Mode of Action of Bi- and Trinuclear Zinc Hydrolases and Their Synthetic Analogues

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Contents

A moon-landing child (born July 19, 1969), and of course fascinated by science, Jennie Weston studied chemistry at Arkansas College (now Lyon College). After completing his B.S. degree in 1990 and a short graduate study stint at Rice University, a transfer to Justus-Liebig-University (Gieβen, Germany) in 1992 not only brought a culture change but also necessitated a repetition of a large part of the basic study of chemistry due to the incompatibility of university rating systems. After finishing with not only an American B.S. but also a German Diplom (1994) and Ph.D. (1998) degrees, postdoctoral studies brought the opportunity to join the Special Research Center 436 ("Metal Mediated Reactions Modeled after Nature") based at the Friedrich-Schiller-University (Jena, Germany). In addition to this ongoing joint research venture (with an individually funded "Teilprojekt" since 2000), he currently heads a Junior Research Group founded in 2002.

1. Introduction

In living organisms it is necessary to be able to perform countless hydrolysis reactions efficiently and, above all, *selectively* to sustain thousands, maybe even millions, of critical biological functions. In many cases nature has solved this problem very elegantly by placing two or even three divalent metal ions (Zn^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}) close to each other (M−M distance 3−5 Å)[1,2] in the active site of an enzyme. These ions work together to bind and activate the substrate and a water molecule, which then undergo hydrolysis under biological conditions (room temperature and neutral pH), conditions where these reactions normally cannot take place.

These enzymes are truly cocatalytic[3,4] with both metal ions working synergistically as a single unit. Through long evolution, nature has optimized the structure of the active site as well as the identity of the metal ions in order to obtain the necessary selectivity for the specific reaction process present in the biological medium. Removal of one or both metal ions from the active site usually causes a significant decrease in and/or total inactivation of the enzyme function. The specificity of these bimetalloenzymes combined with an ever-increasing number of reliable X-ray structures has prompted a flood of mechanistic studies as well as numerous attempts to synthesize and study simple biomimetic complexes based on these enzymes. The synthesis of robust catalysts that function well in aqueous or nonaggressive media and are finely tunable yet highly selective is a very tempting goal not only for academic interest but also for industrial applications. Due to the extreme activity in this field at the present time as well as the massive amounts of data that have been collected, a comprehensive review of the modes of action of all oligometalloenzymes is not possible. This review therefore constrains itself to bi- and trinuclear zinc hydrolases.

Experimental mechanistic studies on zinc enzymes are often hindered by the fact that zinc ions are

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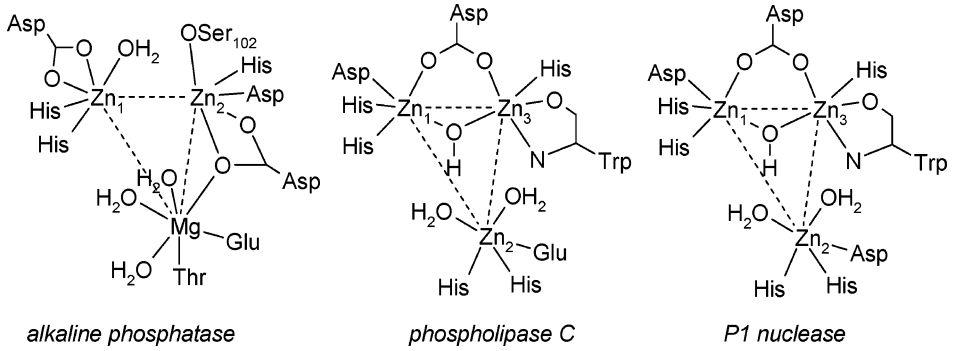

Figure 1. Structure of the active sites of three trinuclear phosphatases.

spectroscopically silent. Fortunately they can often be replaced by other metal ions (especially Co^{2+} and Cu^{2+}) without destroying the catalytic activity.[5] Some replacements can even result in hyperactive forms.[6] Although it is often assumed that most mechanistic data available for Co^{2+}-substituted forms can probably be extended to the physiologically relevant zinc enzymes,[7] care obviously needs to be exercised. Another point that needs to be considered is the fact that inhibitors generally undergo "tight" bonding interactions with the active site of multinuclear enzymes (often bridging both metal ions) to generate inactive enzyme−inhibitor complexes which can often be structurally characterized. It is the belief of the author that one needs to be extremely careful in interpreting facts discovered for inhibitors as being relevant for substrates. Indeed, it could very well be that a prerequisite for effective inhibition is the ability of the inhibitor to tightly bind to the metal ions in the active site, whereas active substrates are possibly not capable of this. Mechanistic studies are also limited by a fundamental characteristic of enzymes—they are *fast*. So fast that most experimental techniques cannot follow the individual chemical steps in the catalytic circle. One must therefore rely on the help of modern quantum chemical techniques to join the isolated experimental facts together into a more or less complete picture of the mode of action of the enzyme.[8]

Significant progress has been made in recent years in applying computational techniques to biochemical processes, and experimental studies are increasingly being supported by computational approaches. There are currently three basic approaches for dealing with larger systems available. Molecular modeling/molecular dynamic approaches (MM/MD) based on force fields generally provide adequate structural accuracy, and it is now possible to calculate complete biological macromolecules using these methods.[9] However, if chemical accuracy is desired, a quantum chemical approach must be employed, which drastically limits the size of the molecule that can be calculated. At the lower end, semiempirical methods can be employed. Depending on their parametrization, more or less structural and/or chemical accuracy can be obtained. At the upper end, density functional techniques (DFT) are rapidly becoming the method of choice for obtaining chemical accuracy, and calculations on systems containing 200 and more atoms are becoming routine.[10]

This review summarizes recent mechanistic investigations of both an experimental and a theoretical nature on the mode of action of bi- and trinuclear zinc enzymes. Recently developed biomimetic models are also discussed. In-depth reviews on the structure of binuclear zinc enzymes are already available.[1−3,11−13] In addition, a recent review on biomimetic mononuclear zinc complexes has been published which also contains a good introduction to synthetic analogues of multinuclear zinc enzymes.[14]

2. Phosphate Ester Hydrolysis

Phosphohydrolases are responsible for the hydrolytic cleavage of phosphoryl groups, a task of essential importance for the control of metabolic processes in countless biological systems. Many of these enzymes employ metal ions as cofactors in order to lower the activation energy for P−O bond fission.[12] These metalloenzymes have a wide structural variance and often possess homo- or heterobinuclear or even -trinuclear active sites.[1,2] In addition to zinc, magnesium ions are often present as seen in *inositol monophosphatase*[15,16] or *fructose biphosphatase,*[17] both of which contain a binuclear Mg^{2+}−Mg^{2+} core. Sometimes nature combines a redox-active metal ion (Fe^{3+}/Fe^{2+}) with a redox-inactive ion (Zn^{2+}, Mg^{2+}) in the active site; the best example of this class of enzymes is perhaps the purple acid phosphatases.[1,2] Reviews of these bimetallic enzymes can be found in several excellent articles,[1,2,11,12] and more are being discovered all the time.

Representative for a binuclear zinc phosphatase, this review focuses first on what is known about the mode of action of phosphotriesterase, which is quite interesting not only because it has no known natural substrate but also because an atmospheric CO_2 molecule is required for assembly of the binuclear active site.[18] Three structurally related trinuclear phosphatases (alkaline phosphatase,[19,20] phospholipase C[21] (PLC), and P1-nuclease[22]) are then discussed. All three possess an ancillary M^{2+} ion in close proximity to a bizinc active site (Figure 1). The three catalytically active metal ions roughly form a triangle, and the chemical identity and arrangement of the ligands in the first coordination spheres of the metal ions are remarkably similar, especially for phospholipase C and P1 nuclease, which differ only in one ligand (Glu or Asp) on Zn2.

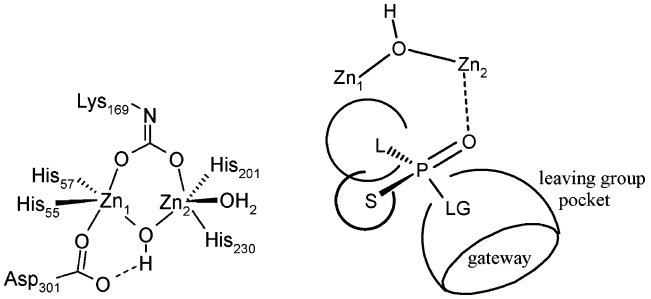

Figure 2. Schematic representation of the active site of phosphotriesterase as well as the three pockets (large, L; small, S; leaving group, LG) for the substrate in the active site.

2.1. Phosphotriesterase

Organophosphate triesters and their related phosphonates are extremely toxic and counted among the most dangerous compounds ever synthesized.[23] Unfortunately, some of them (sarin or soman, for example) are sometimes employed as potent biological warfare nerve agents.[24] Others, paraoxon for example, have found widespread applications as agricultural insecticides. It has been found that several soil-dwelling bacteria, *Pseudomonas diminuta*,[25] *Flavobacterium sp*.,[26] and *Agrobacterium radiobacter P*230,[27] are capable of detoxifying paraoxon and other phosphotriesters by an extremely efficient phosphotriesterase (PTE) mediated enzymatic hydrolysis (nearly diffusion-limited for many substrates).[28] It is a remarkable fact that no naturally occurring substrate for PTE has been found to date,[29] which has led to the assumption that the catalytic activity has only recently evolved due to the widespread use of insecticides throughout the world.[30]

PTE is a homodimer with each subunit containing a bizinc active site (Figure 2).[31] An equivalent of atmospheric CO_2 is necessary for metal binding; it reacts with the side chain of Lys169 to form the carbamate bridge which fixes the two metal ions in the active site.[18,32] Six amino acids (His55, His57, Asp301, Lys169, His201, and His230) are directly involved in metal coordination, and selective mutagenesis of any of these results in either total or partial inactivation, thus demonstrating that all six are needed to maintain the bimetallic structure of the active site.[33]

A μ-OH unit is also present. Although the X-ray data could not differentiate between a bridging OH or H_2O, several independent density functional theory (DFT) and molecular dynamic (MD) studies on active-site models all concur that the hydroxide form is preferred over a water ligand at physiological pH.[34] The calculated pK_a (5.9)[34a] compares very favorably to the experimental value (5.8).[35] X-ray structural analyses are also available for the *apo* form[36] (without metal ions), various heterobimetallic forms,[31,37] as well as several inhibitor complexes.[38,39]

Molecular dynamics (MD) calculations have shown that solvation affects the structure of the active site. When PTE is immersed in water and allowed to relax in a MD simulation, the gateway for the leaving group (Figure 2) is widened (8 Å in the solid state as compared to 11−18 Å in solution).[40] This opening seems to be solvent induced as it is calculated regardless of whether or not a substrate is present.

All available experimental and theoretical data have been recently combined into a picture of the mode of action of PTE (Figure 3).[41,42] As the substrate docks onto the active site, it replaces a water molecule on Zn2 (the more solvent-exposed site) and a strong O(P)−Zn2 contact is formed very early in the course of hydrolysis (intermediate **2**).[43] Already at this point chiral differentiation has taken place. The wild-type enzyme preferably hydrolyzes the S_P enantiomer,[44] and it has been shown that this stereochemical preference is controlled by the structural properties of the active site. Three different pockets (small (S), large (L), and leaving group (LG)) have been identified in the active site which are responsible for the orientation and selectivity of substrates (Figure 2).[44,45] Through rational reconstruction of these pockets by selective mutagenesis[46] it is possible to enhance, relax, or even reverse the inherent stereoselectivity of PTE.[23,47] Once docking has occurred, nucleophilic attack on phosphor by the bridging hydroxide is then suspected to take place. This is accompanied by hydrogen transfer to Asp301 and

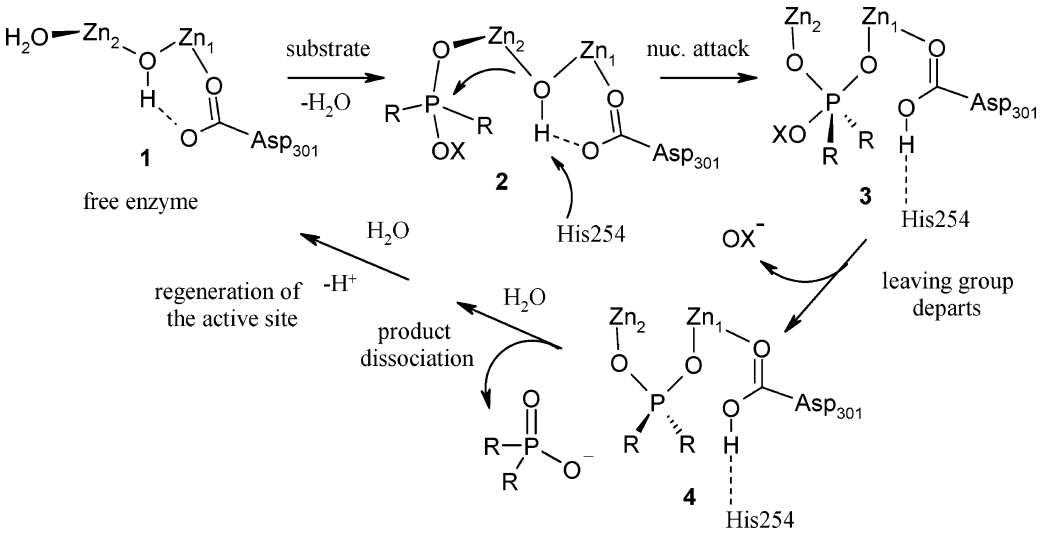

Figure 3. Proposed mode of action of phosphotriesterase.

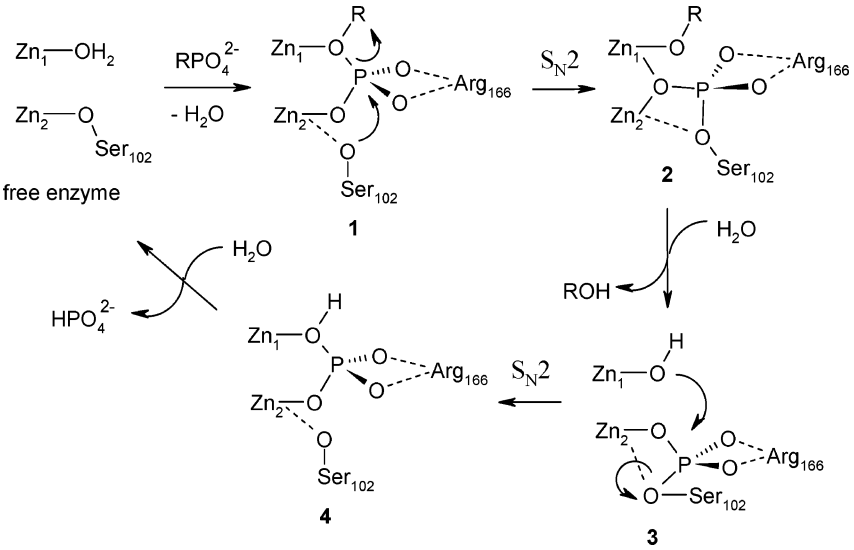

Figure 4. Mode of action of alkaline phosphatase as it is currently understood.

stabilized by a strong hydrogen bond to His254 (which acts as a general base[48]), thus effectively whisking the proton away from the immediate vicinity of the active site. The trigonal bipyramidal intermediate/transition structure **3** then ejects the leaving group. Net inversion of configuration is experimentally observed and probably best mechanistically described in terms of an S_N2-type reaction with an "in-line" nucleophilic attack on phosphor.[49] In addition, ^{18}O isotope effects support an S_N2-like transition structure.[50]

After the departure of the leaving group, a phosphate anion **4** which bridges the two zinc ions is left behind. Solvent molecules then move in to regenerate the bridging hydroxide, and in this process the product is released. Specific details on exactly how this happens are still rather unclear. A theoretical study [Hartree−Fock (HF) method using additional effective fragment potentials to describe secondary environmental effects] suggests that a network of waters bound to ligands in or near the active site may have an important function in the hydrolysis.[51]

2.2. Alkaline Phosphatase

Alkaline phosphatase (AP) hydrolyzes phosphate monoesters nonspecifically under both acidic and alkaline conditions[52,53] and is, without question, one of the best-studied metalloenzymes reported thus far in the literature. Several review articles[1,2,11,14,52,54−56] have devoted extensive chapters to discussion of the mode of action of AP with the most recent one haven been written by Parkin.[14] A summary of the current mode of action (a modified version of the original mechanism postulated by Coleman[52]) is presented in Figure 4, and the above-mentioned review articles are recommended for their extensive discussions on the topic.

The two zinc ions in the active site are essential for activity,[57] whereas magnesium alone is not catalytically active.[58] It, however, enhances the activity of the zinc-containing AP.[59] The role of the Mg^{2+} ion is generally believed to be ancillary[1,52,55] and serves to enhance the enzymatic activity.[59] The magnesium is linked to the bizinc site via a bridging carboxylate (Asp51; Figure 1). Mutation of this rest results in a drastically reduced activity due to the loss of Zn2.[60]

As a phosphate monoester docks onto the bizinc core to build a phosphate bridge, it is believed to displace the zinc-bound water present in the active site of AP (and possibly other waters as well; Parkin assumes that a second Zn2-bound water is present[14]). A bidentate enzyme−substrate intermediate **1** for which solid-state structures are available[20,61] is formed. The phosphate is additionally stabilized by hydrogen bridging to an arginine rest.[62] Mutation of this rest has demonstrated that Arg166 is probably responsible for the preference of AP for phosphate monoesters.[62]

The active nucleophile is believed to be the deprotonated Ser102 rest (most probably stabilized by an interaction with zinc), which has been reported to possess a pK_a of ≤ 5.5 in the free enzyme.[63] Although this presents no problem under acidic conditions (Figure 4), a general base will be needed to deprotonate the Ser102 hydroxyl group (which is most likely not nucleophilic enough by itself) under alkaline conditions. It has been suggested that the role of the third metal ion (Mg^{2+}) in the mode of action of AP is to provide an additional metal-bound water in the vicinity of Ser102 that can function as a general acid/base for regulating the protonation state of Ser102.[20]

In accord with a crystal structure of a vanadate inhibitor complex,[64] an in-line S_N2-type attack by the serine nucleophile over a trigonal bipyramidal transition structure is generally postulated.[52,64] An investigation of linear free-energy relationships indicates that electrostatic interactions of the phosphate with both zinc ions *and* Arg166 are critical stabilizing factors in the transition structure.[65] Reaction-induced IR spectroscopy recently showed that the motion required for this nucleophilic attack is minimal on the part of AP,[66] consistent with AP being an "almost perfect"; i.e., nearly diffusion-limited, enzyme.[67,68] Under acidic conditions (pH < 6), this is the rate-limiting step.[69] Under alkaline conditions (pH ≥ 8), product dissociation becomes rate limiting.[69]

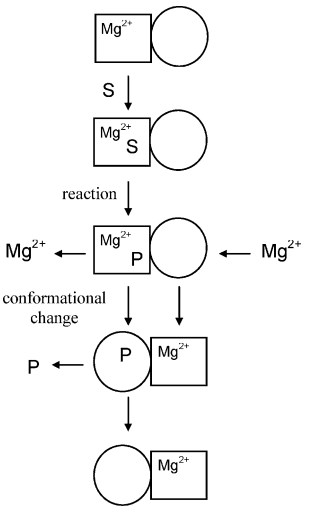

Figure 5. Kinetic switch mechanism for the allosteric function of magnesium ions in the subunits (square and circle) of alkaline phosphatase. The subunit with bound Mg^{2+} is represented as the square.

A covalent phosphoseryl intermediate **2** (known solid-state structure[70]) results. Hydrolysis then sets the alcohol product free, and a Zn1-bound hydroxide (species **3**) is believed to be formed which then attacks the phosphor group in an S_N2 manner in order to generate the enzyme−phosphate product complex **4** (solid-state structure available),[20] thus setting the Ser102 functionality free again.[52] A consequence of this "ping-pong" mechanism is the retention of the absolute configuration at phosphorus upon transphosphorylation, a fact confirmed by isotope-marking (^{18}O) experiments.[71] The hydrolyzed phosphate in **4** must then be released from the zinc core (displaced by water) in order to regenerate the free enzyme, a process which is still not well understood.

Although the AP dimer is clearly symmetric in the solid state,[20,61] early research reported a negative cooperativity between the two subunits[72] as well as considerable deviations from Michaelis−Menten kinetics in solution.[72] These findings have been interpreted in terms of an allosteric mechanism involving two different subunits.[73,74] More recently, in-depth studies have reported that a Mg^{2+} binding/debinding event is directly involved and is most likely responsible for this allosteric activity.[75,76] Fully loaded AP (two Zn and one Mg) undergoes continual structural rearrangements in solution. Two distinct, fully active subunit (unsymmetric dimer) conformations are present and in equilibrium with each other.[77] In the absence of Mg^{2+} only one conformation (symmetric dimer) is observed.[76] Magnesium binding thus seems to be the event which triggers dynamic refolding so that a second conformation becomes possible.[76]

Recently, Pavela-Vrancic et al. developed a model that successfully explains these findings by assuming inherently nonequivalent subunits (Figure 5).[73] Since Mg^{2+} binds to AP with negative cooperativity,[72] a dimer will prefer to have only one magnesium present (the subunit with Mg^{2+} is represented as a square). This subunit has the higher affinity for the substrate S (and the product P), which will then preferentially dock here. Hydrolysis is carried out according to the general mechanism of Figure 4, and the product remains "stuck" to this subunit. A Mg^{2+} ion is then believed to dock onto the second unit, which triggers a conformational change (12 → 21), thus ejecting the Mg^{2+} ion in the subunit with the bound product. The product is not as tightly bound in this new subunit conformation and can be more easily released. This conformation-controlled and Mg^{2+}-assisted dissociation of the reaction product P is postulated to serve as a kinetic switch to accelerate the overall rate of reaction of AP.[73]

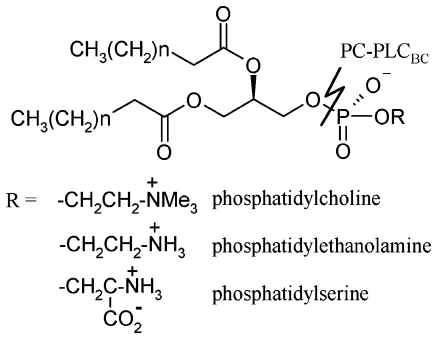

Figure 6. Phospholipids catalyzed by PC−PLC_{BC}.

It is believed that this mechanism could possibly allow simultaneous diffusion of Mg^{2+} and HPO_4^{2-} ions into cells.[73] This is supported by the fact that the transport system for HPO_4^{2-} in *E. coli* is known to cotransport HPO_4^{2-} and Mg^{2+} ions.[78]

2.3. Phospholipase C and P1 Nuclease

Not very much is known about the mode of action of P1 nuclease. Since it[22] is structurally very similar to phospholipase C,[21] differing only in one ligand (Asp vs Glu) on Zn2; Figure 1, one can assume that the mode of action will probably resemble that of phospholipase C to a great extent.

The trinuclear zinc-based phospholipase C isolated from *Bacillus cereus* (PC−PLC_{BC}) catalyzes the hydrolysis of the phosphodiester bond in phospholipids to provide diacylglycerol (DAG) and a phosphorylated headgroup R (Figure 6).[79] In mammals, DAG is an important secondary messenger molecule in the signal transduction cascade.[80] Since it has not yet proved possible to isolate and characterize a mammalian PLC, there is much interest in studying the bacterial system as a potential model.[81]

A mode of action consistent with all available data has been proposed for the PC−PLC_{BC}-catalyzed hydrolysis of phospholipids (Figure 7).[82] The phosphatidylcholine substrate binds at the enzyme active site **1,** thus displacing the bridging OH moiety and the Zn2-bound water to give an enzyme−substrate complex **2** which is stabilized by ionic attractions between the three zinc ions and the phosphodiester functionality.[82] Since the solid-state structure of PLC_{BC} bound to a phosphonate substrate analog[83] reveals that there are no water molecules in the first coordination sphere of any of the zinc ions, it is believed that the primary role of the zinc triad is to bind and activate the substrate toward nucleophilic attack through charge neutralization rather than to provide a zinc-bound nucleophile.[82] This is in direct contrast to the mode of action of alkaline phosphatase in which the

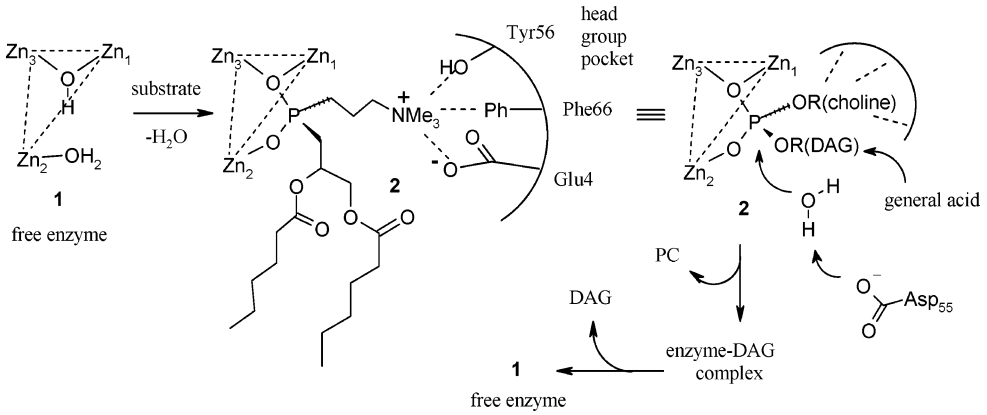

Figure 7. Mode of action proposed for the hydrolysis of phospholipids in the active site of phospholipase C.

third metal is believed to activate the Ser_{102} nucleophile[20] (discussed above).

The choline moiety binds in a headgroup pocket comprised of Glu4, Tyr56, and Phe66 (Figure 7).[83,84] All three rests are directly involved in substrate binding,[85,86] and their specific mutation considerably changes the substrate specificity.[84] Hydrolysis most probably commences when a proton is abstracted from an active-site water molecule by a general base.[82] Rests that have been postulated to fulfill this function are Glu4,[87] Glu146,[83] and Asp55.[82] Selected mutagenesis has ruled out Glu4[86] and Glu146[88] and suggests that Asp55 is the general base.[86,89] Proton transfer and not substrate binding/product release is the rate-limiting step in the catalytic circle.[90] The hydroxide thus generated then attacks the zinc-bound phosphor. This is believed to occur via an in-line, associative mechanism that runs over a trigonal bipyramidal transition structure.[91] However, stereochemical investigations confirming this are not yet available. Collapse of the pentacoordinate transition structure leads directly to two products, phosphorylcholine (PC) and diacylglycerol (DAG).[82]

The identity of the general acid needed to protonate DAG is still unknown.[82] Molecular modeling studies have proposed that Asp55 fulfills this role.[91] However, experimental evidence overwhelmingly implicates that Asp55 is the general base,[86,89] and kinetic data conclusively rule out the possibility that this single residue functions as both a general acid and a general base.[82] No other residues in the general vicinity are likely candidates, and the possibility that a zinc-bound water serves in this capacity must be considered.[82]

Product release follows a two-step kinetic mechanism, neither of which is rate determining.[82] Considering the fact that DAG is a moderately strong competitive inhibitor and PC an extremely weak, noncompetitive inhibitor of $PC-PLC_{BC}$, it is to be expected that PC will be released first.[82] This corresponds to the order predicted by molecular modeling calculations.[92]

3. Metallo-β-lactamases

Antibiotics based on β-lactams (penicillins, carbapenems, and cephalosporins, for example) account for more than one-half of the commercially available antibiotics worldwide.[93] Bacteria have unfortunately evolved a defense mechanism by employing a class of enzymes, the β-lactamases,[94] which inactivate these antibiotics by hydrolytically cleaving the β-lactam ring. Many of these β-lactamases can be spread by both plasmid and integron-borne mechanisms,[95] and bacterial resistance to these antibiotics has reached such a level that successful therapy can no longer be taken for granted.[96] At present, there are no clinically useful inhibitors for this class of enzymes,[97] but a concerted effort is currently being made in the scientific community to understand the mode of action of these enzymes and thus hopefully find effective inhibitors.

As more and more β-lactamases have been discovered, their extreme diversity has become increasingly evident, both biochemically and genetically.[98] This has resulted in attempts to develop a classification scheme capable of describing all of them.[99−101] Currently, β-lactamases are divided into two major classes,[102] the serine β-lactamases and the metallo-β-lactamases (formerly class A and class B enzymes, according to the older nomenclature of Ambler[99]). The serine β-lactamases, which employ an active-site serine for hydrolysis, are then subdivided into three groups A, C, and D which retain their names from the former classification of Ambler, who considered them to be major classes.[99,102] Both of these classes hydrolyze β-lactams but are structurally and mechanistically completely unrelated.

The metallo-β-lactamases contain at least one metal ion, usually zinc, in their active sites. In 1997 Rasmussen and Bush divided these enzymes into three subclasses B1, B2, and B3,[100] a classification which has been universally accepted and incorporated into a general numbering scheme for reporting amino acid sequence homologies.[101] Although it has recently been suggested that two subclasses "B1+B2" and B3 would be more logical,[102] this review retains the nomenclature of Rasmussen and Bush.

The most populated subclass of the metallo-β-lactamases to date is B1, which exhibits a very broad substrate profile[103] and possesses a binuclear zinc binding site. Three solid-state structures are available for subclass B1, CcrA[104] isolated from *Bacteroides fragilis*, BcII[105] from *B. cereus II,* and the plasmid-borne IMP-1[106] (lower resolution; therefore, not illustrated in Figure 8) from *Pseudomonas aeruginosa*. Most of the mechanistic studies on metallo-β-

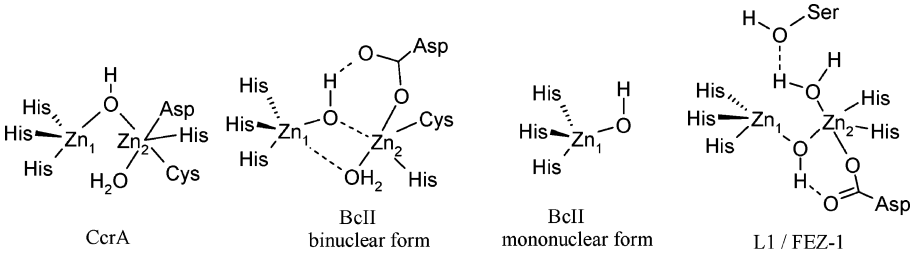

Figure 8. Solid-state structures of the active sites of metallo-β-lactamases.

lactamases to date have concentrated on CcrA and BcII. In CcrA and the binuclear form of BcII, the Zn1 site is coordinated to three histidines and a μ-OH ligand in a distorted tetrahedral arrangement. This half of the active site very closely resembles the active site of carbonic anhydrase (CA)[107,108] (a mononuclear zinc enzyme). In addition, the UV spectrum of a monocobalt-substituted BcII is almost identical to that of the high-pH form of CA, a resemblance that may or may not extend to the mode of action of this enzyme.[109] The structural similarity between the β-lactamases is also visible in the Zn2 site; Zn2 is distorted trigonal bipyramidal coordinated and an Asp and a His residue is conserved in all three enzymes.

BcII differs noticeably from all other B1 enzymes in that the Zn2 is labile.[110,111] Solid-state structures are available for both mononuclear and binuclear forms.[105] The μ-OH bridge in the binuclear form is asymmetric and is best likened to a Zn1−OH bond with a with a further electrostatic contact to Zn2. A carboxylate group (Asp) stabilizes this linkage. There is no doubt that BcII, in contrast to CcrA, is capable of effectively functioning in its mononuclear form, although two equivalents are required for maximum efficiency.[112] These findings have prompted ongoing controversial discussions as to which form (binuclear, mononuclear, or metal-free state) is the proper target for inhibitor design.[97]

Metallo-β-lactamases belonging to subclass B2 do not possess a broad substrate profile and are efficient only for the hydrolysis of carbapenems.[103] They are active in their monometallic form; binding of a second zinc ion causes noncompetitive inhibition.[113] Although a solid-state structure of a B2 enzyme is not yet available, spectroscopic and mutation studies have determined that one of the binding sites contains two His and one Asp and the other an Asp, a His, and a Cys residue.[114]

Two solid-state structures have recently become available for subclass B3—L1[115] isolated from *Stenotrophomonas maltophilia* and FEZ-1[116] from *Legionella gormanii* (Figure 8). Their active sites are isostructural, and both enzymes are active in their binuclear forms.[117,118] They exhibit broad substrate profiles. The general conformation of the zinc binding site is quite similar to subclass B1-type enzymes. Site 1 has been completely conserved, as has one Asp (which stabilizes the bridging hydroxide) and one His in site 2. Instead of Cys, a second His is present in site 2 and a Ser residue provides important hydrogen bonding to the Zn2-bound water. Mechanistic (selected mutagenesis[119] and kinetic[120]) investigations are beginning to be performed on L1 and FEZ-1; however, a clear picture of the mode of action of subclass B3 β-lactamases is not yet available.

3.1. CcrA from *B. fragilis*

The mode of action of CcrA isolated from *B. fragilis* has been the subject of considerable experimental and theoretical interest. CcrA binds two zinc ions with equal affinity, and there is little doubt that the binuclear form is catalytically active.[121] Mutation experiments could show that the active-site cysteine (Cys181) is responsible for ensuring the binding of two zinc ions.[122] If this amino acid residue is exchanged for serine, only one Zn^{2+} ion (in site 1) is bound and the second coordination site remains unoccupied.[122]

The mode of action of CcrA using nitrocefin as the substrate (it is hydrolyzed slowly enough for mechanistic investigations) has been intensively investigated using stopped flow kinetic methods, and a mechanism for the hydrolysis reaction has been postulated[123,124] which has then been modified by extensive density functional and Hartree−Fock calculations on model systems[125] (Figure 9). The original mechanism postulated that Asp103 was deprotonated in the active site **1**;[123] the calculations indicate that a neutral Asp103 is more likely.[125] As nitrocefin docks, the Zn1 expands its coordination sphere in order to undergo a Lewis acid−base interaction with the ring carbonyl group (intermediate **2**). The substrate carboxylate group, which is necessary for binding all good substrates,[126] is postulated to be stabilized by a salt bridge to Lys184 (located on the edge of the active-site pocket), thus serving to position and stabilize the β-lactam in the initial docking complex **2**.[123] Several molecular dynamics (MD) studies on the docking mechanisms of imipenem, ampicillin, and captopril onto CcrA also assign an important stabilizing role to Lys184.[127−129] The polarization of the carbonyl group by Zn1 increases the negative charge on the nitrogen atom, which then interacts with Zn2 to form the bridged species **3**. A pseudorotation on Zn2 ruptures the Zn−Zn interaction, thus activating the zinc-bound hydroxide in **4**. In addition to the DFT study,[125] molecular dynamics docking studies using impenem also proposed that the enzyme initiates catalysis via this conformational change into the nonbridged form.[127] Nucleophilic attack under simultaneous C−N bond rupture then takes place to form an acyl intermediate **5** (spectroscopically observed[130]) in which the negatively charged nitrogen is stabilized by Zn2.

The breakdown of **5** requires protonation of the nitrogen atom in the hydrolyzed β-lactam and in-

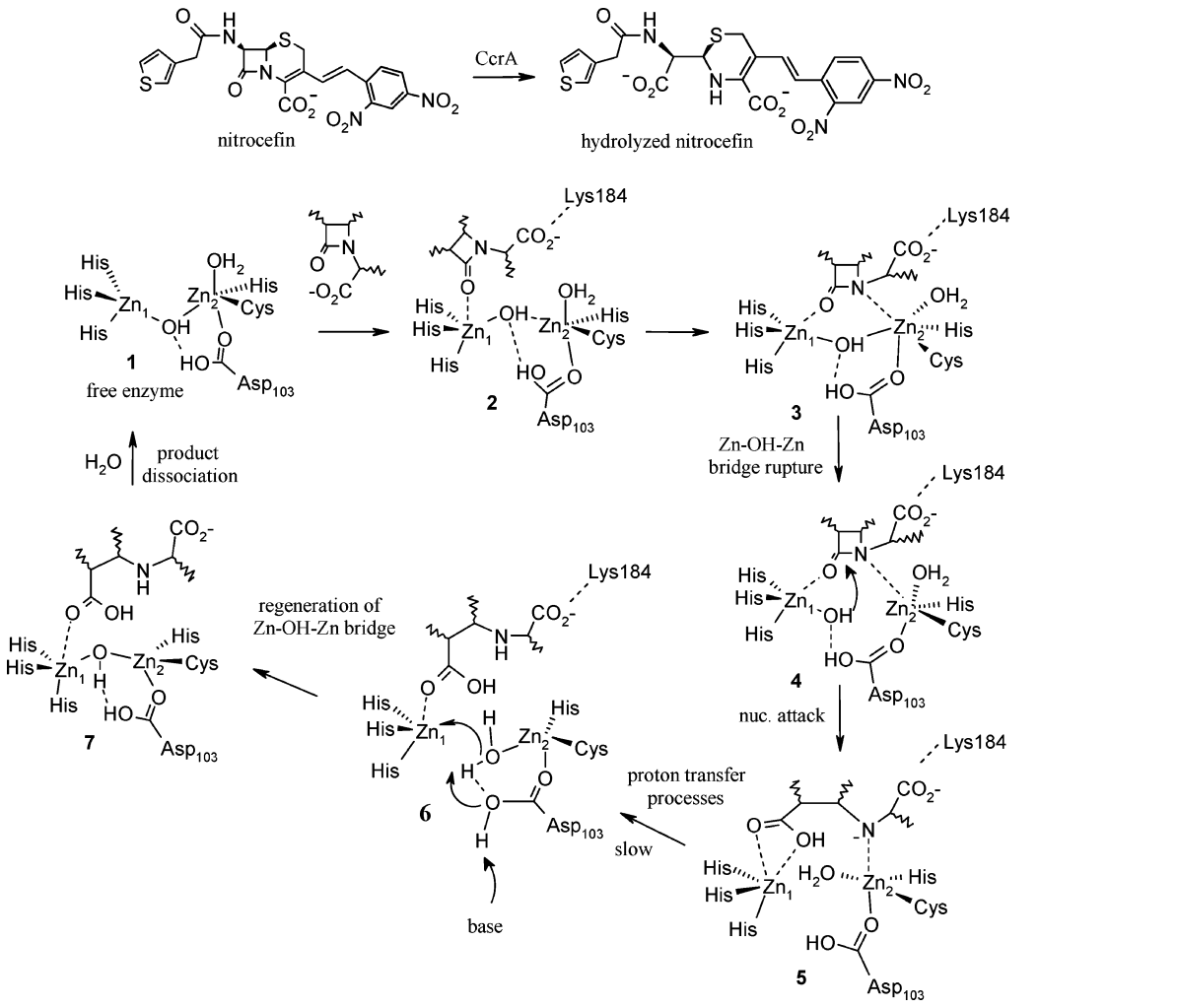

Figure 9. Mechanism of the CcrA (*B. fragilis*) mediated hydrolysis of nitrocefin.

volves several little understood proton-transfer processes. This is the rate-limiting step in the catalytic turnover.[123] A proton shuttle involving a general base and Asp103 (**6**) is a likely possibility for regenerating the Zn−OH−Zn bridge (**7**).[125] Product release is probably assisted by Zn2 accepting a water molecule from the surrounding medium to regenerate the initial state of the active site.

There is currently a bit of controversy as to the actual conformation of the active site of CcrA in vivo. A combined density functional, Hartree−Fock, and molecular dynamics study indicates that four different configurations are fundamentally possible (Figure 10).[131] These differ in the protonation state of the Asp103 residue and the presence or absence of a Zn−OH−Zn bridge. If a neutral Asp103 is present, the active site can readily (ca. isoenergetically) interchange via a change in the coordination number of Zn2 between a bridged **A(H)** and a nonbridged **B-(H)** state. A deprotonated Asp103 results in a rigid Zn−OH−Zn linkage (**A**), and the corresponding open form **B** is much more unstable. The authors of this study[131] postulate that Asp103 is neutral in vivo and cite the fact that mutation of Asp103 to other neutral polar residues (Asn, Ser) leads to mutants that are still able to bind two zinc ions and hydrolyze penicillin G, albeit considerably less effectively than the wild-type CcrA,[132] as support for their hypothesis.

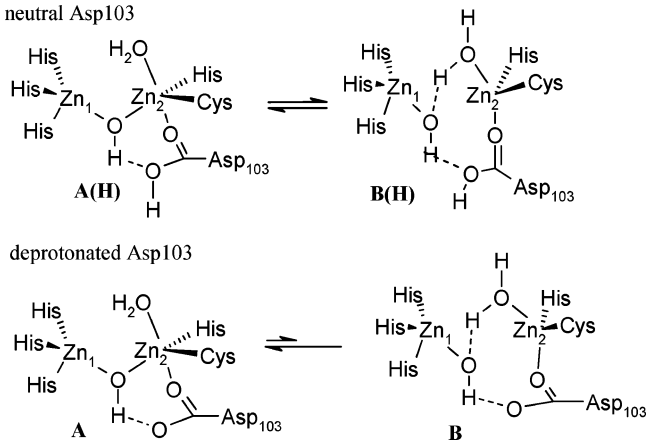

Figure 10. Possible conformations of the active site of CcrA (*B. fragilis*).

In contrast, another density functional study claims that the bonding situation of Asp103 in the solid-state structure can only be reproduced by assuming that it is deprotonated.[133] This independent study uses the same mutation experiments[132] to support their claims.[133] They argue that replacing Asp120 with Asn or Ser disrupts the hydrogen-bond network needed to generate the active nucleophile which leads to decreased efficiency in the mutants. A third independent theoretical study (using Hartree−Fock and

molecular mechanics methods) also concluded that the active site can easily assume conformations that differ from the solid-state structure.[134] Experimental evidence for at least two different conformations of the active site in solution is also available for the cobalt-substituted form of CcrA.[124,135]

A notable feature of CcrA is the flexibility of a region of 11 residues in the N-terminal domain which forms a β-sheet "flap" in the immediate vicinity of the active site.[136] A solid-state structure of an inhibitor complex showed that this flap had closed down on the inhibitor and clamped it into the active site.[136] This prompted NMR investigations of the dynamic changes associated with the binding of tight inhibitors. These studies concluded that dynamic flap motion is an important component of the mode of action of CcrA.[137,138] Upon binding, the flap collapses down around the substrate, effectively locking it in an induced-fit fashion.[137] Molecular dynamic simulations of inhibitor binding also conclude that this flap acts as a "lid" for the active site and, when it is in place, the enzyme−inhibitor complex is much less flexible than the enzyme with an empty active site.[139]

3.2. BcII from *B. cereus*

The presence of at least one metal ion in the active site of the β-lactamase isolated from *B. cereus II* (BcII) is essential for catalytic activity; the apoenzyme is not capable of hydrolyzing or even binding β-lactams.[140] There are two metal binding sites in BcII which exhibit negative cooperativity with respect to metal binding (Figure 8).[110] When the metal-ion concentration is sufficiently low, a single metal ion is distributed between both binding sites[141] and rapid exchange significantly hinders the docking of a second metal ion. This leads to the measurement of two different dissociation constants for BcII.[142] Dialyzing BcII against a large concentration of Zn^{2+} always results in formation of the binuclear form.[143]

There is no doubt that BcII, in contrast to CcrA, is capable of effectively functioning in its mononuclear form even though two equivalents are required for maximum efficiency.[112] This led Fabiane et al. to suggest that a tighter Zn2 binding could be an evolutionary advantage for the more efficient β-lactamases isolated from pathogenic bacteria.[105a] Indeed, the coordination environment of Zn2 is the most variable structural feature within the active site of metallo-β-lactamases.[98] Attempts to tailor the Zn2 site of BcII via selected mutagenesis showed that site 2 can tolerate significant modifications without substantial loss of activity[144] whereas modification of site 1 ligands drastically affects the catalytic ability.[145] It is suspected that the structure of site 1 mainly determines the activity and the role of site 2 is ancillary, helping to guarantee broad band activity for a wide palette of substrates.[144]

The coordination environment of the two zinc ions in BcII closely resembles that found for CcrA (Figure 8). However, in contrast to CcrA, BcII has an Arg121 rest located near the active site. Arg121 seems to be conserved in most other known mononuclear β-lactamases and may be the reason BcII is active in its mononuclear form.[146] In addition, the mononuclear form requires the Cys residue in site 2 to be present, whereas this rest is not essential for the binuclear form, thus indicating that the mono- and binuclear forms function by different mechanisms.[143]

The low affinity for a second Zn^{2+} in BcII has raised the question of whether physiological conditions offer sufficiently high metal ion concentrations to maintain the active metal-bound state.[97] It is suggested that the prevailing state of most β-lactamases in the absence of substrates is the *apo* form since substrate availability induces a spontaneous increase in the binding affinity of zinc ions, which results in formation of mononuclear enzymes even at very low zinc concentrations.[147] Studies of thiomandelic acid (a broad spectrum inhibitor of β-lactamases[148]) inhibited Cd-substituted BcII as a function of Cd^{2+} concentration clearly showed a positive cooperativity in the presence of the inhibitor (which promptly organized two metal ions in the active site).[149] This is in direct contrast to the negative cooperativity observed for the free enzyme.[110] The physiologically active zinc form of BcII is suspected to exhibit a similar cooperativity, although direct measurements are not yet available.

A number of computational studies have been performed on the mononuclear form of BcII, and the currently postulated mode of action is illustrated in Figure 11. It is generally accepted that the zinc is localized in site 1 during catalysis. Density functional calculations[150] as well as molecular dynamics simulations[151] have shown that a zinc-bound hydroxide (**1**) is present in the active site, which is stabilized by a complex hydrogen-bond network to Cys221 and a deprotonated Asp120. Further hydrogen bonding of Asp120 to Arg121 and His263 determines the relative position of Asp120 in the active site.

When a β-lactam docks onto the active site it is believed that the carbonyl group attaches itself to the zinc ion (species **2**), after which the zinc-bound hydroxide attacks the double bond.[152,153] This is generally assumed to be the rate-limiting step, which has an experimentally measured barrier of 15 kcal/mol.[154]

There are several rests in the immediate vicinity of the active site that can possibly serve as proton shuttles for H transfer to the β-lactam nitrogen atom. Four of these possibilities (His263, Asp120, Cys221, as well as an active-site water molecule) have been considered in theoretical studies; DFT,[155,156] Hartree−Fock,[156] and QM/MM[157] studies have shown that all four are fundamentally possible and occur via two basic mechanisms (Figure 11). If the proton originates from His263 or Asp120, hydrogen transfer accompanies the nucleophilic attack and C−N bond cleavage occurs simultaneously. No stable intermediate occurs. If the proton shuttle involves Cys221 or an active-site water molecule, H transfer occurs after nucleophilic attack has generated a stable tetrahedral intermediate.

Very recent Car−Parinello QM/MM calculations have indicated a further possibility for a proton-donor mechanism; Zn1 is capable of accepting an additional water molecule under expansion of its coordination sphere.[158] This activates the water, which can then function as a proton donor.[158]

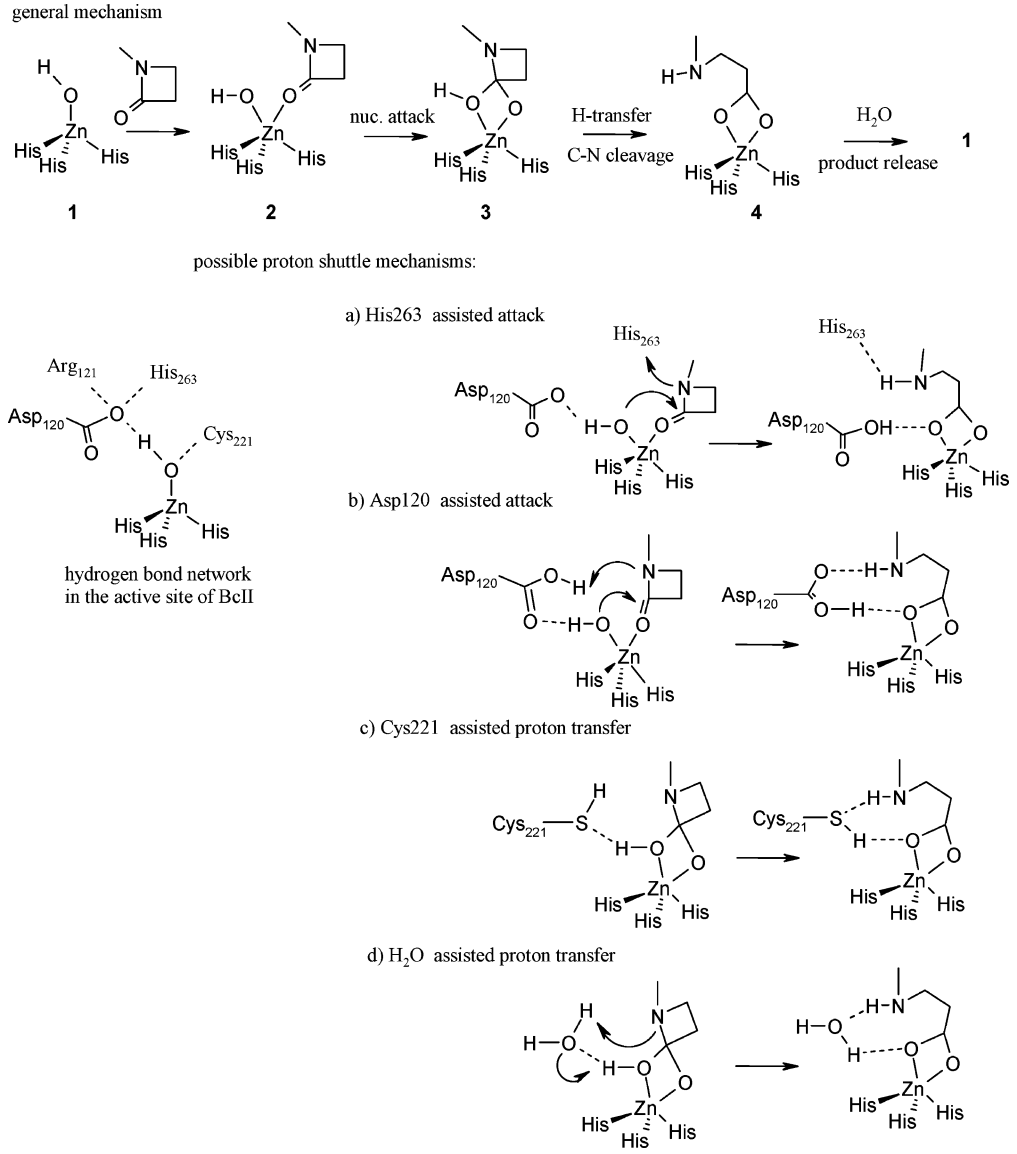

Figure 11. General mode of action of the mononuclear form of BcII.

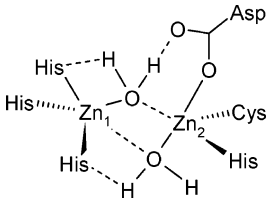

Figure 12. Proposed binuclear form of BcII.

The proton-transfer mechanism (and whether or not a stable intermediate is generated) is probably quite dependent on the identity of the substrate (orientation/size, presence of side groups, etc). The possibility of several effective shuttle mechanisms undoubtedly contributes to the broad band activity of BcII toward most β-lactams.

Less attention has been paid to the binuclear mode of action of BcII. Molecular modeling studies suggest that both waters bound to zinc are possibly protonated (Figure 12).[159] Zn1 seems to play a structural role in maintaining both water molecules through direct contact and histidine-maintained hydrogen bonding. Binding of the β-lactam hardly modifies the active-site geometry. Only the carbonyl group of the lactam is believed to make a direct contact to Zn1 with secondary interactions in the pocket of the active site helping to fix the β-lactam.[159] The Zn1−carbonyl interaction polarizes the β-lactam, thus allowing one of the waters to attack the carbonyl function. The Zn2-bound Asp can accept a proton from the water, thus increasing its nucleophilicity. An acyl intermediate similar to species **5** found for CcrA (Figure 9) is most likely formed.[159]

4. Metalloaminopeptidases

This family of enzymes, which possess either one or two metal ions per active site, specifically cleaves the N-terminal residues of polypeptide chains and thus plays a critical role in protein modification/degradation as well as metabolic cycles in both eucaryotes and procaryotes.[11,13] It is an interesting fact that nature seems to prefer zinc for the hydrolysis of peptide bonds, and quite a few metalloaminopeptidases (MAPs) contain zinc in their active sites. This is in contrast to other metalloenzymes such as phosphatases where magnesium or manganese is often preferred instead of zinc.[1,2,11] When a

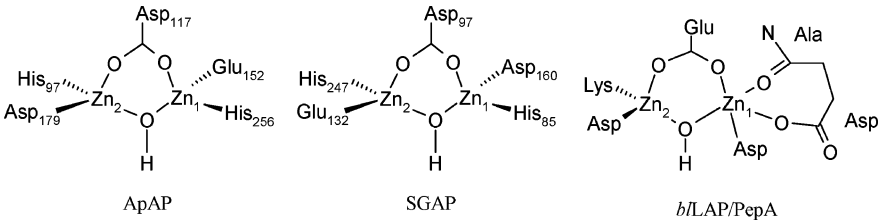

Figure 13. Active-site structures of binuclear zinc aminopeptidases.

binuclear site is present, MAPs tend to be cocatalytic; activity is usually observed only when both metal binding sites are occupied. Two excellent reviews on this topic are available, one on MAPs in general[13] and one concentrating on the aminopeptidase from *Aeromonas proteolytica* (ApAP), which is perhaps the best understood MAP to date.[7] In addition, several reviews on metalloenzymes in general contain excellent subchapters on this theme.[1,2,11]

The solid-state structures of four binuclear enzymes representative of "broad range" MAPs have been well characterized (native structures as well as diverse inhibitor complexes). Broad range MAPs are capable of cleaving N-terminal residues without strict requirements for either the terminal or penultimate residue.[13] The active site in all four enzymes is held together by a bridging carboxylate, and in addition, a μ-OH bridge is present (Figure 13). The two aminopeptidases isolated from *A. proteolytica*[160] and *Streptomyces griseus* (SgAP),[161] although their overall sequence identity is very low (30%),[162] possess almost identical coordination geometries in their active sites. The two metal ions are essentially symmetric, each coordinating an additional His and Asp/Glu in a tetrahedral arrangement. Note that the assignment of Zn1 and Zn2 is reversed in ApAP as compared with SgAP. The site 1 metal ion is defined as that closest to the mouth of the active site, whereas site 2 is more buried and adjacent to the binding pocket for the side chain of the N-terminal amino acid.[163]

The active site of bovine lens leucine aminopeptidase (*bl*LAP)[164] is essentially homologous with that of aminopeptidase A (PepA) from *Escherichia coli*;[165] all catalytically important residues are conserved between the two enzymes, and the active sites are even isostructural.[166]

4.1. Aminopeptidase from *A. proteolytica*

The metalloaminopeptidase isolated from *A. proteolytica* has been the focus of numerous studies as a consequence of its small size (32 kDa), ease of purification, heat stability, and activity as a monomer.[7,13] ApAP exhibits broad band peptidase activity and prefers large hydrophobic N-terminal amino acids with Leu being the most efficiently cleaved. It can accommodate all residues in the penultimate position except Pro and Glu.[13]

The spectroscopically silent zinc ions in the binuclear active site of ApAP can easily be replaced by other metal ions (especially Co^{2+} and Cu^{2+}) without destroying its catalytic activity.[167] Indeed, some replacements result in hyperactive forms of the enzyme.[6] Extensive comparisons of Zn^{2+}- and Co^{2+}-loaded ApAP have led to the conclusion that the roles of the two metal ions appear to be quite similar, and most mechanistic data available for Co^{2+}-substituted ApAP can probably be extended to the physiologically relevant zinc form.[7] EPR spectra of both Co^{2+}- and Cu^{2+}-loaded ApAP clearly show that the metal ions are coupled with each other and are quite capable of modulating each others behavior, thus providing direct experimental evidence for the intrinsic cocatalytic nature of the active site.[168−170]

The first step in peptide hydrolysis is the recognition of the N-terminal acid side chain by a hydrophobic pocket adjacent to the binuclear active site (Figure 14). A significant amount of the overall binding interaction is believed to be provided by tyrosine residues (two are located in this pocket), which help by providing hydrogen bonds to assist in positioning the substrate.[7] Experimental evidence is available for this substrate recognition step. A series of aliphatic alcohols (competitive inhibitors of ApAP) clearly bind to the hydrophobic pocket and not to the active site, which is consequently sterically blocked.[171] Although this hydrophobic pocket was not directly considered in a very recent, quite extensive QM/MM study,[172] an initial docking intermediate was reported which did not directly involve the metal ions (species **2** in Figure 14). A hydrogen-bond network to Glu151 recognized the substrate and oriented it optimally for a later interaction of the N-terminal amino group with Zn1. The presence of side chain interactions with the pocket probably enhances this positioning effect of Glu151.

According to this QM/MM study, Glu51 then deprotonates the N-terminal amino group, thus initiating binding to Zn2 (species **3**).[172] This step differs considerably from all previously postulated mechanisms for ApAP.[7,13,173] Until the QM/MM study on the mode of action of ApAP became available earlier this year, all mechanistic proposals were based on the binding modes found in inhibitor complexes.[7,13,172] Five solid-state inhibitor structures are available, and all clearly show that inhibitors bind to the active site via interactions with *both* zinc ions (Figure 15).[170,173,174]

Quite a few mechanistic studies of inhibitor binding have been performed for ApAP. For example, spectroscopic monitoring of the binding of phenylurea (a competitive inhibitor) revealed that the oxygen atom of the urea functionality directly interacts with the binuclear active center.[175] Kinetic studies of 1-butane boronic acid (BuBa) inhibition shows that it interacts primarily with Zn1, which, together with its solid-state inhibitor complex,[174b] has led it to be described as a "substrate analog".[176] In contrast, L-leucine phosphonic acid inhibits via a phosphonate bridge.[173,177] L-Leucine thiol and other peptide thiols are slow binding inhibitors that bind a thiolate moiety to one

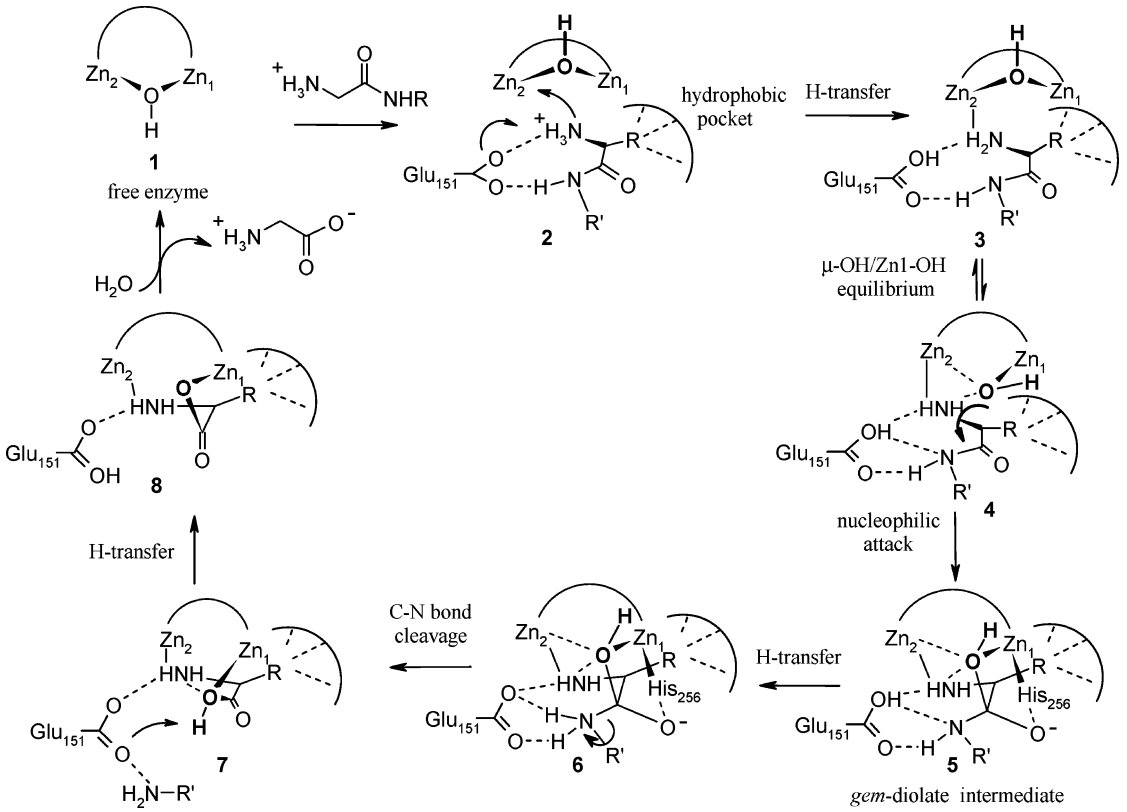

Figure 14. Mode of action of ApAP as it is currently understood.

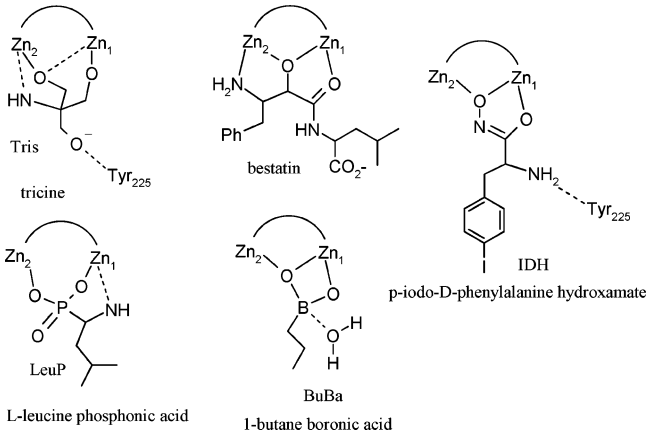

Figure 15. Solid-state structures of ApAP inhibitor complexes.

of the metal ions, likely replacing the μ-OH bridge in the active site.[178] The sulfur, however, does not seem to bridge the binuclear cluster.[178]

On the basis of the inhibitor complexes, it has been assumed that the carbonyl group first binds to Zn2 and then the N-terminal amino group to Zn1 to form a bridged intermediate in a two-step process.[7,13,173] The μ-OH bridge is given up, and a terminal Zn1−OH results in the substrate−enzyme complex which then attacks the carbonyl group bound to the same zinc ion.[7,13,173] However, not a single stable bridged structure or even a Zn1-bound carbonyl intermediate was found on the QM/MM hypersurface.[172] Similar results have been calculated for the mode of action of *bl*LAP (discussed below), which help support the idea that the carbonyl group of substrates does not directly interact with the metal ions.[202,206]

The QM/MM study indicated that N-terminal amino group binding to Zn2 loosens the μ-OH bridge in **3** (Figure 14), and an equilibrium with a terminally bound Zn1−OH species **4** becomes possible.[172] Spectroscopic studies of BuBa-inhibited cobalt-substituted ApAP showed that such an equilibrium is fundamentally possible since inhibitor binding broke up the μ-OH bridge.[169] Fluoride ions also inhibit ApAP—after substrate binding—which also suggests that a terminally bound Zn−OH is present in the substrate−enzyme complex that acts as the nucleophile.[179] Indeed, the QM/MM study shows that the Zn1-bound hydroxide in **4** is quite capable of attacking the carbonyl bond that has been properly oriented by Glu151.[172] A tetrahedral *gem*-diolate intermediate **5** results in which His256 helps to stabilize the negative charge on oxygen.

Protonation of the peptide nitrogen is then accomplished by Glu151. This elongates the C−N bond and facilitates decomposition to **7**. Although this is represented as a two-step mechanism in Figure 14, the QM/MM study does not exclude the possibility that proton transfer may be coupled with C−N bond cleavage in a concerted manner.[172] Experimental mutation experiments,[180] isotope rate effects,[181] and proton inventory studies[180] also suggest that a single proton-transfer step occurs during the C−N bond-breaking step of peptide hydrolysis. There is little doubt that Glu151 functions as a general acid/base in the mode of action of ApAP.[170,180] The QM/MM study[172] suggests that the cleaved residue remains bound to the active center. The peptide (RNH_2) is now bound only by hydrogen bonds to active-site residues and can now easily either diffuse out of the active site or wait until the cleaved rest dissociates before

moving in again to cleave the next N-terminal residue. Product dissociation is believed to again involve Glu151, which now acts as a general base to deprotonate the carboxylic acid rest of the bound product (**8**).[172] Uptake of an active-site water molecule then regenerates the μ-OH bridge and protonates the amino group of the leucine, which can then dissociate as a zwitterion from the active site.[172]

4.2. Aminopeptidase from *S. grieseus*

Similar to ApAP, the aminopeptidase isolated from *S. grieseus* (SgAP) is a broad band MAP with a preference for large hydrophobic N-terminal residues. Since the active site of SgAP is nearly identical to the one observed in ApAP, the major steps of its catalytic mechanism are likely to be quite similar.[182] For example, fluoride ions inhibit SgAP in an analogous manner as ApAP, which indicates that a terminally bound Zn−OH is probably the active nucleophile in the mode of action of SgAP.[183] Furthermore, a recent mutation study combined with kinetic investigations has shown that Glu131 (analogue to Glu151 in ApAP) also acts as a general base.[184]

However, SgAP differs from ApAP in several important aspects. It possesses a unique Ca^{2+} binding site (ca. 25 Å away from the binuclear active center) which stabilizes the enzyme and clearly modulates its activity and affinity toward substrates and inhibitors.[185] The presence of this calcium ion significantly affects the metal binding properties of SgAP in a manner not yet understood.[186] In addition, an arginine (Arg202) and a tyrosine (Tyr246) are located near the active site. Chemical modification of Arg202[187] as well as solid-state structures of various inhibitor complexes[188] indicate that both rests play an important role in substrate/product binding and orientation. SgAP is capable of catalyzing the hydrolysis of not only peptide chains but also phosphate diesters.[189] Since an Arg side chain is involved in the catalytic action of several metallophosphoesterases and phosphotransferases,[2] it has been postulated that Arg202 enables this unique phosphodiesterase behavior.[187] However, ApAP (which does not contain an Arg in its active site) has also demonstrated esterase capability.[190] A satisfactory explanation of how SgAP and ApAP hydrolyze diesters is not yet available.

4.3. Bovine Lens Leucine Aminopeptidase

Bovine lens leucine aminopeptidase(*bl*LAP), prefers to hydrolyze, as its name implicates, a N-terminal leucine in a peptide chain.[13] However, it is also capable of hydrolyzing (albeit somewhat slower) most of the other amino acids as well.[13] This enzyme is extremely widespread and found in the metabolism of mammals where it plays a central role in the degradation and modification of proteins and in sight processes (the concentration of this enzyme in cow eyeballs is high enough to allow it to be conveniently isolated[191]). If the activity of this enzyme is interrupted or disturbed, several pathogenic effects in humans including cancer, leukemia, and cystic fibrosis occur.[192] It has even been implicated in human HIV pathophysiology.[193]

*bl*LAP has a hexameric structure[194] which ensures that each binuclear[195] active site (one per monomer and each subunit functions independently of the others[196]) is sitting on the edge of two water channels—a small one located just above Zn1 and a much larger one for the substrate located below the two metal ions as solid-state structures of native *bl*LAP[164] and several of its inhibitor complexes[197,164] have demonstrated. The zinc ions occupy different coordination sites (Figure 13) and are truly cocatalytic; activity is only observed when both metal binding sites are occupied.[192] The tight binding site ion, Zn2, cannot be replaced by other metal ions except for Co^{2+}, and that is only when absolutely no zinc in the culture medium is present when the enzyme is expressed.[198] Zn1, which possesses an unusual pentacoordinate geometry, can be replaced by Mg^{2+}, Mn^{2+}, or Co^{2+} without destroying the catalytic activity.[164b,198] A flexible lysine side chain (Lys262) is located on the edge of the larger water channel. The amino group in this side chain has a radius of allowed motion of ca. 2.5 Å and is positioned directly beneath Zn1. In many of the inhibitor complexes this group has moved in ca. 2 Å to help stabilize the tetrahedral structure of the transition-state analogue via a direct hydrogen bond.[197,164] Selected mutagenesis has shown that this amino acid is directly involved in the mechanism of hydrolysis.[199] Computational analyses of inhibitor−Lys262 binding interactions also confirm the importance of this interaction,[200] and computer-aided structure-based design and selective synthesis is now being employed to try and develop novel inhibitors for *bl*LAP.[201]

Several catalytic mechanisms based on the binding pattern observed for inhibitor complexes have been proposed for *bl*LAP, all of which have assumed that the reaction runs through a *gem*-diolate intermediate and is stabilized by contacts to Zn1 and Lys262.[164,197] It has been simply assumed that the terminal amino group in the peptide docks onto Zn2 and the carbonyl group onto Zn1, although several docking permutations are theoretically possible. A QM/MM study demonstrated that the terminal amino group does indeed dock onto Zn2; however, neither the carbonyl oxygen nor the NHR group docks onto Zn1 (Figure 16).[202] According to the QM/MM calculations, the substrate backbone must be unfavorably twisted in order to achieve bidentate docking.[202] Instead, the Lys262 group in the pocket of the enzyme stabilizes the docking intermediate via a hydrogen bond to the carbonyl group, thus ideally positioning the peptide function for nucleophilic attack. This is quite similar to the mode of action of ApAP where Glu151 performs a similar function.[172] Upon docking, a proton is probably transferred from the terminal amino group (assumed to be protonated) to the bridging μ-OH functionality which then becomes a terminally bound Zn1−OH_2 group in **2**.[202] This water molecule is not reactive enough to directly attack the carbonyl bond (binding a neutral water to a zinc ion reduces the already weak nucleophilic character of the water[203]). Lys262 is then postulated to deprotonate this water to generate an activated Zn1−OH species **3**. Subsequent nucleophilic attack on the carbonyl bond which

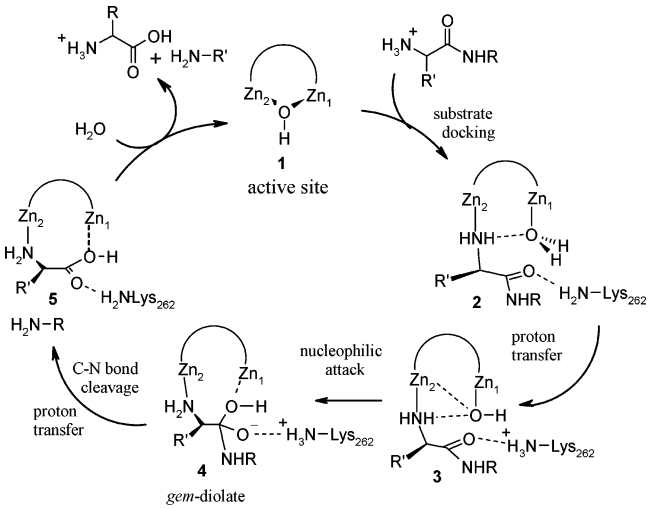

Figure 16. Catalytic cycle for *bl*LAP according to QM/MM calculations.

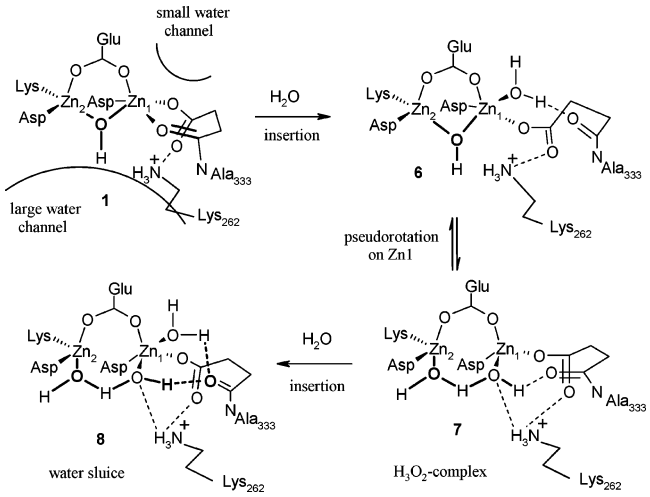

Figure 17. Generation of a water sluice in the active site of *bl*LAP according to DFT calculations.

is polarized by a strong hydrogen bond to the protonated Lys262 leads to the formation of a *gem*-diolate species **4**. The Lys262 then protonates the amino group about to be eliminated. This paves the way for cleavage of the C−N bond, which is the rate-limiting step. Release of the hydrolysis product **5** is most likely achieved by protonation of the N-terminus by an active-site water molecule with the resulting hydroxide ion regenerating the μ-OH bridge found in the free enzyme.

Perhaps the largest problem with this QM/MM study is the size of the calculated barrier for nucleophilic attack. With the best estimate being ca. 50 kcal/mol, this is much too large for an enzymatic process.[189] Although this could possibly be due to insufficiencies in the computational method employed, it is far more likely that fundamental details in the mode of action of *bl*LAP have been missed.

For example, Zn1 features an unusual trigonal bipyramidal coordination. It is extremely unlikely that a neutral ligand (the carbonyl originating from the amide bond to Ala333) can bind to zinc if all other ligands are "hard", e.g., negatively charged oxygen.[204] Yet nature does precisely this in the Zn1 site of *bl*LAP. A density functional study of the active site of *bl*LAP demonstrated that nature manages to "glue" the carbonyl group in place via an extended hydrogen-bond network originating from the peptide backbone in the vicinity of Zn1.[205] It is quite interesting that the second, smaller water channel in *bl*LAP is located just above and to the left of this very loosely coordinated carbonyl group. A more recent DFT study has shown that placing a water molecule in this general area results in its spontaneous insertion into the Zn1−O bond (Figure 17).[206] Zn1 then undergoes an extremely facile pseudorotation, which causes the bridging μ-OH contact to be given up, thus lowering the coordination number on Zn1 to four. A metal-bound H_3O_2 functionality results, which is again capable of spontaneously organizing another water molecule from the smaller water channel. The weakly bound carbonyl ligand functions as a "traffic cop" to direct water molecules coming from the small channel into the heart of the active site.[206]

These calculations provide a plausible mechanism for the continual regeneration of a water sluice capable of acting as an active nucleophile in the *bl*LAP-mediated hydrolysis of amide bonds in peptide chains.[206] Support for the plausibility of this mechanism is independently offered by several solid-state structures of biomimetic zinc complexes with H_3O_2 functionalities.[207,243] Kinetic investigations on biomimetic complexes have also reported that zinc-bound H_3O_2 species are intrinsically more reactive than μ-OH units.[242]

5. Biomimetic Models On the basis of Bi- and Trizinc Cores

Numerous research groups have taken up the challenge to synthesize biomimetic model compounds containing various metal ions that are capable of mimicking the activity of hydrolase enzymes with varying degrees of success. In the zinc sector much effort has been dedicated to the synthesis of *mono*nuclear biomimetic zinc complexes, which is now being expanded to *bi*- and even *tri*nuclear zinc compounds. These synthetic analogues have recently been reviewed in an excellent article by G. Parkin,[14] and this review therefore restricts itself to the presentation of multinuclear zinc compounds which have been reported to exhibit hydrolase-like activity. Special attention will be paid to those complexes for which mechanistic investigations are at least partially available. Their mechanisms are discussed in light of the "natural" modes of enzymatic action presented above.

The general strategies for obtaining stable multinuclear complexes are the spacer and the macrocyclic strategy. In the spacer strategy two simple ligands, each capable of fixing one zinc ion, are glued together by a short, usually rather stiff spacer (also termed a bridging or scaffold or template) unit. A distinct disadvantage of this method is that these complexes often react with the substrate under cleavage and separation of the metal centers. Another problem is that the zinc ions do not necessarily "have" to interact with each other, and as a consequence, the metal−

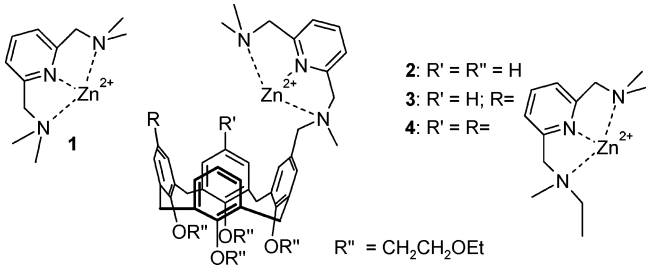

Figure 18. Biomimetic zinc complexes based on calix[4]-arenes.

metal distance is quite flexible and obviously not optimal for catalysis.

In the macrocyclic strategy two metal ions are coordinated within a single macrocyclic framework. This should protect the complex from cleavage by substrate molecules. However, the synthesis of large macrocycles is not by any means trivial; expensive and time-consuming synthetic methods often involving large-scale dilution procedures coupled with low yields makes this method rather unattractive.

In the very recent past second-generation "natural" ligands capable of building binuclear zinc complexes with carboxylate and phosphate linkages (thus directly mimicking the active site of many enzymes) have been synthesized.

5.1. Spacer and Compartment Techniques

Calix[4]arenes that are functionalized with Zn^{2+} complexes **1** at the distal positions of the upper rim (Figure 18) are capable of catalytically cleaving phosphate diesters (a RNA model).[208] Extensive kinetic studies have shown that the binuclear complex **3** increased the rate of reaction by a factor of 23 000 with turnover as compared to the uncatalyzed reaction.[209] A high degree of cooperation between the two zinc centers is present; the mononuclear catalyst **2** is less active by a factor of 50 than the binuclear one.[209] It could be demonstrated that a certain conformational flexibility is essential; tying the calix-[4]arene together at the lower rim, thus making it rigid, inhibited its catalytic activity.[208] The calix[4]-arene is more than just a molecular scaffold since its presence causes a 6-fold increase in the rate as compared to the simple zinc complex **1**.[209] This has been attributed to a lowering in the pK_a of a zinc-bound water molecule due to the hydrophobic aromatic surface of the calix[4]arene unit.[208] Modification of the model by introducing two amino groups on the upper rim of the calix[4]arene (thus making a general base directly available to assist in the deprotonation step) did not improve its performance, a fact probably due to steric overloading in the vicinity of the "active site".[210]

The binuclear complex **3** is very effective in catalyzing the cleavage of activated phosphate diesters. However, it is inactive toward natural substrates such as RNA dinucleotides.[211,212] Modification of the calix[4]arene model to add an additional metal ion resulted in a trinuclear complex **4** that was capable of hydrolyzing RNA dinucleotide substrates such as UpU and GpG.[212] Kinetic investigations indicate that a three-ion cooperativity is present.[208] Interestingly

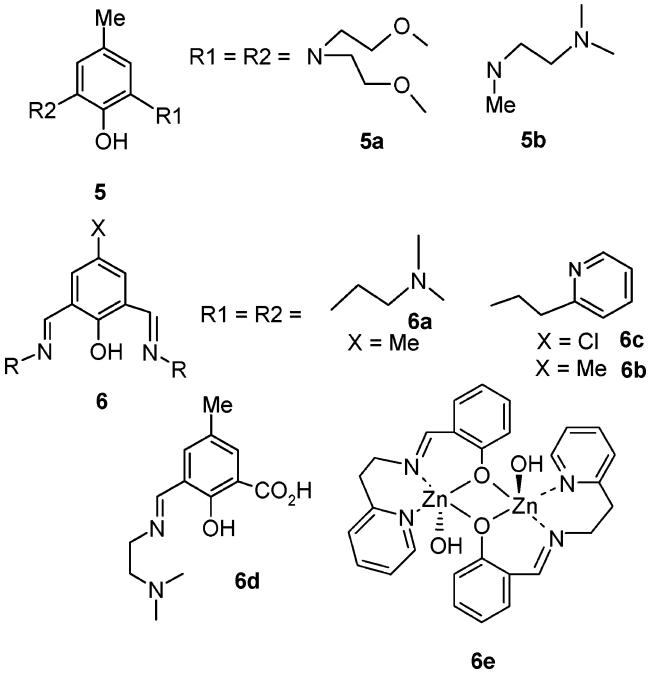

Figure 19. Compartment ligands and one of their binuclear zinc complexes.

enough, a heterotrinuclear Zn_2Cu complex is more active than the corresponding trizinc species.[208] Some naturally occurring metallophosphoesterases also employ mixed metal ions, undoubtedly the result of nature's optimization of the catalytic activity.[1,2,11]

In an attempt to overcome the flexibility problem inherent in the spacer strategy, many researchers are employing spacers with a built in donor heteroatom intended to bridge the two metal ions, thus fixing them 3−5 Å apart from each other. Phenol-based "compartment" ligands (Figure 19) have been quite successful in modeling bimetallic active sites containing transition-metal ions (Mn^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}),[213] but they tend to build M_2L_2 moieties when zinc is employed. With careful control of the zinc ion concentration it is, however, possible to generate the desired Zn_2L complexes.

Some of these complexes are capable of hydrolyzing substrates, among them the first bizinc model reported to exhibit an aminopeptidase capability (species **5a**).[214] There are very few effective functional models for aminopeptidases reported in the literature.[215] Peptide bond cleavage induced by zinc complexes, whether mono- or binuclear, has rarely been observed.[216] Free zinc ions binding to peptides may even inhibit the cleavage of the peptide bond.[217] A related compartment complex **5b** synthesized by Lippard et al. is a functional model for metallo-β-lactamases as it is capable of hydrolyzing nitrocefin.[218] Extensive kinetic and spectroscopic studies show that the reaction mechanism is quite similar to that postulated for the CcrA (*B. fragilis*) mediated hydrolysis of nitrocefin illustrated in Figure 9, including the direct observance of an acyl intermediate.[218]

Okawa,[219] Erxleben,[220] and Ye[221] exchanged the amino arm for an imine (compounds **6**). Some of their ligands yield binuclear zinc complexes that are also capable of aminopeptidase-like (ligand **6a**) and phos-

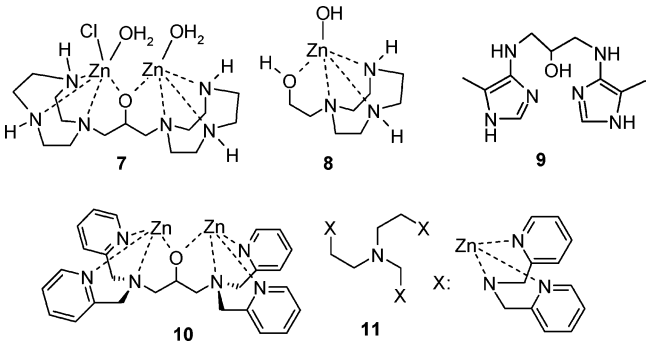

Figure 20. More biomimetic zinc complexes based on spacer/compartment techniques.

phatase-like (**6b**, **6c**) activity. Substitution of one of the chelate arms by a carboxylic acid in an attempt to introduce asymmetry (ligand **6d**) led, however, to either Zn_2L_2-type complexes or tetranuclear arrays.[222] Döring et al. removed one of the sidearms. Although this led to a L_2Zn_2 complex (**6e**), the solid-state structure revealed that two terminal Zn-bound hydroxides are present—a fact which is promising for possible hydrolysis activity.[223]

It has been demonstrated that a phenol is not necessary for the bridging unit; a simple alcohol functions just as well (Figure 20). Recently, Richard and Morrow et al. performed extensive kinetic studies on complex **7,** which catalyzes the hydrolysis of an activated phosphate diester.[224] This complex is also capable of hydrolyzing a chemical model for the 5′-cap of mRNA.[225] The binuclear **7** shows a clearly enhanced cooperative rate of reaction as compared to the mononuclear **8**. This is attributed to a greater ease of formation of an essential zinc-bound hydroxide (pk_a 7.2 for **7** and 9.2 for **8**).[224] The alcohol linker has a very low pK_a and is coordinated as an alkoxide to the two zinc ions. In the mononuclear **8** the alcohol remains protonated and a zinc-bound water is deprotonated at pK_a 9.2. Although the compartment ligand is symmetric, the solid-state structure of **7** demonstrates that the zinc ions have different coordination numbers and geometries. It is believed that a μ-OH bridge is not formed.

Although a solid-state structure of the zinc complex formed from ligand **9** is not yet available, a kinetic study has demonstrated that a binuclear Zn_2L unit is responsible for the hydrolytic activity of this ligand.[226] In the presence of Zn^{2+}, **9** efficiently hydrolyzes uridine 2′,3′-cyclic monophosphate (a RNA model).[226]

A related binuclear complex **10** with pyridine arms is capable of hydrolyzing the simple diribonucleotide ApA.[227] Better results were obtained when the O-linkage in **10** was replaced with an amine linkage to yield the trinuclear zinc complex **11**. This species is capable of hydrolyzing a whole series of ribonucleotide dimers at pH 7 and 50 °C.[228] Kinetic investigations confirmed that the trinuclear species was indeed responsible for the hydrolysis activity.[228] When two units of **11** are bound via a benzene linkage to a phosphor−amidit monomer, a sequence-selective RNA hydrolyzer can be obtained.[229]

Kimura et al. discovered that two cyclene zinc complexes held together by a *p*- or *m*-xylyl spacer (**12**) quite selectively and efficiently recognize thymidine and uridine nucleotides in aqueous solutions at physiological pH (Figure 21).[230] They are also good receptors for barbiturates.[231] If appropriate lipophilic carriers could be developed, such complexes would possibly yield novel transport agents for dT-nucleotide drugs.[230] The complexes **12** also inhibit the photo[2+2]cycloaddition of thymidilyl(3′-5′)thymidine, which is a typical UV-light-catalyzed damage mechanism (potentially carcinogenic) in cellular nucleic acids.[232] These bis-Zn^{2+}−cyclenes may possibly be valuable as a new prototype of chemical blocker against photodamage by UV light.[232]

Mechanistic investigations on *m*-**12** demonstrated that linking the two units together induces a differential pK_a with values of 7.2 (first) and 7.9 (second deprotonation) for the bound water molecules as compared to the mononuclear reference compound **13** (pK_a = 7.6).[233] In addition, *m*-**12** shows a dynamic behavior on the ^{1}H NMR time scale between pH 6.7 and 8.5 which has been attributed to an equilibrium between a "closed" (more stable) and an "open" monohydroxy species.[233,234] Introducing a third cyclene unit (species **14**) generated a biomimetic model for enzymes containing three zinc ions (phospholipase C/P1 nuclease).[233] The trinuclear complex **14** cooperatively recognizes phosphate dianions in aqueous solution.[234]

A completely different tactic based on a spacer approach developed by Scrimin et al. employs artificial heptapeptides which have been modified to carry two zinc-complexing pendant ligands.[235] An example (species **15**) is illustrated in Figure 21. These peptides possess a stable helical conformation (exactly two turns) in solution. The helical twist positions the pendant ligands close enough together so that they can cooperatively hydrolyze RNA and even DNA model substrates.[236,237] If three of these zinc−pendant-bearing peptides are linked with each other via a tris(2-aminoethyl)amine platform, an allosteric supramolecular catalyst (not illustrated) is obtained that readily catalyzes the transphosphorylation of phosphate esters.[238] Kawai et al. also managed to build two zinc chelating units into a cyclic β-sheet peptide (not illustrated).[239] The resulting binuclear complex is capable of hydrolyzing a RNA model substrate.[239]

Attempts to synthesize binuclear zinc compounds containing a phosphate bridge have been successful with a naphthyridine-based ligand BPAN[240] (Figure 22). This ligand organizes a water and a phosphate molecule to form a phosphate-bridged binuclear zinc species **16** containing a μ-hydroxide-bridge with a pK_a of 6.8.[240] This seems to be the first *functional* phosphate-bridged biomimetic complex reported in the literature since it effectively promotes the hydrolysis of phosphodiester and β-lactam substrates, thus mimicking the enzymes P1 nuclease and β-lactamase, respectively.[240] Kinetic investigations on the hydrolysis of bis(*p*-nitrophenyl)phosphate indicate that the bridging hydroxide in **16** acts as the active species, functioning as a general base to deprotonate a water molecule.[240] A pthalazine-based ligand (BDPTZ) is

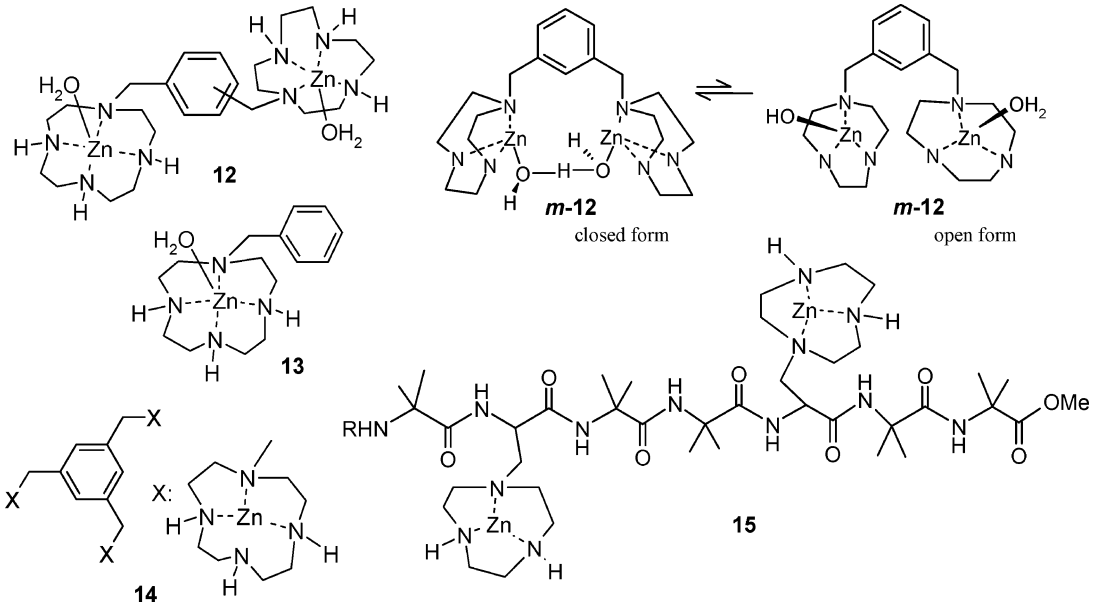

Figure 21. More biomimetic zinc complexes based on spacer/compartment techniques.

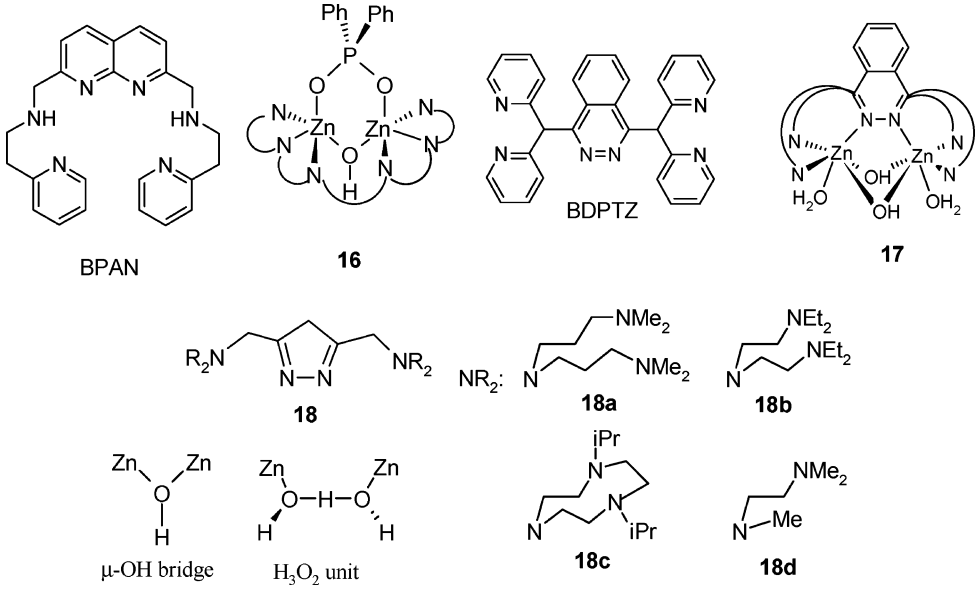

Figure 22. More biomimetic zinc complexes based on spacer/compartment techniques.

also capable of building a discrete bimetallic complex **17** with μ-hydroxide bridges (Figure 22).[241]

If the spacer is a pyrazolate functionality, a series of linker ligands **18** can be employed to obtain discrete bizinc complexes.[242] Depending on the length of the linker, either a μ-OH unit or a bridging H_3O_2 species can be isolated.[243] The longer linkers **18a/c** prefer a μ-OH bridge, whereas the shorter ligand sidearms in **18b/d** pull the two zinc ions apart, thus inducing incorporation of an additional solvent (water or methanol) to give a H_3O_2 unit. Extensive comparative kinetic and spectroscopic studies on the series **18** has led to the realization that a μ-OH unit is a relatively poor nucleophile and either a terminal Zn−OH unit or a H_3O_2 bridge is much more reactive.[242]

5.2. Macrocyclic Approach

In 1994, Bencini, Bianchi, and Paoletti reported the synthesis of a macrocyclic ligand that organizes two Zn^{2+} ions and two water molecules to form a $Zn_2(\mu\text{-}OH)_2$ cluster **19** (Figure 23).[244] Unfortunately, this complex is relatively inert, and although **19** readily complexes with simple substrates such as 2-hydroxypyridine or cytosine,[244] no hydrolysis activity was observed. Kimura et al. synthesized a very similar complex **20** (eight instead of seven nitrogen atoms and NH instead of NMe) which proved capable of cleaving an activated monophosphate ester; however, the hydrolysis product remained stuck to the complex.[245]

Bencini et al. then turned to a simpler macrocycle **21** which is a successful biomimetic complex for the mononuclear zinc enzyme carbonic anhydrase[246] and expanded the ring to make room for two zinc ions (ligand **22**).[247] This ligand readily builds a stable binuclear zinc complex **23** with a μ-hydroxide linkage in aqueous solution.[247] Measurement of the pK_a of the bound water in **23** yielded a value of 7.6.[248] In more

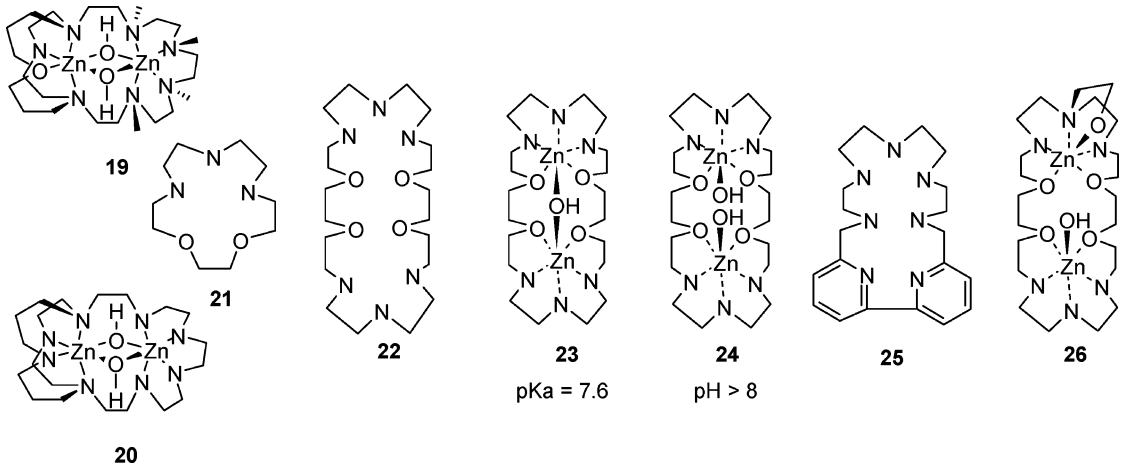

Figure 23. Selected macrocyclic ligands and their zinc complexes.

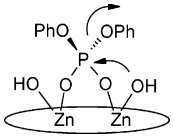

Figure 24. Postulated mechanism of phosphate hydrolysis promoted by complex **24**.

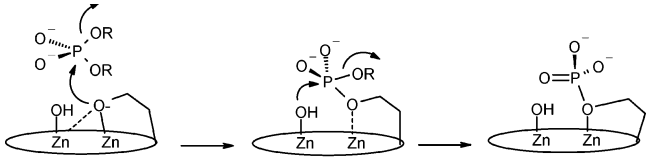

Figure 25. Postulated mechanism of phosphate hydrolysis promoted by the binuclear zinc complex **26**.

basic solutions (pH > 8) a second water is bound and deprotonated (complex **24**).[248]

The bihydroxy species **24** promotes the hydrolysis of bis(*p*-nitrophenyl) phosphate (BNP)[248] as well as adenylyl(3′-5′)adenosine (ApA).[249] Kinetic studies show that the hydrolysis occurs via a bimolecular mechanism and that the monohydroxy species is inactive.[248] In particular, complex **24** is 10 times more active that the mononuclear zinc complex based on ligand **21**. It has been proposed that the two zinc ions work cooperatively to fix the phosphate diester through a bridging interaction with both electrophilic Zn^{2+} centers. One of the nucleophilic Zn−OH functionalities is then transferred to phosphor (Figure 24). In support of this mechanism, Bencini et al. succeeded in obtaining a crystal structure of diphenyl phosphate complexed with **24** in which the phosphate unit does indeed bridge the two metal ions.[248]

In an attempt to analyze the effect of different coordination environments of the Zn ions, Bencini et al. simply enlarged the macrocycle **22** by introducing additional heteroatoms.[250] However, the ability of the resulting binuclear zinc complexes to hydrolyze BNP decreased and the pK_a of the active bihydroxy species increased.[250]

Introduction of bipyridine units in the macrocycle in hopes of slightly increasing the rigidity of the ligand resulted in a system with a complex coordination behavior of the zinc ions that is dependent on both the pH and the Zn^{2+} ion concentration.[251] An example of this is an ion-hopping mechanism observed for ligand **25**. Depending on the pH of the solution and the Zn^{2+} ion concentration, monoadducts are formed either inside or outside of the ring cavity; under alkaline conditions and higher Zn^{2+} concentrations a binuclear adduct is observed.[251]

Bencini et al. then went on to synthesize a monohydroxide complex by adding an alcohol-pendant to the macrocycle (complex **26** in Figure 23).[252] This compound contains both a Zn-bound alkoxide and a Zn−OH nucleophilic function and is thus a biomimetic model for alkaline phosphatases where both a deprotonated serine and a Zn−OH function are involved in phosphate ester hydrolysis. This complex hydrolyzes both *p*-nitrophenyl acetate (NA) and BNP at an even faster rate than that found for **24**.[252] NMR tracking experiments identified species **27** (Figure 25) as an intermediate step toward generating the final hydrolysis product **29**.[252] Compound **29** is extremely stable and exceedingly inert. Release of the hydrolyzed phosphate, thus enabling a catalytic process, could not be realized.[252]

If either thymine or uracil is added to a solution containing either **23** or **24**, these nucleobases are deprotonated and used to replace the OH in the first coordination sphere of both Zn ions in the binuclear cluster.[253] In this manner a stable supramolecular structure can be formed.[254] A binuclear zinc complex also based on a macrocyclic ligand that exhibits a similar binding ability toward thymidylylthymidine (TpT) has also been reported by Martell et al.[255] In vivo investigations of Martell's complex indicates that it is capable of inhibiting tumor cell growth. Binuclear zinc complexes with macrocyclic ligands are thus potential synthetic receptors for nucleobases, nucleosides, and nucleotides. Due to the biological nontoxicity of Zn, medical applications could be promising in this sector.

The bizinc complex of a related macrocycle containing acetylene spacers (species **30** in Figure 26) is capable of hydrolyzing activated carboxy esters (aminopeptidase model) with catalytic turnover. Potentiometric equilibrium measurements and X-ray structural analysis indicate that both a μ-OH bridge and a terminal ZnOH unit are present in the catalytically active form.[256] Another zinc macrocycle **31** containing

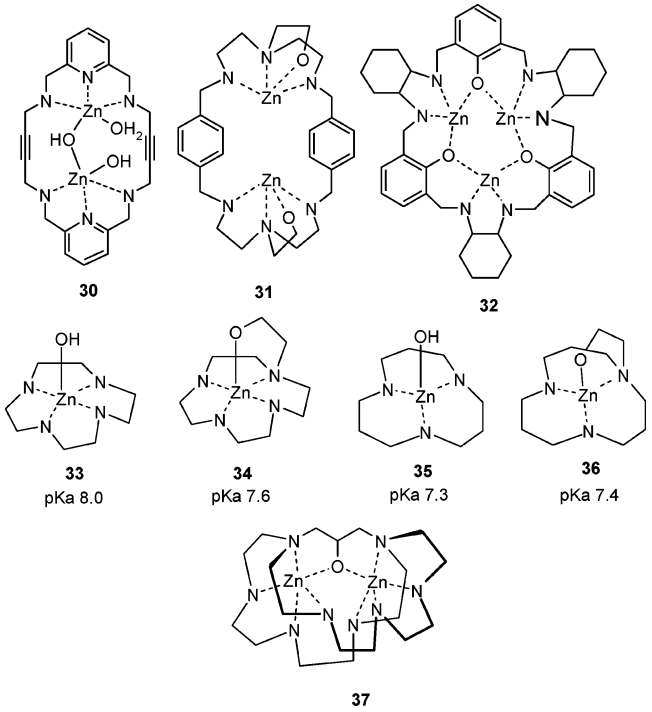

Figure 26. More biomimetic zinc complexes based on polyamine macrocycles.

phenyl spacers and two alcoholate pendants crystallizes with an acetate ion bound to each zinc ion. It is capable of hydrolyzing 4-nitrophenyl acetate and is believed to function via a ping-pong mechanism similar to the mode of action of alkaline monophosphatase.[257]

Not many biomimetic complexes are available for trinuclear zinc enzymes such as phospholipase C or P1 nuclease. However, Zacharias et al. managed to tailor a macrocycle that complexes with three zinc ions (species **32**) and is capable of cleaving calf thymus DNA.[258]

Kimura et al. performed a series of studies on macrocyclic polyamine complexes of zinc as biomimetic compounds for alkaline phosphatase (Figure 26). Initially they employed mononuclear complexes without[259] (**33**, **35**) and with[260] (**34**, **36**) alcohol pendants in order to investigate the role of Ser_{102} in the catalytic mechanism. Many of these complexes are able to hydrolyze tris(*p*-nitrophenyl) phosphate (TNP), bis(*p*-nitrophenyl) phosphate (BNP), and *p*-nitrophenyl acetate (NA).[259,259,260] Kinetic studies demonstrated that the Zn-bound OH or alkoxide group acts as a nucleophile to attack the phosphor or acetate group while the zinc anchors the substrate through a Zn−O=P or Zn−O=C contact.[260] These model studies also showed that the Zn-bound alkoxide is a much better nucleophile than the Zn−OH.[260]

Extending their model system to the binuclear case, Kimura et al. then synthesized a related macrocycle that complexes with two zinc ions to build a discrete dimer **37**.[261] An X-ray crystal structure of **37** showed that the two Zn^{2+} ions are equivalent, each with a distorted trigonal bipyramidal coordination.[262] Both zinc ions work cooperatively to selectively react with phosphomonoesters (but not neutral phosphotriesters or monoanionic phosphodiesters) in aqueous solution. The monophosphate binds irreversibly to both zinc ions under nucleophilic cleavage (one of the apical amines dissociates and attacks the phosphor atom) of a P−O ester bond. A bimetallic phosphate-bridged complex results that is thermodynamically quite stable.[261,262]

5.3. Complexes On the basis of Natural Ligands

A large fraction of all multinuclear biomimetic complexes contains ligands other than those used by nature such as amines, pyridines, and alkoxides. This is often a very important synthetic advantage and quite appropriate for developing practical biomimetics. However, one would like to have biomimetics containing "natural" ligands (carboxylates and imidazoles) for direct comparison with the enzymatic systems. Many binuclear enzymes (not only zinc-based) contain two metal ions surrounded by carboxylate groups (at least one bridging both metal ions), a μ-OH linkage, and one or more histidine residues.[263] The synthesis of a discrete bimetallic core that mimics these enzymes is, however, no trivial task due to either unwanted oligomerization reactions or hydrolysis of the bimetallic core itself.

A partial solution to this problem has been presented by Lippard et al., who employed the "preorganized dinucleating carboxylate ligand"[264] *m*-xylylenediamine bis(Kemp's triacid imide), H_2XDK, **38,** for the remarkably successful generation of carboxylate-bridged bimetallic complexes (not just zinc).[264,265] An example of such a complex (species **39**) is illustrated in Figure 27. Addition of diphenyl phosphate ions to solutions of these complexes causes the (in most cases irreversible) formation of stable phosphate-bridged species,[266] which have been postulated as structural models for phosphate ester hydrolysis in alkaline phosphatase and DNA polymerase I. However, the thermodynamic stability and relatively limited solubility of these addition products unfortunately inhibits catalytic applications. Extension of the XDK system to include a D-glucopyranosyl unit allows complex homo- and heterotrimetallic complexes (crystals) to be synthesized.[267]

Moving away from the XDK system, Lippard et al. succeeded in reacting a smaller, sterically hindered carboxylate ligand with zinc ions in the presence of pyridine to form a doubly carboxylate-bridged bimetallic complex **40**.[264] No hydrolysis data is yet available for **40**. A very interesting, doubly carboxylate-bridged thiolate complex **41** has been reported by Yam et al. in which the bridging and terminal thiolates readily undergo fluxional exchange.[268] Perhaps the most interesting property of **41** is that it is luminescent.[268] A solution of benzhydroxamic acid, tetramethylethylenediamine, and $Zn(OAc)_2$ undergoes a spontaneous self-assembly to form **42**, whose solid-state structure closely resembles the hydroxamate−inhibitor complex of ApAP.[269]

Anslyn et al. recently reported a binuclear zinc complex **43** containing "all natural" ligands (per zinc two imidazoles and one carboxylate) that is capable of hydrolyzing HPNPP (2-hydroxypropyl-*p*-nitrophenyl phosphate), Figure 28.[270] Complex **43** is, however, not capable of cleaving RNA.[270] Another imidazole-

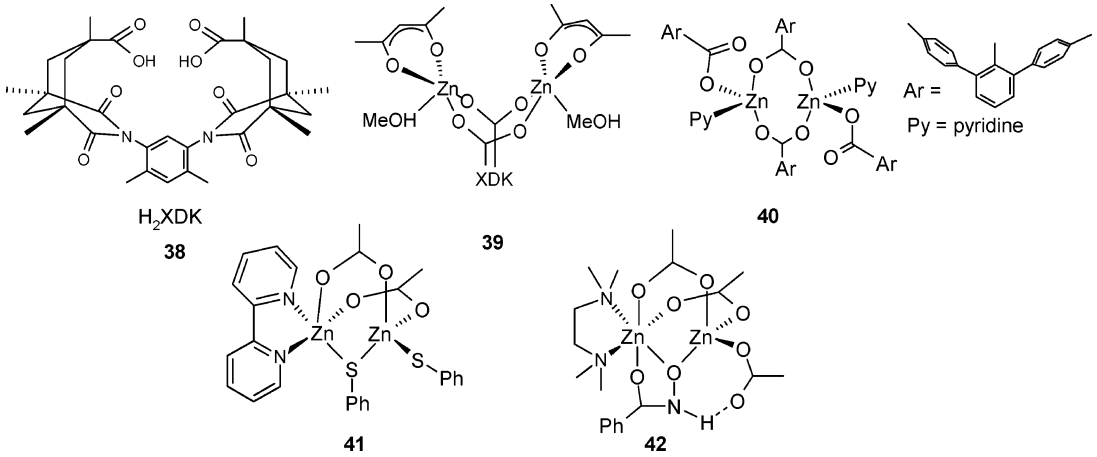

Figure 27. Synthetic binuclear zinc complexes containing carboxylate bridges.

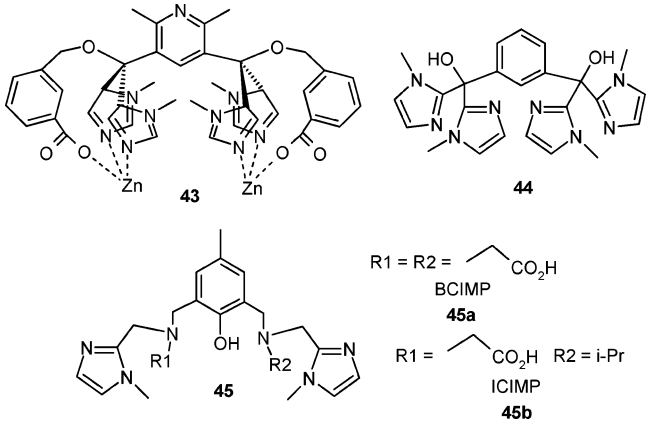

Figure 28. Synthetic binuclear zinc complexes containing imidazole ligands.

based ligand capable of cleaving activated phosphate diesters in the presence of two equivalents of Zn^{2+} is **44**.[271]

Nordlander et al. recently attached pendant arms containing imidazole and carboxylate functionalities to toluene to obtain the BCIMP **45a** (symmetric) and ICIMP **45b** (asymmetric) ligand (L^1) systems.[272] These complex with zinc ions and diphenyl acetate (coligand L^2) to form tetramers (dimers of $Zn_2L^1L^2$ dimers) in the solid state.[272] The tetramers dissociate in solution to yield biomimetic models for the active site of phosphotriesterase.[272] The asymmetric complex **45b** is capable of hydrolyzing 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP).[272]

6. Summary

Understanding the mode of action of binuclear zinc hydrolases is a very challenging field of research which requires a great amount of interdisciplinary cooperation. Traditional molecular and structural biology lays the foundation upon which mechanistic investigations follow. Computational investigations then help to tie the experimental information together and help illuminate the individual chemical steps involved (which often cannot be directly observed). The knowledge thus gained often leads to new questions, thus prompting new investigations. Parallel to investigations on the enzymatic systems themselves, the synthesis and mechanistic studies of smaller inorganic bizinc complexes (biomimetic systems) promises invaluable insight into the chemical processes taking place in the heart of the enzymatic active sites.

The *rational* development of binuclear zinc compounds capable of functioning as biomimetic hydrolases is, as has been shown in this review, by no means a simple task. It assumes knowledge of not only of the structure of the active site but also the enzyme's mode of action. Once known, essential structural principles must then be incorporated into synthetically accessible discrete bi- or trizinc compounds. These potential catalysts must be soluble and stable in the desired medium (preferably water) and capable of organizing the reactants. Once hydrolysis has occurred, the products must be able to leave the first coordination sphere of the zinc ions in order to induce a catalytic circle.

The different steps in this procedure have been focused on by various research groups, each with their own area of expertise and each working on specific problems encountered in the understanding of natural systems or in the design and synthesis of working biomimetica. This review has attempted to gather all major work done in this area and present it in a logical fashion that clearly shows the evolution from understanding the natural systems to successful synthetic biomimetica just beginning to be developed. As evident from the text, this field of research is undergoing rapid development.

7. Acknowledgments

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