ($2J = -11$ to -22 cm^{-1}).[102−104] ^{1}H NMR data have been particularly informative about the ligands, initially indicating His coordination and identifying Tyr as a Fe(III) ligand in the reduced enzyme.[102] NMR measurements later provided evidence for Nϵ coordination of His to the Fe(III) and Nδ coordination of His

to the Fe(II) in the reduced enzyme and for carboxylate coordination to the metal ions.[105−107] Advanced EPR techniques, including ENDOR,[108] ESEEM,[108,109] and LEFE,[109] have confirmed His ligation and a trapped valence description of the Fe(III)Fe(II) site in the reduced enzyme and have demonstrated solvent accessibility and probable coordination to the metal ions. X-ray absorption and EXAFS measurements[110−112] ruled out sulfur ligation, indicated metal−ligand bond lengths, provided metal−metal distances in the reduced (3.5 Å)[111] and oxidized (3.0 Å;[110] 3.2−3.3 Å[111]) forms, and suggested bridging ligand coordination. With regard to the latter point, the strong antiferromagnetic coupling reported initially for oxidized PAP and comparisons[85,86] to the binuclear iron sites in hemerythrin, methane monooxygenase, and ribonucleotide reductase suggested an oxo bridging ligand in the Fe(III)Fe(III) site. However, recent EXAFS data,[111] the absence of a symmetric ν(Fe−O−Fe) band at ∼500 cm^{-1} in the resonance Raman spectrum,[99] and a comparison of the reduction potentials of binuclear ferric metalloproteins[113] all suggest the absence of a Fe−O−Fe structural unit in oxidized PAP.

The high-spin Fe(II) of reduced PAP is labile and can be substituted by other dipositive metal ions, in some cases retaining substantial catalytic activity;[114,115] in particular, the Fe(III)Zn(II) form has been studied to unambiguously characterize the ferric iron coordination and identify contributions from the binuclear iron exchange interaction.[116] This FeZn form is similar to the native enzyme from kidney bean (kbPAP),[89] which has a high-spin Fe(III) but does not have an inactive oxidized form. In this PAP, the Zn(II) is labile and can be substituted by Fe(II) to form a catalytically active enzyme with a Fe(III)-Fe(II) active site.[117] Comparisons have now shown that spectral and kinetic properties of the FeZn form of the binuclear iron enzymes are similar to those of native kbPAP,[95,118] and the properties of the FeFe form of the kidney bean enzyme are similar to those of the native binuclear iron PAP,[119,120] thus indicating similar active sites. Although there is low overall sequence homology between mammalian and plant PAP, secondary structure predictions suggest a similar overall protein topology and a homology of the metal ligating residues has been noted.[121]

Purple acid phosphatases exhibit product inhibition by phosphate and are also inhibited by other oxyanions, including arsenate, vanadate, molybdate, and tungstate. Both phosphate and arsenate show mixed but predominantly competitive inhibition (K_i = 0.2−14 mM), while the larger metal oxyanions show stronger but noncompetitive inhibition (K_i = 2−10 μM).[118,122] An initially confusing factor, however, was an increased susceptibility of the Fe(III)Fe(II) site to oxidation (lower reduction potential) upon binding phosphate.[123,124] This was further complicated by the absence of an EPR signal for the phosphate complex of the Fe(III)Fe(II) enzyme, although Mössbauer data indicated that a high-spin Fe(II) was still present[125] and an EPR-detectable Kramers doublet ground state was expected; later it was shown that the phosphate-inhibited Fe(III)Fe(II) site had a weaker antiferromagnetic coupling ($2J$ = −6 cm^{-1}) and a broad

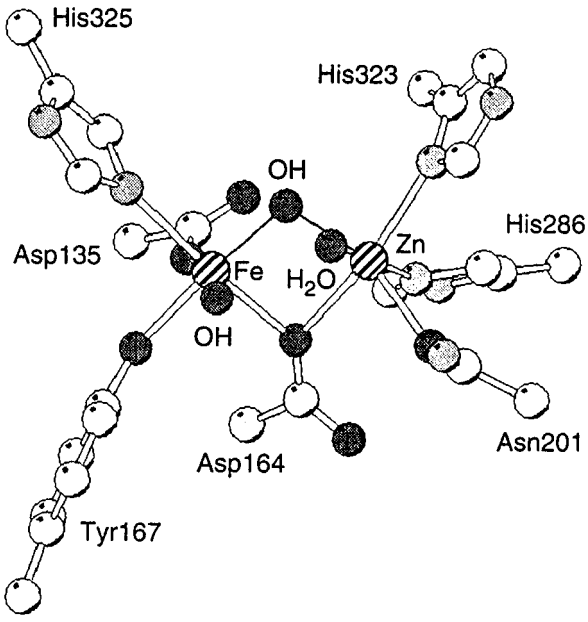


Figure 8. Crystallographically determined structure of the kidney bean purple acid phosphatase Fe(III)Zn(II) site. (Reprinted with permission from ref 128. Copyright 1995 American Association for the Advancement of Science.)

previously unidentified EPR signal.[103] EPR studies have provided evidence for direct binding of phosphate[118] and molybdate[108] to the metal ions, and spectral evidence suggested different binding modes for competitive and noncompetitive inhibitors.[126] EPR evidence has now been provided for a ternary enzyme−phosphate−molybdate complex.[127] Although Mössbauer data suggested that there was a direct interaction of phosphate with only the Fe(III) in the reduced enzyme,[125] both magnetic[103] and NMR[105] data indicated that phosphate perturbs the Fe(III)−Fe(II) magnetic coupling and may be affecting the bridging ligand(s). Electrochemical studies have quantified inhibitor effects on the reduction potential of the PAP irons and provided evidence that proton transfer accompanies oxidation.[113] Finally, EXAFS data and analysis indicate that phosphate and arsenate bridge the two Fe(III) ions in oxidized PAP,[111] which is consistent with their observed tight binding.[123,124]

Recently, the X-ray crystal structure of kidney bean PAP, which has a cystine-bridged homodimeric structure and one FeZn site in each 55.5 kDa subunit, has been reported, initially to 2.9 Å resolution[128] but now to 2.65 Å resolution.[129] The FeZn site (Figure 8) lies at the bottom of a broad pocket, the general accessibility of which correlates with the broad substrate specificity of the enzyme. Each metal ion has four protein ligands, Tyr167, Nϵ of His325, Asp135, and Asp164 for the Fe(III), and Nϵ of His286, Nδ of His323, the amide oxygen of Asn201, and Asp164 for the Zn(II). Aspartate-164 provides a monoatomic carboxylate bridge between the Fe(III) and Zn(II), which are separated by 3.26 Å. While the protein tertiary structure and the protein ligands of the metal ions are easily identified at this resolution, solvent and other small ligands could not be identified, even at 2.65 Å resolution. Based on the "geometry of the coordination sphere around the metal ions",[128] three additional ligands were added to the

active site structure, a bridging hydroxide, a terminal water on the Zn(II), and a terminal hydroxide on the Fe(III). Thus, this model suggests that both metal ions are 6-coordinate, each with an exchangeable solvent ligand. A number of nearby polar residues (His202, His295, His296, Asp169, Asp369, Glu299) may be important in the enzyme hydrolysis mechanism.

Crystal structures of the phosphate and tungstate complexes of kbPAP have now been reported.[129] Both inhibitors bind in a very similar bridging geometry, displacing the putative terminal solvent ligands on each metal ion and interacting with nearby His202 and His296, but apparently not displacing the putative bridging hydroxide or significantly affecting the Fe−Zn separation. This bridging coordination appears to be associated with noncompetitive inhibition found exclusively with tungstate and raises structural and chemical questions about the difference between competitive and noncompetitive inhibition of PAP.

It has now been established that PAP hydrolysis of phosphate esters occurs with inversion of stereochemistry at the phosphorus.[130] This makes a phosphorylated enzyme intermediate highly unlikely,[131] suggests direct transfer of the phosphoryl group to water, and supports a mechanism involving attack of a metal-bound hydroxide ligand on the phosphate ester and a trigonal-bipyramidal phosphorus intermediate. A mechanistic hypothesis has been proposed[132] that is based on three pH-dependent properties of the reduced active enzyme: (a) evidence in the $g \sim 1.7$ EPR spectrum for low- and high-pH forms of the mixed-valent site;[99] (b) higher affinity for phosphate at lower pH;[125] (c) a shift of the Tyr → Fe(III) CT band of the phosphate-bound enzyme to longer wavelength at lower pH.[125,132] All of these phenomena are associated with an apparent pK_a in the range 4.4−4.9, and this correlates well with the lower pK_a obtained from enzyme kinetic data,[132] which show a typical bell-shaped dependence on pH with apparent pK_a's of 4.8 and 6.9. The former is assigned as the pK_a of an aquo ligand of the Fe(III), while the latter is attributed to the pK_a of the aryl phosphate substrate, although it could be associated with a mechanistically important His in the active site. This mechanistic model and others[133] include phosphate ester binding to the more labile Fe(II) or Zn(II) and attack by a hydroxo ligand of the Fe(III). At lower pH the hydroxide is protonated and displaced by the phosphate ester, while at higher pH the deprotonated phosphate ester dianion has decreased electrophilicity and is not attacked by the Fe(III)-bound hydroxide. This mechanism is consistent with and has now been correlated to the kbPAP active site structure (Figure 9).[129] Further, it is consistent with the bridging phosphate coordination, either through displacement of a solvent ligand or attack by the Fe(III)-bound hydroxide nucleophile; the latter possibility is supported by kbPAP catalysis of ^{18}O exchange between phosphate and solvent.[131] Finally, stopped-flow measurements of the rate of formation of the phosphate complex (i.e., E·P) upon phosphate binding or phosphate ester hydrolysis are consistent with this model.[134]

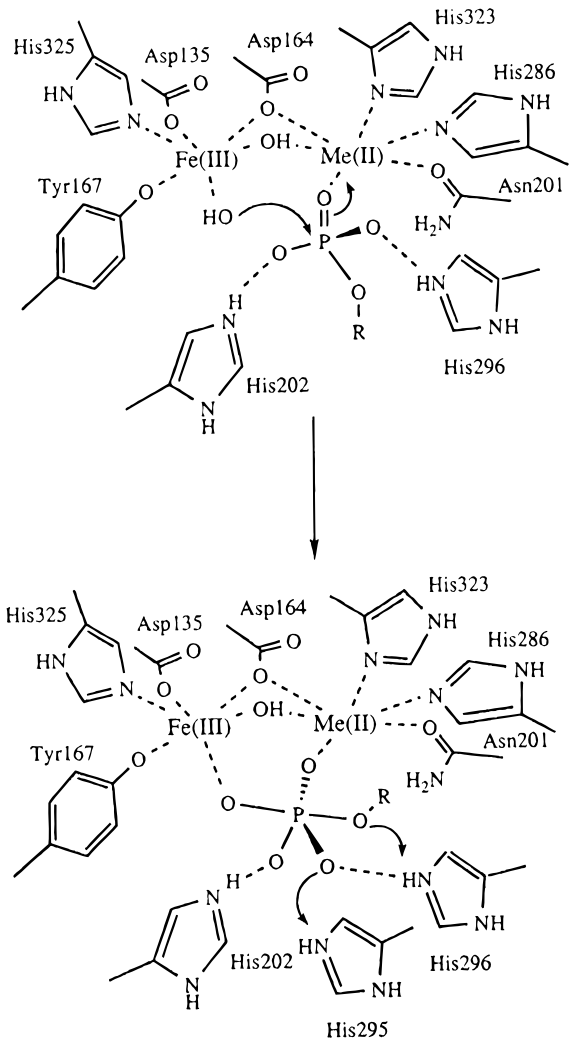


Figure 9. Proposed mechanism for phosphate ester hydrolysis at the kidney bean purple acid phosphatase Fe(III)Zn(II) site. (Reprinted with permission from ref 129. Copyright 1996 Academic Press.)

Alkaline Phosphatase (Orthophosphoric-monoester Phosphohydrolase (Alkaline Optimum); EC 3.1.3.1)

Phosphatases with optimal activity at higher pH (>7.5), low substrate specificity for phosphate monoesters, and a requirement for Zn(II) are found in eucaryotes and procaryotes, although their physiological role remains poorly understood. The best studied of these alkaline phosphatases (AP)[135−137] is that from *E. coli*, which has structural and mechanistic similarity with mammalian AP and consists of a homodimeric protein with two Zn and one Mg in each 47 kDa subunit. Early evidence for a phosphorylated enzyme intermediate led to a mechanism involving nucleophilic attack by Ser102 on the phosphate monoester,[138] and this is consistent with the observed retention of phosphorus stereochemistry,[139] which suggests consecutive nucleophilic attacks by Ser102 and solvent, each occurring with inversion at the phosphorus.

The two Zn(II) ions of alkaline phosphatase are required for phosphatase activity, but the Mg(II) ion plays an ancillary role, enhancing the catalytic activity.[140] Co(II)-substituted AP retains ~30% activity,[141] while Cd(II)[142] and Mn(II)[143] sustain only very low levels of enzymatic activity. However, the phospho-

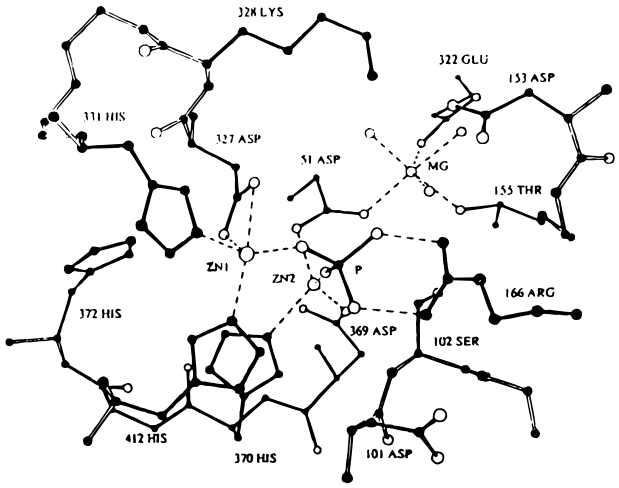


Figure 10. Crystallographically determined structure of the phosphate complex with the *E. coli* alkaline phosphatase $Zn(II)_2Mg(II)$ site. (Reprinted with permission from ref 147. Copyright 1991 Academic Press.)

rylated enzyme intermediate (E−P) can be obtained with all three of these metal-substituted forms, and spectroscopic data indicate an interaction of these metal ions with bound phosphate.[143,144] The first evidence that the metal ions of AP are in close proximity was low-resolution X-ray diffraction data, which showed metal ion separations of 4, 5, and 7 Å.[145] Refinement of this data to 2.8 Å resolution identified the metal ion coordination and ligands, indicating that the two Zn(II) ions are 3.9 Å apart, share no bridging ligand, and are located near the nucleophilic residue Ser102.[146]

A 2.0 Å resolution X-ray crystal structure of the phosphate-bound enzyme (E·P) (Figure 10), a 2.5 Å resolution structure of the phosphorylated Cd(II)-substituted enzyme (E−P) and re-analysis of the 2.8 Å resolution native Zn_2Mg enzyme structure now provide detailed structural information about the AP active site.[147] One zinc (Zn2) is less exposed to solvent and has $\sim T_d$ coordination with His370, Asp51, Asp369, and one phosphate oxygen in E·P; in the native enzyme the alcohol of Ser102 replaces the phosphate oxygen. The other more exposed zinc (Zn1) is 5-coordinate in E·P with His331, His412, both oxygens of Asp327, and another oxygen of the phosphate constituting the first coordination sphere; in the native enzyme there is some indication of a solvent or other small ligand located 2.2 Å from this Zn. In the Cd(II)-substituted E−P structure, there is no major change in the metal ion coordination to the protein, except for the expected increase in metal−ligand bond lengths. The oxygen of Ser102, which forms the phosphate ester linkage, remains bonded to the more deeply buried Cd(II), and one phosphate oxygen remains bound to the more exposed Cd(II), possibly with some weaker bonding interaction with the other Cd(II). In both E·P and E−P, Arg166 is involved in electrostatic stabilization of the phosphate.

These X-ray structures of E−P (Figure 11, middle right) and E·P (Figure 11, middle left) form the basis of a phosphate ester hydrolysis mechanism at the AP active site.[147] Several roles are suggested for the two Zn(II) ions, which are held ~4 Å apart but without any bridging ligand in the native enzyme.

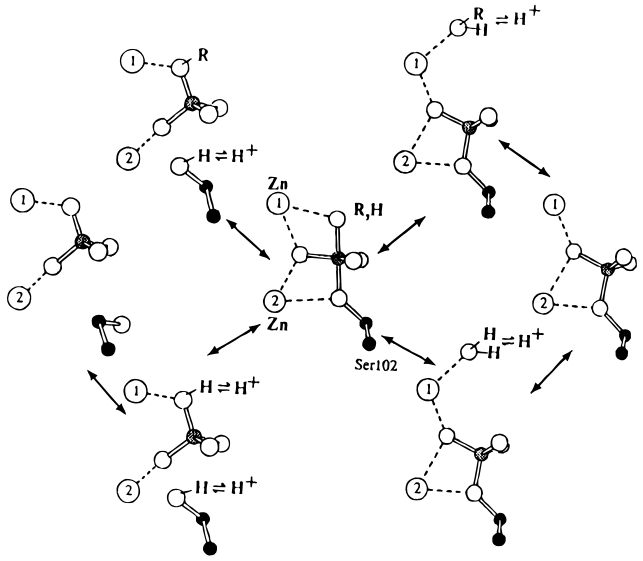


Figure 11. Proposed mechanism for phosphate ester hydrolysis at the *E. coli* alkaline phosphatase binuclear Zn-(II) site. (Reprinted with permission from ref 147. Copyright 1991 Academic Press.)

As indicated in Figure 11, these include the following: (a) binding the phosphate monoester substrate (upper left), (b) stabilizing the first trigonal-bipyramidal phosphorus intermediate formed upon attack by Ser102 (middle), (c) assisting in release of the first product (alcohol) (upper right), (d) binding and lowering the pK_a of a water nucleophile (lower right), (e) stabilizing the second trigonal-bipyramidal phosphorus intermediate formed upon attack by the Zn(II)-coordinated water (hydroxide) (middle), and (f) assisting in release of the second product (phosphate) (lower left). Enzyme kinetic data have shown that the rate-limiting step at higher pH is the release of phosphate, while at lower pH the turnover rate is limited by hydrolysis of the phosphorylated intermediate.[148] This change in the rate-limiting step appears to correlate with a pK_a of 7.4, indicated by the pH dependence of k_{cat}, and may relate to protonation of a Zn(II)-bound hydroxide, which is suggested by electron density near the more accessible Zn(II) in the native enzyme and E−P crystal structures. Finally, site-specific mutagenesis has been used to obtain variant enzymes with nonnucleophilic Leu or Ala replacing Ser102, and these have been shown to retain low levels of activity that may involve direct attack by a metal-bound solvent nucelophile at the binuclear Zn(II) site.[149]

Inositol Monophosphatase (1L-*myo*-Inositol-1-phosphate Phosphohydrolase; EC 3.1.3.25)

Inositol 1-phosphate (I-1-P) is the product of inositol biosynthesis from glucose 6-phosphate, and I-1-P, I-3-P, and I-4-P are produced in signal transduction pathways involving phosphatidylinositol.[150] The enzyme inositol monophosphatase (IMP),[151] which is a homodimer of 30 kDa subunits that requires Mg-(II) but does have partial activity with Mn(II), Zn-(II) or Co(II), catalyzes hydrolysis of the phosphate ester bond of these inositol monophosphates. Because of its role in a neurochemical signaling pathway and its uncompetitive inhibition by Li(I),[152] IMP

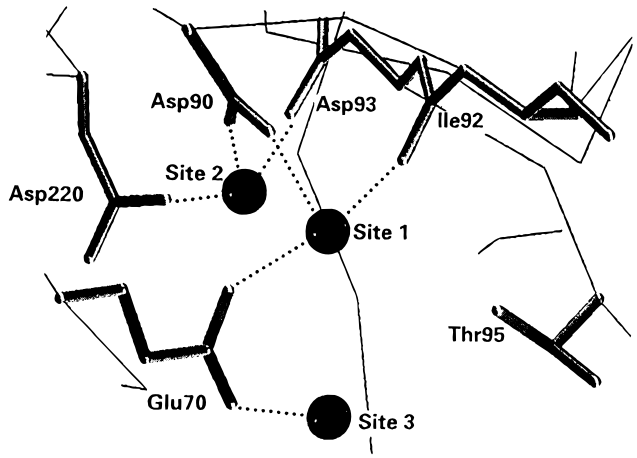


Figure 12. Crystallographically determined structure of the human inositol monophosphatase Mn(II) site. (Adapted with permission from ref 158. Copyright 1994 National Academy of Sciences.)

is a putative target of Li therapy for manic−depressive disorders.

Inositol monophosphatase binds two metal ions with different rates and exhibits cooperative Mg(II) activation of enzyme activity (Hill coefficient of 1.9), although this was initially associated with intersubunit interaction upon binding a single Mg(II) at each phosphatase site.[153] Recent 2.6 Å resolution X-ray crystal structures of human IMP in the presence of 5 mM $MnCl_2$, both with and without phosphate, have elucidated the metal ion coordination and revealed a binuclear Mn(II) site (Figure 12; note, this figure shows only the protein ligands).[154] In the native enzyme, one Mn(II) (site 1) has a 5-coordinate geometry that is distorted between trigonal bipyramidal and square pyramidal, with ligands consisting of Glu70, the carbonyl oxygen of Ile92, a water, Asp90, and Cl^-, the latter two of which bridge to the second Mn(II) (site 2), whose $\sim T_d$ coordination also consists of Asp93 and Asp220. A third Mn(II) (site 3) is found coordinated to the other oxygen of Glu70 and three waters but appears to be only weakly bound to the protein. However, site-specific mutagensis has been used to show that Glu70 is not essential for tight metal binding but, like Thr95, is catalytically important.[155] In the phosphate complex, the site 3 Mn(II) and the Cl^- are displaced and the phosphate bridges the two tightly bound Mn(II) ions with one oxygen and coordinates to the site 1 Mn(II) with another oxygen, providing $\sim O_h$ coordination and retention of the water (hydroxide) ligand on this metal ion.

Using these structural results, structural data on D- and L-*myo*-inositol 1-phosphate binding to the Gd(III)-inhibited form of the enzyme[156] and kinetic,[157] mutagenesis,[155] and modeling[158] studies, a mechanism for I-1-P hydrolysis involving two Mg(II) ions has been proposed (Figure 13). Evidence for a phosphorylated enzyme intermediate is lacking,[157] and metal ion participation in binding substrate, activating a solvent nucleophile, stabilizing the trigonal-bipyramidal phosphorus intermediate, and interacting with product(s) is suggested. This mechanism proposes that one Mg(II) remains bound tightly throughout the catalytic cycle, while the other Mg(II) comes on with substrate to form a catalytically

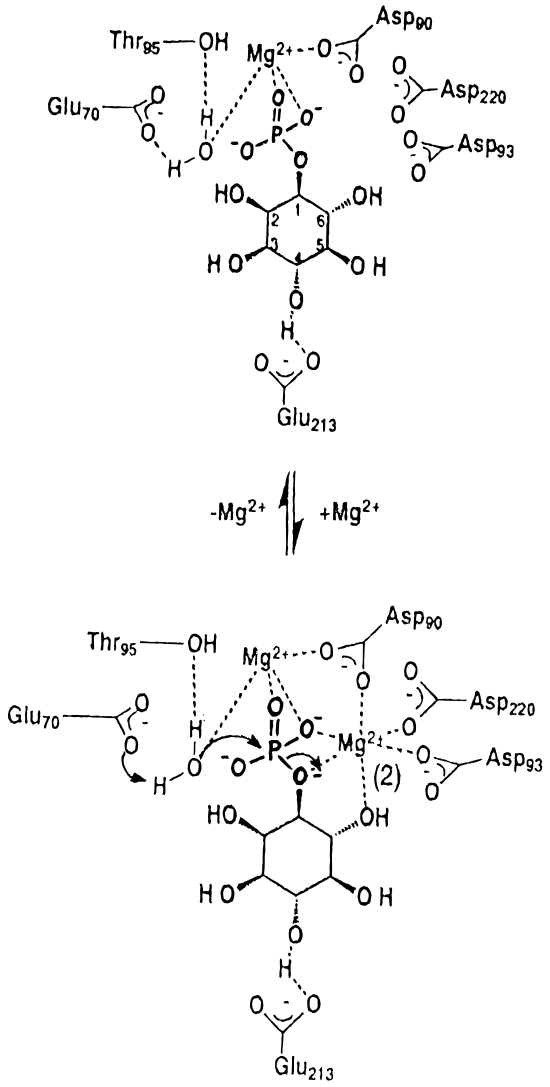


Figure 13. Proposed mechanism for inositol 1-phosphate hydrolysis at the inositol monophosphatase active site. (Adapted with permission from ref 151. Copyright 1995 Federation of European Biochemical Societies.)

competent site but then is released with products. Since Li(I) or higher concentrations of Mg(II) are uncompetitive inhibitors with respect to I-1-P, it is suggested that they bind at this exhangeable site and inhibit phosphate release, although recent kinetic studies indicate that Li(I) inhibition is more complex.[159]

The functionally similar enzyme inositol polyphosphate 1-phosphatase (1-Ptase) hydrolyzes phosphate from the 1-position of inositol 1,4-bisphosphate and inositol 1,3,4-trisphosphate and is also involved in phosphatidylinositol signaling pathways.[160] This monomeric 44 kDa enzyme also requires Mg(II), exhibits cooperative metal binding with a similar Hill coefficient for enzyme activation, and is inhibited by Li(I), but has low sequence homology with IMP. However, the recent 2.3 Å resolution X-ray crystal structure of 1-Ptase in the presence of Mg(II) shows a similar protein topology and binuclear Mg(II) site, with the two Mg(II) ions 3.88 Å apart and bridged by Glu79 and a solvent (hydroxide), as found for IMP.[161] Thus, it appears that 1-Ptase may catalyze the hydrolysis of phosphate from inositol by a similar mechanism at a binuclear Mg(II) site.

Fructose-1,6-bisphosphatase (D-Fructose-1,6-bisphosphate 1-Phosphohydrolase; EC 3.1.3.11)

In a key regulatory step in the gluconeogenesis pathway, fructose-1,6-bisphosphatase (Fru-1,6-Pase)[162] catalyzes the hydrolysis of 1-phosphate from D-fructose 1,6-bisphosphate to give D-fructose 6-phosphate. This enzyme is a homotetramer of 36.5 kDa subunits that exhibits allosteric inhibition by AMP and requires dipositive metal ions (Mg(II), Mn(II), Zn(II), or Co(II)). Kinetic data indicate that more than one Zn(II) or Mn(II) is bound to each subunit in the presence of substrate, inhibitors or product,[163] although Mg(II) stoichiometry is less clear. The substrate exists in solution in an equilibrium among different forms, primarily the α (15%) and β (81%) anomers, and kinetic data suggest that only the α anomer is hydrolyzed by Fru-1,6-Pase.[164]

X-ray crystallography of Fru-1,6-Pase complexes with Mg(II) and the competitive inhibitor fructose 2,6-bisphosphate, the allosteric inhibitor AMP and the product fructose 6-phosphate have shown a single Mg(II) bound to Glu97, Asp118, Asp121, and Glu 280 in the so-called negatively charged pocket, which is adjacent to the substrate and competitive inhibitor binding site.[165,166] Further, it was noted in structures of the metal-free forms that a significant displacement of these Mg(II) ligands accompanied AMP binding and the R → T allosteric transition of the quaternary protein structure, and this appears to correlate with altered metal ion affinity.[167]

Recently X-ray crystal structures at 2.5−3.0 Å resolution have been reported for Fru-1,6-Pase complexes with substrate, fructose 1,6-bisphosphate (Fru-1,6-P_2), and with the inhibitors 2,5-anhydro-D-glucitol 1,6-bisphosphate (AhG-1,6-P_2, an analog of the α anomer of Fru-1,6-P_2) and 2,5-anhydro-D-mannitol 1,6-bisphosphate (AhM-1,6-P_2, an analog of the β anomer of Fru-1,6-P_2) in the presence of Mg(II), Mn(II), or Zn(II).[168] These structures reveal a binuclear metal ion site for the Fru-1,6-Pase complex with AhG-1,6-P_2 and Mn(II) or Zn(II) but only a single metal ion bound to this complex with Mg(II) (even in the presence of 20 mM $MgSO_4$) or the Fru-1,6-Pase complex with AhM-1,6-P_2 and Mg(II) or Mn(II). In the binuclear Mn(II) and Zn(II) complexes, three bidentate ligands, Glu97, Asp118, and the 1-phosphate of AhG-1,6-P_2, bridge the metal ions that are 3.6−3.8 Å apart, while Glu280 and the carbonyl oxygen of Leu120 provide a fourth ligand to the two metal ions, which have $\sim T_d$ coordination. In the complexes with a single metal ion, it occupies the site with Glu280 but also has Asp121 as a ligand in a distorted square-pyramidal structure.

Since AhG-1,6-P_2 is an analog of the α anomer of Fru-1,6-P_2, the Mn(II) and Zn(II) complexes with this competitive inhibitor provide a basis for predicting substrate binding (Figure 14A) and a mechanism for hydrolysis of the 1-phosphate of Fru-1,6-P_2 (Figure 14B) at a binuclear metal ion site.[168] This model proposes that the metal ions are involved in binding and positioning the substrate, activating a water nucleophile and stabilizing the trigonal-bipyramidal phosphorus intermediate; it is consistent with the reported inversion of phosphorus stereochemistry[169] and the observed shift in optimal pH with different metal ions, which implicates a mechanistically important metal-bound solvent nucleophile. Alternatively, it has been suggested that bidentate bridging coordination of the 1-phosphate of AhG-1,6-P_2 may indicate a dissociative mechanism that involves metal ion stabilization of a metaphosphate (PO_3^-) intermediate.[168] The lack of a binuclear Mg(II) complex with AhG-1,6-P_2, however, leaves open the question of whether hydrolysis of Fru-1,6-P_2 occurs at a mononuclear or a binuclear Mg(II) site.

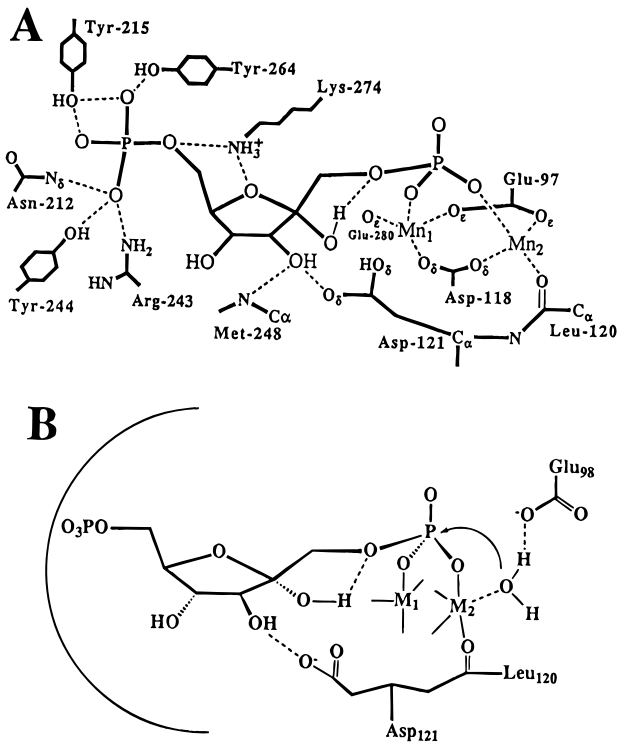


Figure 14. (A) Representation of the proposed binding of fructose 1,6-bisphosphate to the fructose-1,6-bisphosphatase binuclear Mn(II) site; (B) proposed mechanism for fructose 1,6-bisphosphate hydrolysis at the fructose-1,6-bisphosphatase binuclear metal ion site. (Reprinted with permission from ref 168. Copyright 1993 American Chemical Society.)

Ser/Thr Phosphatases (Phosphoprotein Phosphohydrolase; EC 3.1.3.16)

The phosphorylation and dephosphorylation of protein residues is important for signal transduction in all living organisms, and enzymes that hydrolyze Ser−, Thr−, or Tyr−phosphate bonds play key roles in essential signaling events.[170] These phosphatases typically have specificity for either alkyl−phosphate (Ser/Thr) or aryl−phosphate (Tyr) bonds and several examples of the former have a requirement for metal ions. One of the more extensively studied Ser/Thr phosphatases is calcineurin (protein phosphatase-2B),[171] which consists of a 60 kDa catalytic subunit that contains Zn(II) and Fe(III),[172] but which can be activated by Ni(II), Mn(II), Mg(II), or Co(II), and a 19 kDa calmodulin-like regulatory subunit that binds Ca(II). Calcineurin is inhibited by the immunosuppressants cyclosporin and FK506 when they are bound to their respective receptor proteins (immunophilins) cyclophilin and FKBP12,[173] and this inhibition was used to demonstrate an essential role for calcineurin in T cell activation.[174,175] Structural information on Ser/Thr phosphatases is now available from the recently reported X-ray crystal struc-

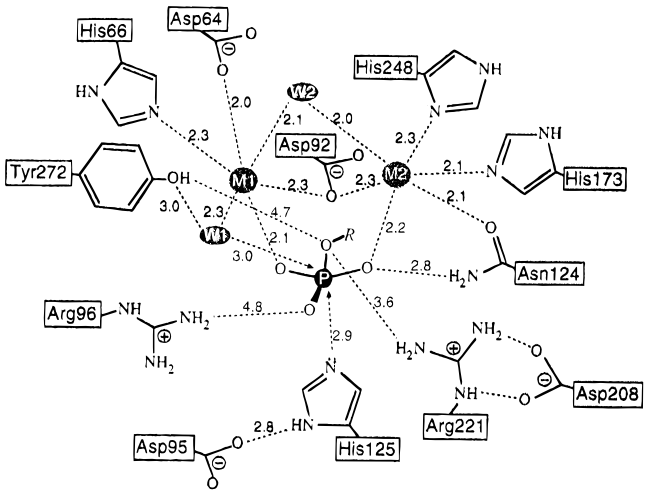


Figure 15. Model of phosphate ester binding to the crystallographically determined structure of the mammalian protein phosphatase-1 binuclear metal ion site. (Reprinted with permission from ref 176. Copyright 1995 Macmillan Magazines.)

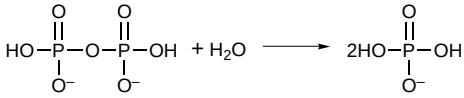
tures of rabbit muscle protein phosphatase-1 (PP-1),[176] which has a catalytic domain homologous to that of calcineurin and certain other Ser/Thr phosphatases, and bovine[177] and human[178] calcineurin and their complexes with FKBP12-FK506.

The 2.1 Å resolution structure of PP-1 complexed with the cyclic hexapeptide inhibitor microcystin revealed two metal ions, most likely Mn(II), bound in close proximity to several protein residues[176] that are conserved in all known eucaryotic Ser/Thr phosphatases and bacteriophage λ and are required for metal binding and catalysis.[179] Each metal ion is 5-coordinate, and they are bridged at a 3.3 Å separation by an oxygen of Asp92 and a water (hydroxide). One metal ion has a square-pyramidal geometry and additional coordination to Nϵ of His66, Asp64 (axial ligand), and another solvent molecule, while the other has a distorted trigonal-bipyramidal geometry and additional coordination to Nϵ of His173, Nδ of His248, and the carbonyl oxygen of Asn124. The peptide inhibitor interacts with the metal ions through a hydrogen bond between the terminal solvent ligand of the first metal ion and the carboxylate of the γ-linked D-glutamate of microcystin. Based on this structure, a model (Figure 15) for the interaction of alkylphosphate substrates with the two metal ions suggests bridging coordination of the phosphate to both metal ions, and attack by either a metal-bound water (hydroxide) or a protein nucleophile (His125), since the stereochemical outcome of phosphoester hydrolysis is not known.

The 2.5 Å resolution structure of the ternary complex of a fragment of the bovine calcineurin catalytic subunit, the bovine calcineurin regulatory subunit, FKBP12 and FK506,[177] and the 2.1 Å resolution structure of the heterodimer of the two human calcineurin subunits[178] reveal very similar Fe(III)Zn(II) phosphatase sites. These in turn are structurally very similar to the kidney bean purple acid phosphatase (kbPAP) active site,[128] except for the substitution of a solvent for the characteristic Tyr ligand of the Fe(III). This similarity between Ser/Thr phosphatases and PAP has been shown to include the core protein topology and homology of the metal-binding residues[129] and has also been shown for the EPR spectral properties of Fe(III)Zn(II) and Fe(III)Fe(II) forms of these enzymes.[180] The bovine calcineurin sample crystallized with a bound phosphate (i.e., E·P complex) and is structurally very similar to the recently reported kbPAP complex with phosphate.[129] The Fe(III) and Zn(II) are 3.0 Å apart and bridged by Asp118 and the phosphate; in addition, a bridging solvent (hydroxide) was modeled into the site to complete $\sim O_h$ coordination for both metal ions. Histidine-151 is located within hydrogen-bonding distance of one phosphate oxygen and in turn is hydrogen bonded to Asp121, suggesting a proton donor role in alcohol product release. In the higher resolution human calcineurin structure, three metal-bound solvent ligands, one bridging the two metal ions and two more on the Fe(III), were located. In this native phosphatase site, His151 is hydrogen bonded to one of the Fe(III) solvent ligands that appears to have been displaced by microcystin in the PP-1 structure and by phosphate in the bovine calcineurin structure. This is a likely candidate for a metal-bound solvent nucleophile, since kinetic and other experimental data suggest direct phosphoester hydrolysis without a phosphorylated enzyme intermediate.[181] Absence of the Tyr ligand and variation in the identity and placement of other residues in the Fe(III)Zn(II) site appear to contribute to an increase in the optimal pH of Ser/Thr phosphatases relative to PAP.

Inorganic Pyrophosphatase (Pyrophosphate Phosphohydrolase; EC 3.6.1.1)

Ubiquitous cytoplasmic enzymes that hydrolyze pyrophosphate (eq 5) are essential for maintaining



(5)

intracellular levels of phosphate and for removing the pyrophosphate product of nucleotide coupling reactions, thereby favorably affecting the thermodynamics of RNA and DNA synthesis. Inorganic pyrophosphatases (PPase)[182] from *Saccharomyces cerevisiae* and *E. coli* are the best studied examples and consist of homodimeric (32 kDa per subunit) and homohexameric (20 kDa per subunit) proteins, respectively. Although there is only modest sequence homology between the yeast and bacterial proteins, there is a striking conservation of a cluster of polar residues (Asp, Glu, Lys, etc.) among all PPases, and substitution of nearly all of these residues by site-specific mutagenesis results in decreased enzymatic activity, suggesting that they form a conserved active site.[183,184]

Metal ions are required by all pyrophosphatases, with Mg(II) providing the highest levels of activity (10^{10} rate enhancement), but Zn(II), Mn(II), and Co(II) sustain appreciable rates of pyrophosphate hydrolysis.[185] Physical characterization of PPases with different transition metal ions provided early evidence for multiple binding sites in close proximity and for two metal ions that bind to the protein with higher affinity, in addition to metal ions that accompany the pyrophosphate substrate. In particular, EPR studies of the active Mn(II) form of PPase

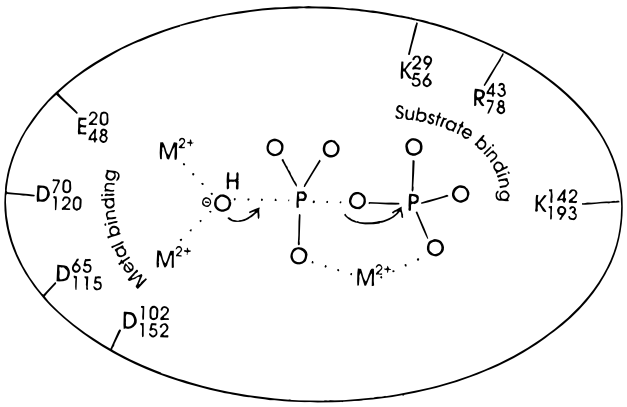


Figure 16. Proposed model for pyrophosphate hydrolysis at the inorganic pyrophosphatase active site (lower and upper numbers indicate *S. cerevisiae* and *E. coli* PPase residues, respectively). (Reprinted with permission from ref 197. Copyright 1996 Federation of European Biochemical Societies.)

showed magnetic interaction between the two high-affinity Mn(II) ions,[186,187] and both EPR and NMR data indicated phosphate interaction with the metal ions.[188]

A 2.35 Å resolution X-ray crystal structure of yeast PPase with three Mn(II) ions and two bound phosphates confirmed the binuclear Mn(II) unit, with a 3.5 Å separation and $\sim O_h$ coordination for both Mn(II) ions to Asp and Glu residues and the two phosphates.[189] The third Mn(II), located 4.2 and 5.3 Å from the first two, interacts with one phosphate but with only one protein residue (Glu58) and appears to be a metal ion that is bound only in the presence of phosphate or pyrophosphate. Crystal structures of *Thermus thermophilus* PPase[190] and *E. coli* PPase[191,192] have confirmed the clustering of conserved residues in an active site similar to that of yeast PPase, and a recent 2.3 Å resolution crystal structure of *E. coli* PPase has shown that the binding site for the highest affinity Mg(II) ($K_D \sim 60$ μM at pH 7.5) consists of Asp102, Asp65, and Asp70.[193] A recent crystal structure of yeast PPase with four Mn(II) ions and two bound phosphates indicates that the latter conserved residue (Asp120 in yeast PPase) and a solvent (hydroxide) also coordinate the second Mn(II) ion, which is bound to Glu48 (Glu20 in bacterial PPase) in the active site; the two additional Mn(II) ions are associated with the bound phosphates.[194] (See note added in proof.)

A mechanism for pyrophosphate hydrolysis by PPase[182] is based on evidence for metal ion interaction with the substrate, participation of residues known to contribute to the optimal activity, and the observed inversion of phosphate stereochemistry,[195] which suggests direct attack by water on pyrophosphate. In addition, recent kinetic measurements on PPase variants with altered active site residues have shown that all changes result in a similar alkaline shift of the pK_a of an essential basic group, thereby suggesting that it is a metal-bound hydroxide that is essential for catalysis,[196] either as the attacking nuclophile or as a general base. A structural and mechanistic model for PPase hydrolysis of pyrophosphate is illustrated schematically in Figure 16, which identifies residues known to bind the two metal ions and residues known to be involved in substrate binding.[197] This model suggests bridging coordination for a metal-bound solvent (hydroxide) nucleophile.

Phospholipase C (Phosphatidylcholine Cholinephosphohydrolase; EC 3.1.4.3)

Hydrolysis of phospholipids is catalyzed by a variety of phospholipases and is an important reaction in phospholipid metabolism, a number of other biochemical pathways, and the toxicity of certain reptile and insect venom. In the case of the soil bacterium *Bacillus cereus*, high phosphate levels supress the biosynthesis of both a monomeric 28.5 kDa phopholipase C (PLC)[198] and alkaline phosphatase, suggesting these two enzymes have a role in phosphate recycling.[199] While certain phospholipases use a protein nucleophile and/or general acid−base catalysis to hydrolyze phospholipids, the *B. cereus* PLC and others contain and require metal ions. This PLC preferentially hydrolyzes phosphatidylcholine to 1,2-diacylglycerol and phosphorylcholine and has similarities to phosphatidylcholine-hydrolyzing PLCs from higher organisms.

Initially it was reported that the *B. cereus* PLC contained two Zn(II) ions,[200] but it is now known to bind a third metal ion more weakly. High-resolution X-ray crystallography[201] provided the first evidence for a binuclear Zn(II) unit in this PLC (Figure 17), showing that the two metal ions are 3.3 Å apart and bridged by Asp122 and a water (hyroxide). Both Zn(II) ions have trigonal-bipyramidal coordination, with His69, His118, and Asp55 completing the coordination of one (Zn1) and His14 and the N-terminal amine and carbonyl of Trp1 completing the coordination of the other (Zn3). The third zinc (Zn2), which has lower site occupancy even in the presence of $\sim$10 μM Zn(II), is located 6.0 and 4.7 Å from the other two metal ions and has a trigonal-bipyramidal coordination consisting of His128, His142, Glu146, and two solvent (water) ligands.

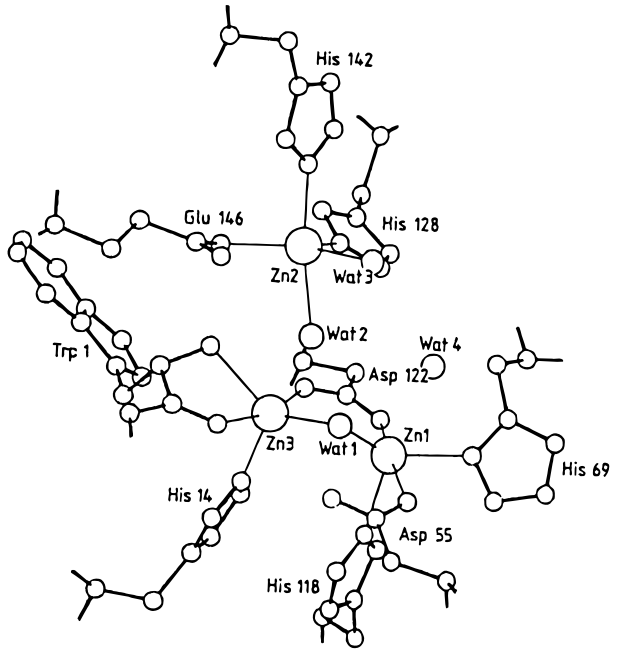


Figure 17. Crystallographically determined structure of the *B. cereus* phospholipase C Zn(II) site. (Reprinted with permission from ref 201. Copyright 1989 Macmillan Magazines.)

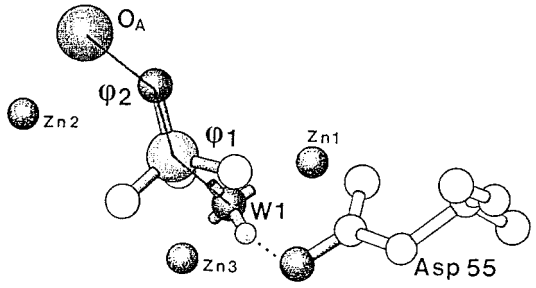


Figure 18. Model for bridging hydroxide attack on the phospholipid phosphorus at the *B. cereus* phospholipase C Zn(II) site. (Reprinted with permission from ref 205. Copyright 1994 Oxford Univeristy Press.)

X-ray crystallography of phosphate- and iodate-inhibited PLC[202] and the PLC complex with the phosphonate competitive inhibitor [3(*S*),4-dihexanoylbutyl]-1-phosphonylcholine[203] provide insight about interaction of the Zn(II) ions with the phosphate group of the substrate, phosphatidylcholine. Phosphate binds to all three Zn(II) ions, displacing one water from Zn2 and the bridging water (hydroxide), which is replaced by a single phosphate oxygen that bridges the 3.5 Å separation between Zn1 and Zn3. However, in contrast to the bridging solvent that it displaces, the phosphate oxygen forms an asymmetric bridge between the two Zn(II) ions. Very similar coordination to the three Zn(II) ions is found for the phosphate group of [3(*S*),4-dihexanoylbutyl]-1-phosphonylcholine in its complex with PLC.

A reaction mechanism for phospholipid hydrolysis by PLC has been proposed, based on the crystal structures of phosphate- and phosphonate-inhibited PLC, and molecular mechanics[204] and molecular interaction energy[205] calculations of dipentanoylphosphatidylcholine binding and hydrolysis at the PLC active site. There is no evidence for a phosphorylated enzyme intermediate and direct attack by a metal-bound solvent is proposed, although the phosphorus stereochemistry has not yet been determined for this enzymatic hydrolysis reaction. There is more than one possibility for a metal-activated solvent nucleophile, but calculations of model substrate interactions at the PLC active site suggest that the bridging hydroxide is best positioned for attack opposite the diacylglycerol leaving group (Figure 18; note, $\varphi 1 = 180°$ for ideal in-line attack). This model proposes that the metal ions orient the phosphodiester substrate for direct attack by the hydroxide that is bridging the binuclear Zn(II) unit and then help to stabilize the trigonal-bipyramidal phosphorus intermediate. A role in stabilizing the diacylglycerol alkoxide leaving group is proposed for the single Zn2 (O_A coordination site in Figure 18).

Aryldialkylphosphatase (Aryltriphosphate Dialkylphosphohydrolase; EC 3.1.8.1)

Bacterial enzymes have been found that catalyze the hydrolysis of organophosphate triesters,[206] many of which are potent insecticides and neurotoxins. These enzymes, also known as phosphotriesterases, require metal ions (Zn(II), Cd(II), Ni(II), Co(II), Mn(II)) for activity, and the best characterized example, *Pseudomonas diminuta* phosphotriesterase, is a homodimer (39 kDa per subunit) that is isolated with up to 2 equiv of Zn(II) per subunit.[207] EPR characterization of the binuclear Mn(II) form of this enzyme shows ^{55}Mn hyperfine evidence for magnetic interaction, similar to that observed for arginase[60] (see Figure 4), and fitting of the temperature dependence of the EPR signal intensity indicates an antiferromagnetic coupling of $2J = -10$ cm^{-1}.[208] Thus, aryldialkylphosphates are hydrolyzed by *P. diminuta* phosphotriesterase at a binuclear metal ion site, which was confirmed by a 2.0 Å resolution X-ray crystal structure of the binuclear Cd(II) form[209] and now by the 2.1 Å resolution structure of the native binuclear Zn(II) enzyme,[210] both with the competitive inhibitor diethyl 4-methylbenzylphosphonate.

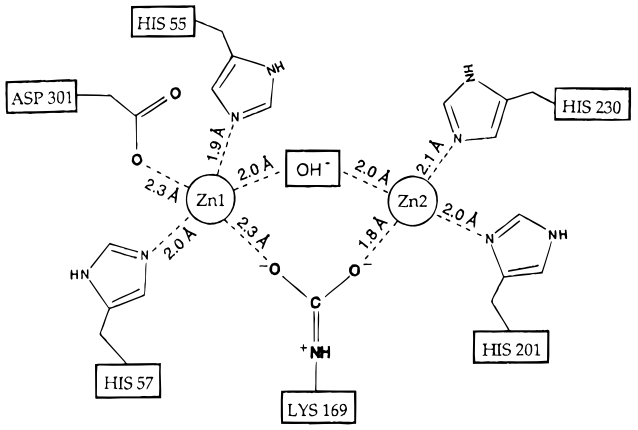


Figure 19. Representation of the crystallographically determined structure of the *P. diminuta* phosphotriesterase binuclear Zn(II) site (indicated bond lengths are the average of the two subunits in the asymmetric unit cell). (Reprinted with permission from ref 210. Copyright 1996 American Chemical Society.)

Many similarities are found between the structure of the phosphotriesterase active site (Figure 19) and that recently determined for urease,[21] (see Figure 2), including a bridging carbamylated Lys, two His ligands for each metal ion, an additional carboxylate ligand for one metal ion, and a bridging solvent (hydroxide) ligand. The two Zn(II) ions are separated by 3.3 Å, with the 5-coordinate trigonal-bipyramidal Zn(II) more buried than the 4-coordinate T_d Zn(II). The substrate analog inhibitor binds near the binuclear Zn(II) site with the methylbenzyl group in a hydrophobic pocket but the phosphoryl oxygen 3.5 Å from the more exposed Zn(II). The catalytically active binuclear Cd(II) site is very similar, except for additional solvent ligands on the more exposed Cd(II) ion. Phosphotriesterase catalyzes phosphoester hydrolysis with inversion of phosphorus stereochemistry,[211] and the mechanism may involve organophosphate binding to the more exposed metal ion followed by nucleophilic attack by the hydroxide ligand that remains bound at least to the more buried metal ion. The kinetically important His[212] with pK_a ~6.1 may be His254 or His257, which are both located near the more exposed metal ion[210] and have been shown through site-specific mutagenesis to be involved in catalysis,[213] but its role as a general base is not included in the current mechanism.

Nucleases

DNA Polymerase I (3′–5′ Exonuclease Site)

The enzyme DNA polymerase I catalyzes the transfer of nucleotides to the 3′ end of template-

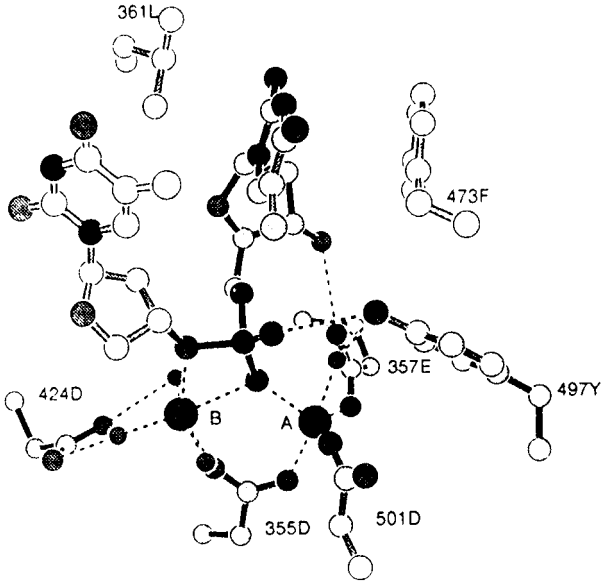


Figure 20. Representation of nucleotide binding to the crystallographically determined structure of the 3′−5′ exonuclease binuclear metal ion site of *E. coli* DNA polymerase I. (Reprinted with permission from ref 221. Copyright 1991 Oxford University Press.)

primer DNA, but also catalyzes hydrolysis of the 3′ terminal nucleotide of single-stranded DNA (3′−5′ exonuclease activity), which corrects errors in DNA replication, and has analogous 5′−3′ exonuclease activity. The well-studied *E. coli* DNA polymerase I can be cleaved into a large C-terminal or Klenow fragment that has the polymerase and 3′−5′ exonuclease activities and a small N-terminal fragment that has the 5′−3′ exonuclease activity.[214] X-ray crystallography of the Klenow fragment has shown that the two catalytic functions occur at different sites separated by >30 Å.[215]

The 3′−5′ exonuclease activity of DNA polymerase I results in a deoxynucleoside 5′-monophosphate and a free 3′ hydroxyl of the shortened DNA polymer. This phosphodiesterase activity requires dipositive metal ions (Mg(II), Mn(II), Zn(II))[216] and has been shown to occur with inverson of stereochemistry at the phosphorus.[217] Only one metal ion is bound tightly to the native protein, but crystallography has shown that the 3′−5′ exonuclease site binds a second metal ion in the presense of a deoxynucleoside 5′-monophosphate product[215] or DNA substrate.[218] This has been confirmed by magnetic resonance measurements of Mn(II) binding to the 3′−5′ exonuclease site of the Klenow fragment, where the tightly bound Mn(II) has $K_D \sim 2.5\ \mu M$ and the affinity for the second Mn(II) increases by 2 orders of magnitude in association with deoxythymidine 5′-monophosphate (dTMP) binding.[219] Futher, cooperative binding of three metal ions has been reported for Co(II), Mn(II), and Mg(II) activation of 3′−5′ exonuclease activity of DNA polymerase I.[220]

Considerable detail about the 3′−5′ exonuclease site of DNA polymerase I has been obtained from high-resolution X-ray crystal structures of a complex of the native Klenow fragment with the product dTMP (E·P) and a complex of the D424A variant Klenow fragment (E*) with the single-stranded DNA substrate deoxythymidine tetranucleotide (E*·S).[221] In the E·P complex (Figure 20), one metal ion (A) is

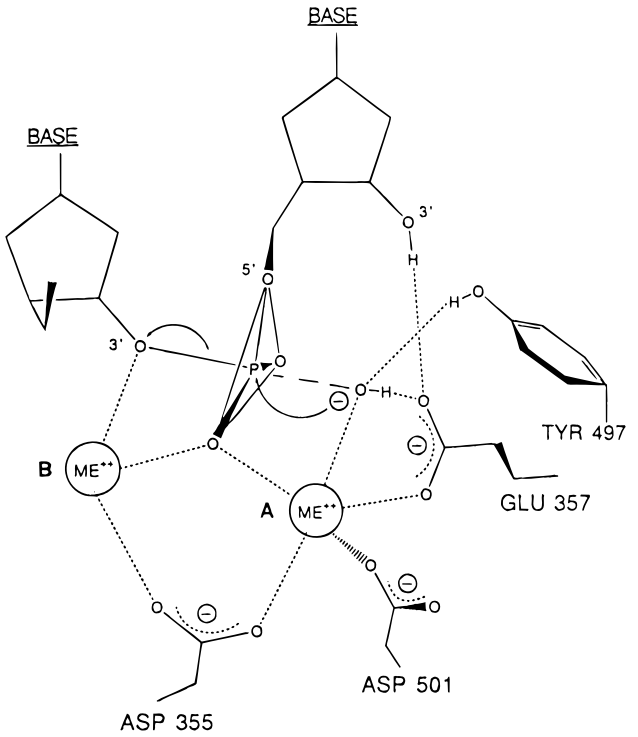


Figure 21. Proposed mechanism for DNA hydrolysis at the 3′−5′ exonuclease binuclear metal ion site of *E. coli* DNA polymerase I. (Reprinted with permission from ref 221. Copyright 1991 Oxford University Press.)

5-coordinate, with the ligands Asp355, Glu357, Asp501, a phosphate oxygen, and addition electron density consistent with a water (hydroxide) ligand that also interacts with Glu357 and Tyr497. The second metal ion (B) has $\sim O_h$ coordination provided by the bridging carboxylate of Asp355, two of the phosphate oxygens, and three water molecules, which also are involved in hydrogen bonds to Asp424 and amides of the polypeptide. Metal ion A appears to be the one that is bound tightly in the absence of substrate or product, and this binding site has a higher affinity for Zn(II) than for Co(II) or Mg(II). The E*·S complex lacks the second metal ion (B), presumably because hydrogen bonding between Asp424 and water ligands of this metal ion is eliminated, or at least altered, with Ala in this position. However, the 3′ terminal thymidine of the tetranucleotide is bound in essentially the same position as the dTMP product is bound to the native enzyme. The spectroscopic properties of Co(II) bound to the 3′−5′ exonuclease site are consistent with a 5-coordinate geometry for the tightly bound Co(II) that can be displaced by Zn(II) and a 6-coordinate geometry for other Co(II) ions that bind less tightly; further, the spectral properties of the 5-coordinate Co(II) are perturbed when dTMP binds to the enzyme, suggesting an interaction between this metal ion and the product.[220]

A mechanism for 3′−5′ exonuclease activity of DNA polymerase I has been proposed,[221] based on these E*·S and E·P structures and on enzyme kinetic data (Figure 21). In particular, the pH dependence of the phosphatase activity shows a lower pK_a of ~9.8, which is attributed to a metal-bound water.[222] This appears to correlate with the solvent ligand of the tightly bound metal ion (A), which is correctly positioned for attack on the phosphate, resulting in

the observed inversion of phosphorus stereochemistry. Interaction of this metal-bound solvent with Glu357 and Tyr497 may help to lower its pK_a when the metal ion is Mn(II) or Mg(II). It is suggested that the two metal ions play roles in enhancing solvent nucleophilicity, stabilizing the trigonal-bipyramidal phosphorus intermediate and assisting in removal of the 3′ hydroxyl of the DNA product. However, recent kinetic data, which disagree with earlier kinetic results,[219,220] and calorimetric measurements of metal-binding affinities indicate that phosphate ester hydrolysis by the Klenow fragment requires only a single Mg(II) or Mn(II), suggesting that the second metal ion binds only at high concentrations of metal ions and is not catalytically relevant.[223] Thus, conflicting metal ion stoichiometries now open the question of whether 3′−5′ exonuclease activity of DNA polymerase I is based on catalytic contributions of one or two metal ions.

Recently the DNA polymerase from *Thermus aquaticus*, which is used in the polymerase chain reaction and which lacks 3′−5′ exonuclease activity, has been structurally characterized by X-ray crystallography.[224] Its 5′−3′ exonuclease site has been found to consist of conserved carboxylate residues that bind three metal ions, two of which share a bridging carboxylate ligand and are separated by 5 Å (the third metal ion is ∼10 Å away), suggesting the possibility of a similar mechanism for both 3′−5′ and 5′−3′ exonuclease activity of DNA polymerase I.

Reverse Transcriptase (Ribonuclease H Domain)

Enzymes known as ribonuclease H (RNase H)[225] hydrolyze the RNA of RNA−DNA hybrids, providing 5′ phosphate and 3′ hydroxyl oligonucleotide products. They require Mg(II) (or Mn(II)) for activity, are broadly distributed, but have poorly understood biological roles. The 17.6 kDa RNase H of *E. coli* is not an essential enzyme, although it catalyzes several known cellular reactions; however, the ribonuclease H activity of reverse transcriptase is essential for replication of retroviruses[226] and requires Mn(II). A 2.2 Å resolution X-ray crystal structure of the metal-free (apo) *E. coli* RNase H has revealed that seven residues that are conserved in all known RNase H sequences, including three carboxylates (Asp10, Glu48, Asp70) shown by site-specific mutagenesis to be essential for catalytic activity,[227] are clustered near the surface of the protein and constitute the active site.[228] A 2.4 Å resolution X-ray crystal structure of the RNase H domain of HIV-1 reverse transcriptase shows a similar active site, and soaking a crystal in 45 mM $MnCl_2$ results in two metal ions bound 4 Å apart to four carboxylate residues, including the three that are essential for activity, with one of these carboxylates bridging the two Mn(II) ions.[229] Thus, RNase H activity of reverse transcriptase may occur at a binuclear Mn(II) site that is similar to the 3′−5′ exonuclease site of DNA polymerase I and involve a similar mechanism for oligonucleotide hydrolysis. In addition, the retroviral enzyme integrase has a metal ion (Mg(II) or Mn(II)) requirement, catalyzes phosphoryl transfer reactions, including 3′−5′ exonuclease activity on viral DNA substrate, and has been shown by X-ray crystallography to have a cluster of conserved carboxylate residues similar to that found for RNase H.[230]

X-ray crystallography of *E. coli* RNase H in the presence of 100 mM $MgSO_4$, however, reveals only one metal ion bound to three active site residues (Asp10, Glu48, and the carbonyl of Gly11).[231] In addition, site-specific mutagenesis has been used to show a nonessential role for Asp134,[227] which corresponds to a Mn(II)-binding residue in the RNase H site of HIV-1 reverse transcriptase. Further, thermodynamic data indicate that RNase H has only one tightly bound Mg(II),[232] and analysis of enzyme kinetic data indicates that only one Mg(II) is required for activity.[233] Thus, hydrolysis of RNA by RNase H involves only one metal ion, which can include substitutionally inert Co(III) complexes.[234] Whether oligonucleotide hydrolysis by the retroviral enzymes reverse transcriptase and integrase is catalyzed by a binuclear metal ion site is equivocal, at best.

P1 Nuclease

P1 nuclease[235] is a 36 kDa glycoprotein isolated from *Penicillium citrinum* that preferentially catalyzes the hydrolysis of single-stranded DNA and RNA and then hydrolyzes the 5′ terminal phosphate from the initial oligonucleotide cleavage. It has a requirement for three Zn(II) ions[236] and hydrolyzes phosphate esters with inversion of phosphorus stereochemistry.[237] The 2.8 Å resolution X-ray crystal structure of this nuclease[238] and a more recent 2.2 Å resolution structure[239] show both a protein structure and an active site (Figure 22) that are very similar to that of phopholipase C (PLC)[201] (see Figure 17), despite low sequence homology. This suggests an evolutionary relationship between these two enzymes. Two Zn(II) ions are bridged by Asp120 and a water (hydroxide) at ∼3.2 Å separation, while the third Zn(II) is nearby with two solvent ligands; other protein ligands provide a 5-coordinate trigonal-bipyramidal geometry for each Zn(II) ion. Although the

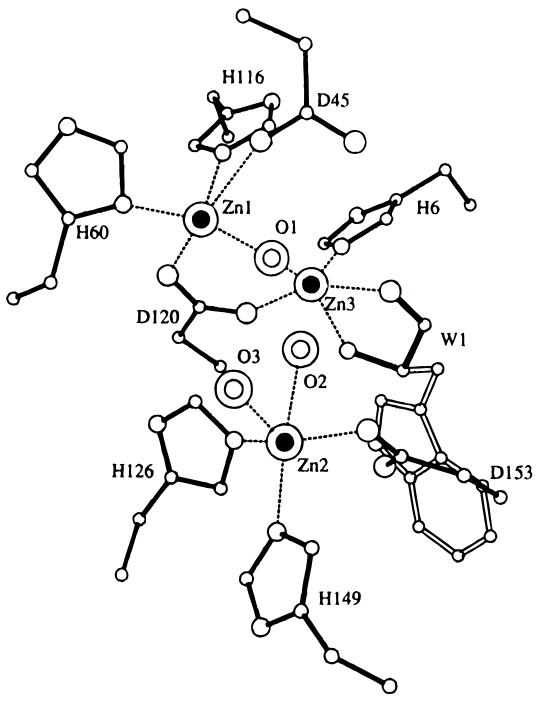


Figure 22. Crystallographically determined structure of the *P. citrinum* P1 nuclease Zn(II) site. (Reprinted with permission from ref 238. Copyright 1991 Oxford University Press.)

binuclear Zn(II) unit is less accessible than the single Zn(II) ion and appears to play a structural role, phosphate binds in the center of the three metal ions, as is found for PLC. This suggests that the binuclear Zn(II) unit participates in oligonucleotide hydrolysis and infers a mechanism analogous to that proposed for PLC.[205] However, P1 nuclease has a low optimal pH (4.5−6, depending on the substrate),[240] similar to that of purple acid phosphatases that use the strong Lewis acidity of Fe(III) to provide a metal-bound hydroxide at low pH. Factors that contribute to the lower pK_a of a metal-bound solvent nucleophile in the P1 nuclease active site, relative to the very similar phospholipase C active site, remain to be determined.

EcoRV Endonuclease

Type II restriction endonucleases catalyze the hydrolysis of the phosphodiester backbone of double-stranded DNA, generally with a high degree of sequence specificity, and many of these require metal ions for activity and/or binding to DNA.[241] Two that have been studied extensively and have been characterized structurally are *Eco*RI and *Eco*RV. *Eco*RI is a 31 kDa protein that is active as a dimer and binds to its double-stranded recognition sequence d(GAATTC) in the absence of metal ions but requires Mg(II) (or Mn(II)) for hydrolysis of DNA between G and A, leaving a 5′ phosphate with inversion of phosphorus stereochemistry.[242] The 3 Å resolution X-ray crystal structure of apo-*Eco*RI with bound oligonucleotides indicates that certain protein residues are positioned to interact with the scissile phosphate;[243] however, Glu111, which has been shown to be essential for hydrolytic activity,[244,245] does not appear to interact with DNA directly but may be involved in binding Mg(II) required for hydrolysis. *Eco*RV has a similar size, is active as a dimer, has a similar recognition sequence (d(GATATC), with cleavage between T and A), hydrolyzes DNA with inversion of phosphorus stereochemistry,[246] and also requires Mg(II). However, it shows little sequence homology with *Eco*RI and has a different tertiary protein structure and DNA−protein contacts than found for *Eco*RI; further, Mg(II) (or Mn(II)) are required for selectively binding its DNA recognition sequence.[247] Nevertheless, a 2.5 Å resolution X-ray crystal structure of *Eco*RV revealed an active site similar to that of *Eco*RI; further, the conserved residues Asp74, Asp90, Lys92, and Glu45, known from mutagenesis studies to be essential for catalytic activity,[248,249] are all found in close proximity to the scissile phosphate in a 3.0 Å resolution structure of an *Eco*RV complex with a DNA decamer containing its recognition sequence.[250]

Recent high-resolution X-ray structures of an *Eco*RV−oligonucleotide complex (E·S) with and without Mg(II) and the *Eco*RV−phosphate complex (E·P) with Mg(II) provide structural evidence for hydrolysis at a binuclear Mg(II) site.[251] Figure 23 shows that a single Mg(II) is bound to Asp74, Asp90, an oxygen of the the scissile phosphate, and three solvent molecules in the E·S complex, while one Mg(II) is bound to both oxygens of Asp74, Glu45, an oxygen of phosphate, and two solvent molecules and another Mg(II) is coordinated to the carbonyl oxygen of Gln69,

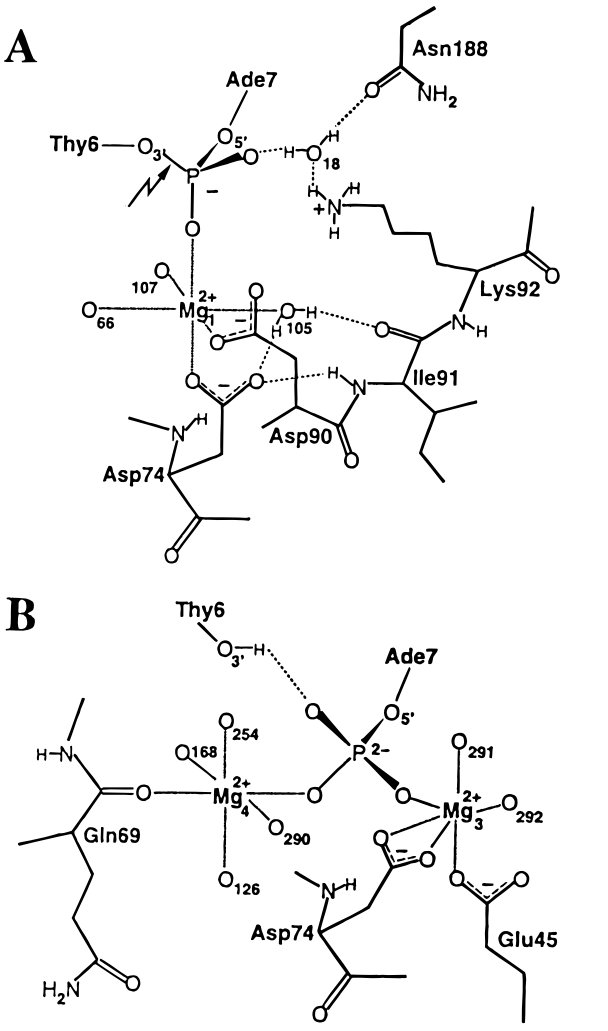


Figure 23. Representations of (A) the crystallographically determined substrate complex and (B) the crystallographically determined product complex of the *Eco*RV Mg(II) site. (Reprinted with permission from ref 251. Copyright 1995 American Chemical Society.)

another oxygen of phosphate, and four solvent molecules in the E·P complex. In each case, ~O_h coordination of the Mg(II) ions is observed. Mechanistic data support the catalytic relevance of these structures; initial rate data from stopped-flow fluorescence measurements indicate that one Mg(II) is bound to the protein prior to DNA binding but hydrolysis requires binding of a second Mg(II).[252] It is suggested that an altered (kinked) DNA structure, which is observed for the recognition sequence when it is bound to *Eco*RV, affects protein affinity for the second Mg(II) and thus discriminates for hydrolysis of the recognition sequence.[250] Finally, the effects of different metal ions (Mg(II), Mn(II), Ca(II)) on DNA cleavage rates by *Eco*RV and *Eco*RI suggest that two metal ions are required for the former but that only one is essential for the latter.[253] However, it remains to be determined whether other type II restriction endonucleases, including *Bam*HI[254] and *Pvu*II,[255] whose recent X-ray crystal structures show active sites similar to that of *Eco*RI and *Eco*RV, use one or two Mg(II) ions in the hydrolysis of DNA.

Ribozymes

Certain sequences of RNA are capable of affecting phosphoryl transfer reactions of RNA, including, in

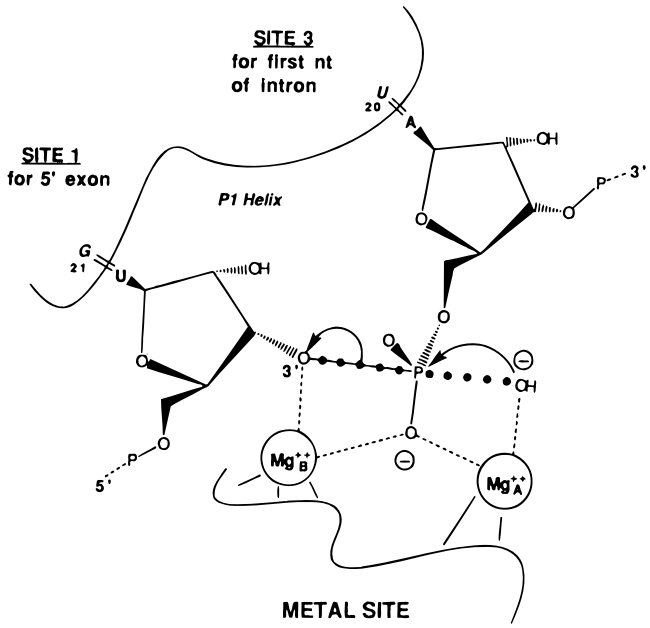


Figure 24. Proposed mechanism for RNA hydrolysis at a binuclear Mg(II) ribozyme site. (Adapted with permission from ref 259. Copyright 1993 National Academy of Sciences.)

some cases, hydrolysis of the phosphodiester bond. These reactions require metal ions (typically Mg(II), but Mn(II), Ca(II), Cd(II), or Pb(II) have been reported to support this activity) and exhibit the salient properties of an enzyme, leading to their designation as ribozymes.[256,257] Little is known about the metal ion stoichiometry for these reactions, and any catalytic role for the metal ion must be distinguished from the well-known role of metal ions in stabilizing RNA structure. Considerable mechanistic data have been reported for different ribozymes, including evidence for inversion of phosphorus stereochemisty,[258] and different roles for Mg(II) ions in ribozyme-catalyzed reactions have been proposed, including a model incorporating the Lewis acidity of two metal ions.[259] This model (Figure 24) is based on the structurally characterized 3′−5′ exonuclease site of DNA polymerase I,[221] where a primary role of the protein appears to be to position two metal ions at a specific distance (∼4 Å) with a particular coordination (see Figure 21), a role that RNA is capable of fulfilling. Aspects of this model are supported by experimental data showing metal ion interaction with certain RNA functional groups during ribozyme-catalyzed reactions of RNA.[260,261]

Pertinent to this review is the RNA−protein complex ribonuclease P (RNase P), which catalyzes the hydrolysis of oligonucleotide sequences from the 5′ end of tRNA[262] and requires Mg(II) (Mn(II) provides limited activity).[263] Since the isolated RNA of RNase P is catalytically active, it is classified as a ribozyme. Kinetic data for Mg(II) activation of RNase P show cooperative binding of at least three metal ions that are required for catalysis, involvement of the substrate 2′ hydroxyl in metal ion binding, and a pH dependence that is best interpreted as direct involvement of hydroxide as a co-substrate.[264] In another relevant case, it is believed that two Mn(II) ions are held in close proximity by RNA functional groups and are involved in Mn(II)-dependent hydrolysis of RNA by small nucleotides.[265] Also of some relevance may be RNA hydrolysis by the yeast mitochondrial group II intron RNA,[266] the *Tetrahymena thermophila* intervening sequence RNA,[267,268] and a small RNA specifically cleaved by Pb(II) in the presence of Mg(II).[269]

Overview

There are now at least 15 confirmed examples of metallohydrolases that contain and require a binuclear metal ion site, and there is evidence that certain nucleases and ribozymes use two or more metal ions in hydrolyzing DNA or RNA. Detailed structural information is available for one or more examples of most of these binuclear metallohydrolases, and structural properties of their binuclear metal ion sites are summarized in Table 1.

Within this class of metalloenzymes there is considerable variability in the metal ion affinity, with some (e.g., urease) exhibiting a high affinity for their native metal ions,[48] but others having readily exchangeable metal ions. In the latter cases (e.g., inositol monophosphatase, pyrophosphatase, and certain nucleases), the catalytic competence of both metal ions must be demonstrated. There is evidence in some cases (inositol monophosphatase, 3′−5′ exonuclease site of DNA polymerase I, *Eco*RV endonuclease) that one metal ion is bound tightly to the protein and the second metal ion binds with substrate to create the catalytically active binuclear metal ion site. In four cases (alkaline phosphatase, phospholipase C, pyrophosphatase, P1 nuclease) there is clear structural evidence for a third (and even fourth) metal ion bound in close proximity to the binuclear metal ion unit; the relative mechanistic contributions of the binuclear metal ion unit and the additional metal ion(s) appear to vary, although this is not yet well understood in some cases. For certain of these metallohydrolases (fructose-1,6-bisphosphatase, ribonucleasease H domain of reverse transcriptase), the metal ion stoichiometry varies with the identity of the metal ion, suggesting that mechanisms using one or two metal ions may occur at the same protein site; this seems to be the case for the type II restriction endonucleases *Eco*RI and *Eco*RV, which have very similar metal binding sites but appear to use one and two Mg(II) ions, respectively.

Similarities in metal ion coordination are found in this class of enzymes (Table 1). Bridging ligands almost always are one or two carboxylates (carbamate in the case of urease and phosphotriesterase) and sometimes a solvent, most likely OH^-. The average separation between the metal ions in the native enzymes is 3.5 Å, but there is a somewhat bimodal distribution with several in the 2.9−3.3 Å range and others in the 3.7−4.0 Å range. As expected, the ligands that bind these relatively hard and borderline metal ions are predominantly carboxylates (Asp, Glu) and solvent, but His, Tyr, amines, and carbonyl oxygens, which modify the Lewis acidity of the metal ion, are also found. Often an asymmetry is observed for the coordination number and/or number of exchangeable solvent ligands of the two metal ions. This is seen in Table 1, where one metal ion (B) has a lower coordination number and/or more solvent ligands in several cases, and appears to correlate with differences in metal ion affinity and suggests

Table 1. Binuclear Metallohydrolases: Structural Properties of the Binuclear Metal Ion Site

enzyme	source	substrate	metal ions[a]	metal A ligands	metal A geometry[b]	metal B ligands	metal B geometry[b]	bridging ligands	bridging distance (Å)	ref
Amidohydrolases and Peptide Hydrolases										
urease	*K. aerogenes*	urea	2 Ni(II)	2 His, Asp, H_2O, μ-Lys*	tbp/sq pyr	2 His, μ-Lys*	trig pyr	carbamate (Lys*)	3.5	21
aminopeptidases										
leucine aminopeptidase	bovine lens	N-terminal Leu	2 Zn(II)	2 Asp, carbonyl, μ-Glu, μ-OH^-	tbp?	Asp, Lys, μ-Glu, μ-OH^-, μ-Asp?	sq pyr?	Glu, OH^-	3.0	79
aminopeptidase	*A. proteolytica*	N-terminal residue (hydrophobic)	2 Zn(II)	His, Glu, μ-Asp, μ-OH^-	T_d	His, Asp, μ-Asp, μ-OH^-	T_d	Asp, OH^-	3.5	80
methionine aminopeptidase	*E. coli*	N-terminal Met	2 Co(II)	His, Glu, H_2O, μ-Asp, μ-Glu	sq pyr	Asp(2), H_2O, μ-Asp, μ-Glu	sq pyr	Asp, Glu	2.9	73
Phosphohydrolases										
purple acid phosphatase	kidney bean	alkylphosphates	Fe(III), Zn(II)	Tyr, Asp, μ-Asp, His, (OH^-), (μ-OH^-)	O_h?	2 His, (H_2O), Asn, μ-Asp, (μ-OH^-)	O_h?	Asp, (OH^-)	3.26	129
alkaline phosphatase	*E. coli*	alkylphosphates	2 Zn(II) + Mg(II)	2 His, Asp(2), H_2O?	$\sim T_d$?	His, 2 Asp, Ser	T_d	none	3.9	147
inositol phosphatases										
inositol monophosphatase	human	inositol 1-phosphate	2 Mn(II) (Mg(II))	Glu, H_2O, carbonyl, μ-Asp, μ-Cl^-	tbp/sqr pyr	2 Asp, μ-Asp, μ-Cl^-	T_d	Asp, Cl^-	*d*	154
inositol polyphosphate 1-phosphatase	bovine	inositol 1,4-bisphosphate	2 Mg(II)	Asp(2), 2 Asp, μ-Glu, μ-OH^-	$\sim O_h$	Asp(2), carbonyl, μ-Glu, μ-OH^-	sq pyr?	Glu, OH^-	3.88	161
fructose-1,6-bisphosphatase[c]	porcine kidney	D-fructose 1,6-bisphosphate	2 Zn(II) or Mn(II)	Glu, μ-Asp, μ-Glu, μ-O(P_i)	T_d	carbonyl, μ-Asp, μ-Glu, μ-O(P_i)	T_d	Asp, Glu, phosphate	3.6−3.8	168
Ser/Thr phosphatases										
protein phosphatase-1	rabbit muscle	Ser-/Thr phosphate	2 Mn(II)?	His, Asp, H_2O, μ-Asp, μ-OH^-	sq pyr	2 His, Asn, μ-Asp, μ-OH^-	tbp	Asp, OH^-	3.3	176
protein phosphatase-2B[c] (calcineurin)	bovine	Ser-/Thr phosphate	Fe(III), Zn(II)	His, Asp, H_2O, (μ-OH^-), μ-Asp, μ-O(P_i)	O_h?	2 His, Asn, μ-Asp, μ-O(P_i), (μ-OH^-)	O_h	Asp, (OH^-) phosphate	3.0	177
protein phosphatase-2B (calcineurin)	human	Ser-/Thr phosphate	Fe(III), Zn(II)	His, Asp, 2 H_2O, μ-Asp, μ-OH^-	O_h	2 His, Asn, μ-Asp, μ-OH^-	tbp	Asp, OH^-	3.14	178
inorganic pyrophosphatase[c]	*S. cerevisiae*	pyrophosphate	2 Mn(II) (Mg(II)) + Mn(II)	2 Asp, H_2O, O(P_i), μ-O(P_i)	O_h	2 Asp, Glu, μ-O(P_i)	O_h?	phosphate	3.5	189
phospholipase C	*B. cereus*	phosphatidylcholine	2 Zn(II) + Zn(II)	2 His, Asp, μ-Asp, μ-OH^-	tbp	His, amine, carbonyl, μ-Asp, μ-OH^-	tbp	Asp, OH^-	3.3	201
phosphotriesterase	*P. diminuta*	aryldialkylphosphates	2 Zn(II)	2 His, Asp, μ-Lys*, μ-OH^-	tbp	2 His, μ-Lys*, μ-OH^-	T_d	carbamate (Lys*), OH^-	3.3	210
DNA polymerase I (3′−5′ exonuclease site)[c]	*E. coli*	DNA (single-stranded)	Zn(II), Mg(II)	Asp, Glu, O(P_i), H_2O, μ-Asp	$\sim T_d$?	3 H_2O, 2 O(P_i), μ-Asp	O_h	Asp	3.9	221
reverse transcriptase (ribonuclease H site)	HIV-1	RNA	2 Mn(II) (Mg(II))	2 Asp, μ-Asp	*d*	Glu, μ-Asp	*d*	Asp	~4.0	229
P1 nuclease	*P. citrinum*	RNA, DNA (single-stranded)	2 Zn(II) + Zn(II)	2 His, Asp, μ-Asp, μ-OH^-	tbp	His, amine, carbonyl, μ-Asp, μ-OH^-	tbp	Asp, OH^-	3.2	238
*Eco*RV endonuclease[c]	*E. coli*	DNA (double-stranded)	2 Mg(II)	Glu, Asp(2), μ-O(P_i), 2 H_2O	$\sim O_h$	carbonyl, μ-O(P_i), 4 H_2O	$\sim O_h$	phosphate	*d*	251

[a] Metal ions in structurally characterized form; native metal ions, if different, are indicated in parentheses. [b] tbp = trigonal bipyramidal; sq pyr = square pyramidal; trig pyr = trigonal pyramidal. [c] Complex with phosphate or phosphate moiety (P_i) of substrate analog or product. [d] Not reported.

differential interaction with substrate. In most cases the protonation state of coordinated solvent is not known; in fact, only reasonable guesses were used to fill in the Fe(III) and Zn(II) solvent coordination in the 2.65 Å resolution X-ray structure of kidney bean purple acid phosphatase.[128]

For many binuclear metallohydrolases, there is reasonable evidence for a metal-bound solvent nucleophile that plays a key mechanistic role. First, a correlation between pK_a's determined from enzyme kinetics and pK_a's associated with the metal ions identifies mechanistically important metal-bound species, and such a correlation has been provided for a Fe(III)−bound OH^- in purple acid phosphatase. Second, for the phosphohydrolases, inversion of phosphorus stereochemistry during the enzymatic reaction suggests direct attack by a solvent nucleophile but retention of phosphorus stereochemistry is attributed to the sequential formation and hydrolysis of a phosphorylated enzyme intermediate, with inversion accompanying each step.[9] Except for alkaline phosphatase, inversion at the phosphorus has been found for all binuclear metallophosphoesterases for which the phosphorus stereochemistry has been determined, implicating direct solvent attack and suggesting a role for metal-bound solvent in the reaction mechanism. Even in the case of alkaline phosphatase, which clearly uses covalent catalysis and a phosphorylated enzyme intermediate, substitution of nonnucleophilic residues for the protein nucleophile (Ser102) results in a new hydrolytic mechanism that may involve direct attack by a metal-bound solvent at the binuclear Zn(II) site;[135] inversion of phosphorus stereochemistry is predicted for these variant enzymes, if this is the case.

Among the binuclear metallohydrolases there appears to be a common mechanistic theme, well illustrated by urease (see above), that one metal ion plays a primary role in substrate coordination while the other delivers the activated solvent nucleophile OH^-. The pH range over which a coordinated hydroxide is available will be determined primarily by the metal ion and its oxidation state but also by other ancillary ligands and the active site dielectric. This is seen in a comparison of the optimal pH of (a) purple acid phosphatase (∼5), with a putative Fe(III)−OH^-, (b) alkaline phosphatase (∼8), with a putative Zn(II)−OH^-, and (c) the 3′−5′ exonuclease site of DNA polymerase I (∼10), with a putative Mg(II)−OH^-; these values parallel the pK_a of water coordinated to the aqueous metal ions Fe(III) (2.2), Zn(II) (8.8), and Mg(II) (11.4).[65] Finally, bridging ligand(s) at the binuclear metal ion site may contribute to the reaction primarily by providing a fixed internuclear separation; however, they may play more important and direct roles in the catalytic mechanism by interacting with substrate, intermediates, or products. This seems to be the case for the aminopeptidases, pyrophosphatase, phospholipase C (and P1 nuclease), phosphotriesterase, and possibly urease, where a bridging hydroxide appears to be the metal-bound solvent nucleophile.

With little doubt, more members of this class of metalloenzymes await to be identified and studied. For example, kinetic data for the MutT protein, which hydrolyzes nucleoside triphosphates to nucleoside monophosphates and pyrophosphate, indicate that two dipositive metal ions are required.[270] However, as in the case of ribonuclease H, *Eco*RI endonuclease, and the recently characterized *E. coli* exonuclease III,[271] where it appears that only a single metal ion is involved in oligonucleotide hydrolysis, each metallohydrolytic enzyme needs to be studied carefully to determine whether or not a binuclear metal ion unit is mechanistically important. This important point has been addressed recently for certain nucleases in a theoretical analysis[272] and in an examination of solution and structural experimental data.[273] Many hydrolysis reactions can be catalyzed by mononuclear metallohydrolases or by enzymes without a requirement for metal ions, suggesting there is nothing unique about a binuclear metal ion site in these cases. However, for certain reactions, such as the hydrolysis of urea, Lewis acidity of two metal ions appears to be necessary for the enzyme-catalyzed mechanism.

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Note Added in Proof

Recently the 2.4 Å resolution X-ray crystal structure of yeast inorganic pyrophosphatase with four Mn(II) ions and two bound phosphates has been reported. (Harutyunyan, E. H.; Kuranova, I. P.; Vainshtein, B. K.; Höhne, W. E.; Lamzin, V. S.; Dauter, Z.; Teplyakov, A. V.; Wilson, K. S. *Eur. J. Biochem*. **1996**, *239*, 220−228.) It is in essential agreement with that reported in ref 194.

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