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Toward the development of metal-based synthetic nucleases and peptidases: a rationale and progress report in applying the principles of coordination chemistry

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Abstract

Recently there has been great interest in designing small metal complexes which are capable of catalytically hydrolyzing deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins. In this review, we attempt to justify this goal, as well as discussing some of the strategies currently being pursued. In addition, because much of the work is based on a few well-studied enzymatic hydrolases and exchange-inert metal complexes, the mechanisms proposed for these systems will also be briefly discussed, with special attention being paid to the relevance of these systems to the design of synthetic metallohydrolases. However, since the ultimate goal is not only to design an efficient hydrolase, but also to completely understand the mechanism, this review will generally be limited to labile metal complexes for which the coordination geometry is known and detailed kinetic and/or mechanistic information is available. © 1998 Elsevier Science S.A.

Keywords: Hydrolysis; Metal complexes; Nucleic acids; Peptides; Phosphodiesters; Proteins

1. Introduction

The human genome consists of 23 chromosomes and approximately three billion base pairs of deoxyribonucleic acid (DNA) [1]. Within this maze of DNA are coded 100 000 genes [1], each of which must be transcribed into messenger ribonucleic acid (mRNA) before the corresponding protein can be synthesized. A single mutation in any one of these genes, loss of the mRNA, or degradation of an essential protein could potentially lead to serious consequences.[2] Given the obvious importance of maintaining the integrity of the genetic code and the proper function of the proteins, nature could hardly have devised more suitable links than the phosphodiester bonds which join the nucleosides in RNA and DNA [3] and the peptide bonds which couple the amino acids in proteins (Fig. 1). The kinetic stability of these biopolymers is remarkable. The half-life for hydrolysis of phosphodiester bonds in DNA at neutral pH and 25 °C has been estimated to be 130 000 years [4]. Due to the presence of the 2′-hydroxyl on the ribose ring in RNA, the phosphodiester bonds in RNA are hydrolyzed much more readily, with an estimated half-life of four years under identical conditions [5]. The peptide bonds which link amino acids in proteins have a half-life of at least seven years at neutral pH and 25 °C.[3] In the case of RNA and DNA, the resistance to hydrolysis is due primarily to the repulsion between the negatively charged backbone and potential nucleophiles [3], while in the case of proteins, delocalization of the electron density in the peptide bond makes the carbonyl a poor electrophile [8].

[2] For example, any one of at least 375 different mutations on chromosome 7 could lead to cystic fibrosis. Other heritable genetic diseases caused by a mutation in a single gene and resulting in nonfunctional or partially functional proteins include Huntington's disease (X chromosome), Beckwith–Wiedemann syndrome (chromosome 11) and familial hypercholesterolemia (chromosome 19) [2].

[3] The half-life of the peptide bond has been estimated by two research groups using different techniques: Kahne and Still [6] estimated the half-life to be approximately seven years, whereas Radzicka and Wolfenden [7] calculated the value to be between 350 and 600 years.

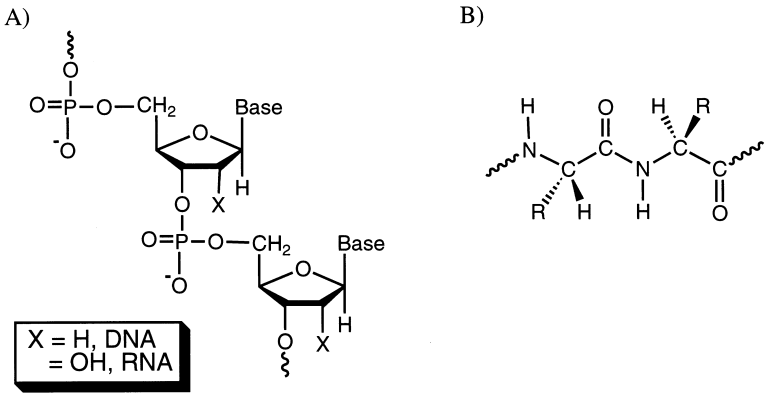


Fig. 1. Structure of chemical bonds found in various biopolymers: (A) phosphodiester bonds link the nucleosides of DNA and RNA; (B) amide (peptide) bonds link amino acids in proteins and polypeptides.

Ironically, the same property which makes these linking bonds so useful in biopolymers, i.e. their kinetic stability, can also be a problem. For example, foreign DNA and proteins, once recognized, need to be destroyed. Mutations in native DNA need to be excised and repaired. Messenger RNA must be hydrolyzed so that the protein it encodes is not synthesized unnecessarily. Finally, native proteins must be degraded into their corresponding amino acids once they have served their function and are no longer needed. Of course, all of this must be accomplished on a physiologically relevant timescale. To accomplish this formidable task, nature utilizes a class of enzymes called hydrolases [9].

Presently, there is considerable interest in developing synthetic hydrolases, i.e. complexes which hydrolyze RNA, DNA or proteins, not merely cleave them (the importance of this distinction will be discussed later). While important reviews detailing various strategies have been published [10–14], less attention has been paid to articulating the rationale for achieving this goal and the mechanistic foundations supporting these strategies. Clearly, work in this field is firmly rooted in both classical coordination chemistry and the insights gained from a few, well-studied hydrolase enzymes (which have since served as a paradigm for this chemistry), but the connection between the enzymes and synthetic hydrolases is rarely discussed. In this review, we attempt to both justify the development of synthetic hydrolases and explain some of the strategies for their development, as well as briefly summarizing the progress in the field to date. Because we are interested not only in designing efficient and catalytic hydrolases, but also in understanding completely the mechanism by which the metal ion promotes hydrolysis, this review will generally be limited to mononuclear labile metal complexes for which the coordination geometry is known and detailed kinetic and/or mechanistic information is available.

2. Why develop synthetic hydrolases?

Although numerous naturally occurring hydrolases are known, the development of synthetic nucleases and peptidases would be of great utility and importance.

Perhaps the most dramatic use of a synthetic hydrolase would be as an artificial restriction enzyme.[4] In theory, sequence specificity could be built into an artificial restriction enzyme either by encoding sequence specificity into the cleavage agent itself or by linking it to an appropriate DNA binding agent [15–17]. Such a molecule would be useful not only to molecular biologists attempting to cut DNA at a site not recognized by current restriction enzymes, but also to those attempting to sequence the human genome. Natural restriction endonucleases, which recognize 4, 6 or 8 base sequences, have sites which are not uniformly distributed in the genome and yield too many fragments to be easily separated. It has been proposed that a reagent which recognizes a 15-base sequence would be exceedingly useful [18]. Such a reagent would yield larger fragments which could be readily isolated and these larger fragments could then be subjected to further hydrolysis by less discriminating restriction endonucleases. The opposite problem exists in determining the sequence of large proteins; because natural peptidases have cleavage sites which are not evenly distributed, they often yield fragments which are too large for sequencing [19]. Moreover, the commonly used chemical peptide cleavage agent, cyanogen bromide, is both volatile and toxic [19]. Thus, it would be desirable and advantageous to create a synthetic peptidase which could be tailored to specific applications. In this way, synthetic nucleases and peptidases would augment, not replace, naturally occurring hydrolases.

Synthetic hydrolases could also find great utility as conformational probes. Information about the three-dimensional structure of nucleic acids or proteins is typically gained from nuclear magnetic resonance (NMR) or X-ray crystallography. While these are two powerful techniques, each requires large amounts of material [18] and the difficulties of obtaining X-ray-quality crystals of proteins and nucleic acids are well known. Moreover, the large and complex nature of biopolymers can make interpretation of NMR spectra exceedingly difficult, especially when little additional information is available. To aid in structure determination, synthetic hydrolases could be designed to recognize certain conformations such as cruciforms, single-stranded regions, left-handed helices or, in the case of proteins, turn regions between two α-helices [18].

Finally, synthetic metallonucleases and metallopeptidases could be helpful in elucidating the role of metal ions in natural hydrolases. Although many hydrolases utilize metal ions, the precise roles of the metal ions in the hydrolysis reactions are not known [18]. Metal ions could promote hydrolysis by providing (1) a scaffold to ensure proper conformation; (2) activation of the carbonyl or phosphodiester bond; (3) charge neutralization to facilitate nucleophilic attack, stabilize charge build-up in the transition state and stabilize the leaving group; and/or (4) a powerful nucleophile at neutral pH [12]. Obtaining a better understanding of the precise function of metal ions in synthetic systems would not only aid in the development of more efficient synthetic hydrolases, but also provide a deeper knowledge of the role of metal ions in natural nucleases and peptidases.

[4] Restriction enzymes are endonucleases which recognize short sequences in double-helical DNA and catalyze the double-stranded hydrolysis of the DNA at those sites.

Oxidative cleavage agents have been utilized in some of the potential applications of synthetic nucleases previously described, providing an important proof-of-concept [20–23]. While oxidative cleavage agents are very efficient, their application has been limited for two reasons. Firstly, oxidative cleavage mechanisms often produce diffusible free radicals. While this is not a problem for some applications, such as DNA footprinting, it can cause difficulties when trying to determine the exact location of targeted secondary structures. Secondly, oxidative cleavage agents do not produce fragments consistent with those produced by natural hydrolases, i.e. 5′-phosphates and 3′-hydroxyls for nucleic acids and free amino termini for proteins. Therefore, nucleic acids cleaved oxidatively cannot be enzymatically religated, limiting the utility of oxidative cleavage agents in molecular biology. Moreover, since protein sequencing via Edman degradation requires free amino termini [24], polypeptides generated from the oxidative cleavage of proteins cannot easily be sequenced. A synthetic hydrolase which does not have these drawbacks is therefore preferable to an oxidative cleavage agent.

3. Strategies for designing synthetic hydrolases

In order to catalyze the hydrolysis of amide or phosphodiester bonds, a synthetic nuclease must by definition be able to manipulate the energy of the transition state to lower the activation energy of the reaction [25,26]. This task is accomplished by enzymes in four different ways: (1) bringing the reactants in close proximity to each other; (2) providing functional groups for acid–base catalysis; (3) inducing bond strain (i.e. binding to the transition state structure more strongly than to either the reactants or the products); and (4) providing an attacking group [27]. Bringing the reactants together in close proximity, thereby decreasing the entropy of activation, has little relevance in aqueous hydrolysis reactions. Moreover, in order to contort a molecule so as to aid in bond cleavage, enzymes rely on multiple substrate-enzyme interactions; small, easily synthesized synthetic hydrolases are unlikely to be able to emulate such interactions. When designing synthetic hydrolases, one should therefore concentrate on providing powerful nucleophiles. In addition, since pure organic compounds cannot provide strong attacking groups at neutral pH in aqueous solutions [26], synthetic nucleases should contain metal ions.

Strategies for designing synthetic metal-based hydrolases can be obtained from the study of natural metallohydrolases. While there are a large number of these enzymes known, the description provided herein will be restricted only to a few of those enzymes for which useful mechanistic information is available. In addition, because much of the mechanistic insight into enzymatic nucleic acid hydrolysis is derived from phosphatases (enzymes which catalyze the hydrolysis of phosphate groups from phosphomonoesters), a brief discussion of phosphatases will also be included.

3.1. Lessons from nature

3.1.1. Nucleases and other phosphohydrolases[5]

Enzymes which hydrolyze phosphodiester and phosphomonoester bonds frequently employ multiple catalytic metal ions. However, the identity of the metal ions used, their specific ligand environments and their mechanistic roles are difficult to discern [29,30]. Many of these proteins do not qualify as true metalloproteins, that is they bind metal ions with low affinity and are isolated in the metal-free or partially-loaded state. The metal ions most frequently employed are Mg^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+}. Their presence in the enzyme, however, is often difficult to determine and their lability makes it difficult to identify the coordination environments. Much of the available information on the mechanistic role of the metal ions comes from crystal structure analyses and the effect of varying the pH and the identity of the metal ion on the enzymatic activity. Even where crystal structures are available, it is often unclear whether the metal ions seen in the crystal are in the native binding sites or even if the specific metal ions bound are the catalytically relevant ones.

The two most thoroughly studied classes of metallophosphohydrolases are the alkaline [31,32] and purple acid phosphatases [33,34], which catalyze the removal of a phosphate group from phosphomonoesters. The physiological importance of these enzymes is not always known, but both classes of enzymes utilize bimetallic active sites to facilitate phosphoester hydrolysis [29]. Alkaline phosphatase is a homodimer with each monomeric unit containing a three-metal cluster comprised of two zinc ions and one magnesium ion [32]. The paired zinc ions are intimately involved in the hydrolysis reaction, while the magnesium serves to enhance the activity but is not absolutely required. The zinc ions are 3.9 Å apart and do not share any common ligands [35]. Alkaline phosphatase catalyzes phosphoester hydrolysis via a two-step nucleophilic displacement mechanism, in which a phosphoenzyme intermediate is formed [36,37]. The zinc ions in turn serve to activate the nucleophile in each of the displacement steps: one zinc activates a serine residue to attack the phosphomonoester and the second zinc activates a water molecule to displace the phosphate group from the serine residue [32]. In each step, the metal ions are proposed to act in concert, with one metal ion activating the nucleophile, the other stabilizing the charge buildup on the leaving group, and both ions anchoring the substrate and providing electrostatic stabilization in the pentavalent transition state. (It should be noted that while the existence of a pentavalent phosphorane intermediate is often proposed for this and related enzymatic reactions, there is no evidence of this species. In fact, recent experiments probing isotope effects suggest that a pentavalent transition state is far more likely for both enzymatic and model

[5] The detailed biochemistry of metallophosphohydrolases and metallopeptidases has been the subject of numerous reviews, many of which are referred to herein. Our purpose here is to present a brief summary of the best known structural and mechanistic information which is guiding the development of synthetic nucleases and peptidases. For more detailed information, the reader is referred to the recent comprehensive reviews by Lipscomb [28] and Wilcox [29].

reactions [38,39].) Each half of the reaction scheme results in inversion at the phosphorus center, leading ultimately to retention of configuration. This proposed mechanism, illustrated in Fig. 2, is based on extensive studies of the pH- and metal-dependent catalytic behavior [32,41], NMR studies demonstrating the interaction of the substrate with both metal ions [41,42], and the crystal structures of the enzyme [40]. Alkaline phosphatase is by far the most thoroughly studied metallophosphohydrolase, and its mechanism serves as a paradigm for all other enzymes in this class.

Purple acid phosphatases also utilize bimetallic catalytic sites in which one Fe^{3+} and a second divalent metal ion, commonly Zn^{2+} or Fe^{2+}, act together to facilitate hydrolysis [29]. The two metal ions are bridged by a single oxygen of an aspartate residue and a putative bridging hydroxide ion (inferred from spectroscopic studies);

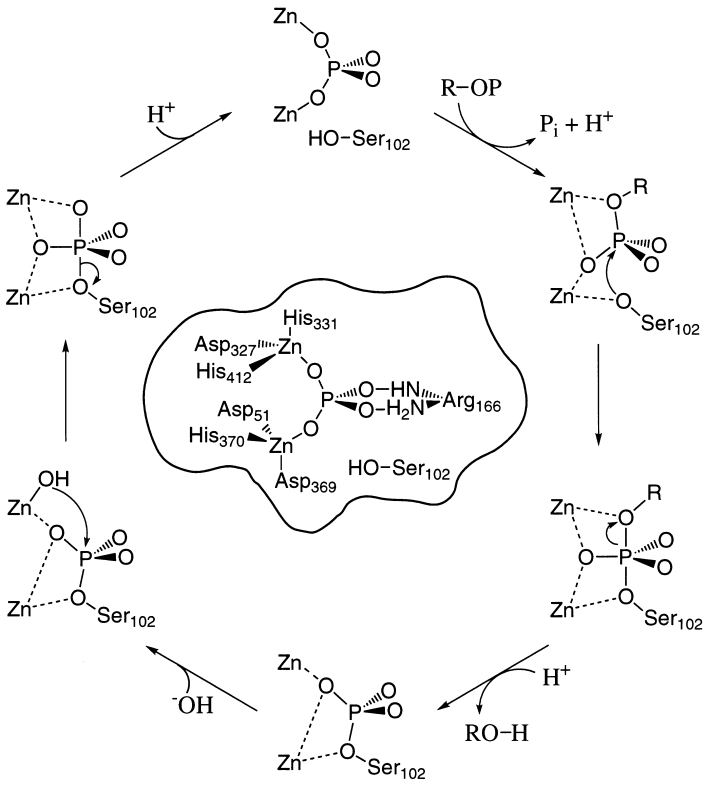


Fig. 2. Active site structure and proposed mechanism for the hydrolase activity of alkaline phosphatase per Kim and Wyckoff [40]. The authors explicitly propose the mechanism for the breakdown of the phosphoenzyme intermediate. However, the mechanism has been generalized to the hydrolysis of the phosphomonoester and formation of the phosphoenzyme intermediate as well [32]. (In a transferase reaction, the zinc-bound hydroxide is replaced with a zinc-bound alkoxide.) It should be noted that the formation of two adjacent, four-membered rings in the putative pentavalent intermediate is exceedingly unlikely based on chemical principles.

each ion also retains one open coordination site which is presumably occupied by solvent water [43]. The acid pH optimum for these enzymes is attributed to the deprotonation of an iron-bound water molecule, forming the catalytic nucleophile [44]. The reaction proceeds with inversion of configuration at the phosphorus as expected for a direct nucleophilic displacement [45]. The divalent ion is proposed to provide electrostatic stabilization in the transition state with the assistance of other key active site residues [46]. An illustration of the proposed mechanism and transition state is shown in Fig. 3. The phosphate ion is a non-competitive inhibitor of the acid phosphatases [47], and a recent crystal structure of the phosphate complex revealed that the phosphate bridged between the two metal ions [46]. Because phosphate does not compete with the substrate, it was concluded that this phosphate-bridged binding mode was non-productive, presumably more representative of the product complex than the enzyme–substrate complex. It was therefore proposed that only the divalent metal ion serves to bind the substrate.

A two-metal-ion mechanism has also been proposed for enzymes catalyzing the hydrolysis of the phosphodiester bonds of DNA. Thorough crystallographic studies of the 3′–5′ exonuclease site of DNA polymerase I have led to the proposal that phosphodiester hydrolysis occurs with facilitation by two Mg^{2+} ions [48,49]. (Although the metal ions used in vivo are not known for certain, Mg^{2+} and perhaps Zn^{2+} are the likely candidates.) One metal ion is tightly bound to the enzyme, while the second associates upon substrate binding [50]. Both metal ions are proposed to function in the catalytic mechanism in much the same way as proposed for the acid and alkaline phosphatases [51]. The metal ions together serve to neutralize the charge on the substrate and activate it for nucleophilic attack, provide a potent nucleophile at neutral pH in the form of a metal-bound hydroxide, stabilize the transition state by neutralizing the negative charge, and facilitate the departure of

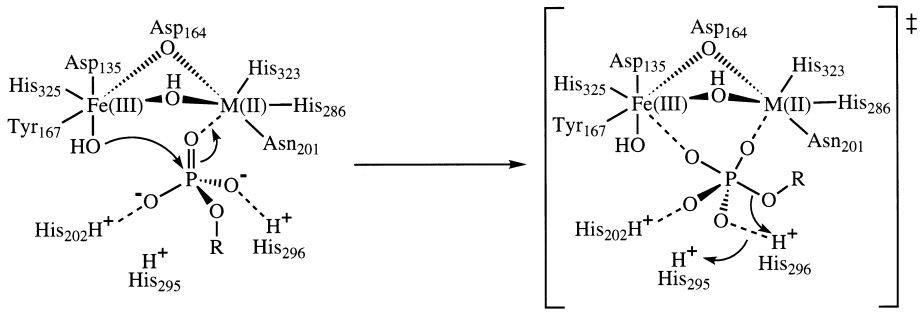


Fig. 3. Proposed mechanism for the hydrolysis of phosphomonoesters by purple acid phosphatases. The active site in the mechanism depicted above is based on the crystal structure of kidney bean purple acid phosphatase (M(II) is Zn), although there is considerable evidence that the active site in mammalian purple acid phosphatase (M(II) is Fe) is similar [43]. His_{202} and His_{296} are believed to activate the phosphomonoester for nucleophilic attack, as well as stabilize the transition state. In addition, His_{296} may act as a general acid catalyst in the breakdown of the phosphorane-like transition state. The bridging hydroxide and the hydroxide bound to the Fe(III) ion were not observed in the crystal structure, but their existence has been postulated based on spectroscopic and kinetic evidence, respectively [46].

the leaving group by lowering its pK_a. A model for the transition state of the phosphodiester hydrolysis reaction catalyzed by DNA polymerase I has been generated using the crystallographically determined structure for the 3′–5′ exonuclease site (Fig. 4): this model does not require any significant changes in the positions of key residues when compared with structures of enzyme–substrate and enzyme–product complexes [51]. This two-metal-ion mechanism is very similar to that proposed for alkaline phosphatase, and an analogous mechanism has also been proposed for the restriction endonuclease *Eco*RV [53] and for RNA cleavage by ribozymes [52].

It should be stressed, however, that two metal ions are *not* a universal requirement, and a number of nucleases, including ribonuclease H [54] and *Eco*RI endonuclease [53], require only a single metal ion for their catalytic activity. Moreover, some researchers have questioned the two-metal-ion mechanism proposed for some Mg^{2+}-dependent nucleases, a mechanism which is based almost solely on the crystal structures of a few enzymes. In a recent review, Cowan [55] stresses that great caution should be used when trying to infer a mechanism from crystallographic data. It was noted that the metal ions used in crystallographic analysis are often not likely to be utilized *in vivo* (i.e. Mn^{2+} or Co^{2+} in place of Mg^{2+}), a substitution used to aid in structure determination. Moreover, when Mg^{2+} is employed, the metal ion concentration utilized in the crystallization of the enzymes often far exceeds physiologically relevant concentrations. In particular, based on various solution studies and basic inorganic coordination chemistry principles, Cowan questions the two-metal-ion mechanism proposed for *Eco*RV, the 3′–5′ exonuclease site of DNA polymerase I, and ribozymes and suggests that perhaps only a single Mg^{2+} ion is utilized in the hydrolytic reaction [55]. Clearly, the evidence for the

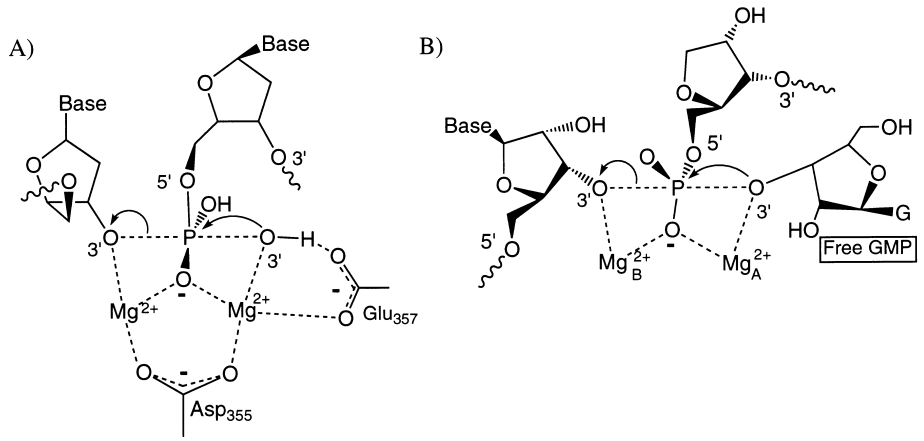


Fig. 4. Proposed two-metal-ion-mechanisms. (A) Proposed mechanism for the hydrolysis of DNA by DNA polymerase I [51]. The metal ions utilized in vivo are not known for certain although two leading candidates are Mg^{2+} and Zn^{2+}. (B) Proposed mechanism for RNA hydrolysis by group I intron ribozymes [52]. Based on chemical principles, the formation of two adjacent, four-membered rings in the putative pentavalent intermediate seems unlikely.

two-metal-ion mechanism is considerably weaker for the nucleases than for alkaline phosphatase, and more extensive enzymological studies are needed.

3.1.2. Peptidases

Metalloproteases, one of the four functional classes of peptidases, most commonly employ a single divalent zinc ion as an obligatory cofactor, although a number of dinuclear zinc enzymes are also known [28]. The best studied enzymes, carboxypeptidase A [56] and thermolysin [57], appear to utilize the catalytically essential zinc ion in substantially similar ways, despite the apparent lack of an evolutionary relationship between the two enzymes [58,59]. Indeed, a similar mechanism has been invoked to explain the catalytic activity of many mononuclear zinc metallopeptidases, despite their diverse substrate specificities, evolutionary origins and biochemical functions [28]. The catalytic zinc ion is typically four-coordinate in a nearly tetrahedral ligand array, in which the protein provides three of the four ligands. The fourth ligand is a water molecule which serves as the nucleophile in the catalytic mechanism. The protein ligands in both carboxypeptidase A [56] and thermolysin [57] are two histidines and a glutamate: other mononuclear peptidases with three histidine ligands are also known [28]. In addition, there are a number of other key residues in the active site, including a glutamate which functions as both a general base and a general acid in the hydrolysis reaction [56,57].

Thermolysin and carboxypeptidase A employ essentially the same catalytic mechanism: the structure proposed for the transition state of both enzymes is shown in Fig. 5 [28]. The nucleophile is a zinc-bound hydroxide ion, generated through deprotonation of a coordinated water molecule. The nucleophilicity of the Zn–OH functionality is enhanced through hydrogen bonding to a nearby glutamate residue. The zinc ion also serves as an Lewis acid catalyst, stabilizing the build-up of negative charge in the transition state by coordination to the carbonyl oxygen. Additional

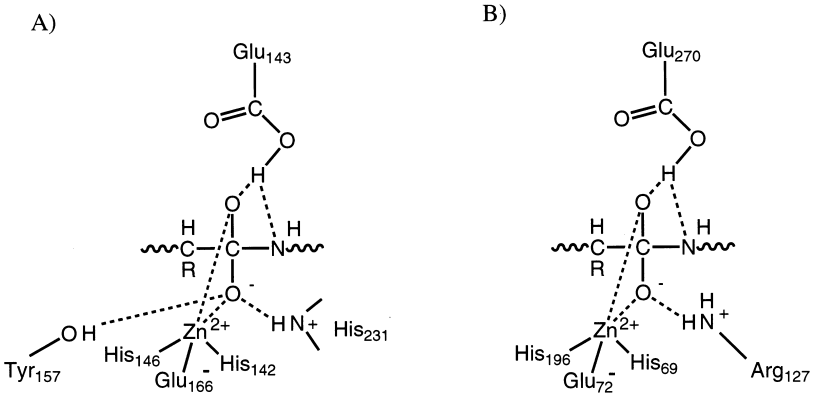


Fig. 5. Proposed transition state for the enzymatic hydrolysis of peptide bonds by (A) thermolysin and (B) carboxypeptidase [56,57]. Note that the two transition states and the active sites of the metallopeptidases are essentially identical.

positively charged residues, Arg_{127} and His_{231}, also provide electrostatic stabilization. The catalytically essential glutamate residue, (Glu_{270} or Glu_{143} in carboxypeptidase and thermolysin, respectively), serves first as a general base to accept the proton from the Zn–OH nucleophile, and then as a general acid to protonate the departing amino group. The most comprehensive evidence in support of this mechanism is derived from crystallography of native and substrate-bound forms of carboxypeptidase [60,61]. Of particular importance have been structural studies on enzyme-bound transition-state analogs [62–64]. Although for many years it was postulated that the primary role of the zinc ion was to polarize the carbonyl group, more recently it has been argued that ground-state effects (i.e. polarization) are less significant than transition-state stabilization through charge neutralization [56]. The well-elucidated role of the metal ion zinc peptidases is therefore similar to that proposed for the metal ions in nucleases.

3.1.3. Nature's choice of metal ions

One common theme in the hydrolases described thus far is that the same metal ions are used continually by different enzymes [65]. What is it about these particular metal ions which make them so suitable to the task of hydrolysis? Nature's choice of Mg^{2+} as the catalytically active metal ion in many nucleases is a logical one. With an ionic radius of 0.6 Å, Mg^{2+} has a higher charge density and is a harder Lewis acid than any other widely available metal ion in biological systems (Table 1). This property makes Mg^{2+} the perfect metal ion for binding to the hard oxygen anions of the negatively charged phosphodiester backbone of nucleic acids [68]. Nature's choice of Zn^{2+} at the catalytically active site of most peptidases is perhaps less obvious. Although Zn^{2+} has a lower charge density than Mg^{2+}, Zn^{2+} is a stronger Lewis acid, based on their respective ionization potentials (Table 1). Therefore, Zn^{2+} is more effective at polarizing carbonyl bonds than Mg^{2+} [69].

Table 1
Physical, thermodynamic, and kinetic properties of some physiologically relevant divalent metal ions

Ion	Charge density (Z/r) ($Å^{-1}$)[a]	I_2 or I_3 (eV)[a]	k_{sub} (s^{-1})[b]
Mg^{2+}	3.0	15.04	$10^{5.1}$
Ca^{2+}	2.0	11.87	$10^{8.4}$
Mn^{2+}	2.5	15.64	$10^{6.6}$
Fe^{2+}	2.7	16.18	$10^{6.1}$
Co^{2+}	2.8	17.06	$10^{5.3}$
Ni^{2+}	2.9	18.17	$10^{4.2}$
Cu^{2+}	2.8	20.29	$10^{9.3}$
Zn^{2+}	2.7	17.96	$10^{7.4}$
La^{3+}	2.8	19.18	$10^{7.9}$
Ce^{3+}	2.9	20.20	$10^{7.9}$
Eu^{3+}	3.2	24.92	10^{7}–10^{8}

[a]Data obtained from Ref. [66].
[b]Rate constant for the substitution of water on the aquo ions [67].

Moreover, not only does Zn^{2+} exchange ligands very rapidly, an important consideration in catalysis [68], but there is no energetic barrier to changing the geometry of Zn(II) complexes since its filled d-shell provides no ligand field stabilization energy [69]. This ability of the zinc ion to adopt different geometries with no cost in energy may very well be important in stabilizing the transition state [69].

3.2. Exchange-inert model systems

Further insight into the requirements of a metal ion in synthetic hydrolases can be obtained from previous work on the hydrolysis of activated phosphodiesters by substitutionally inert metal complexes. Using an Ir(III) complex containing a coordinated phosphodiester and a coordinated water, Hendry and Sargeson [70] determined that when the water was deprotonated, the coordinated hydroxide attacked the bound phosphodiester, resulting in hydrolysis. The mechanism proposed for this reaction (Fig. 6) has since become a paradigm for metal-ion promoted phosphodiester hydrolysis. Further mechanistic work by Chin et al. with LCo(III) complexes (where L is one of a variety of tetradentate ligands) [71,72] demonstrated that when these complexes were incubated with activated phosphodiesters, the rate of hydrolysis was optimized in a pH range which was between the pK_as of the first and second

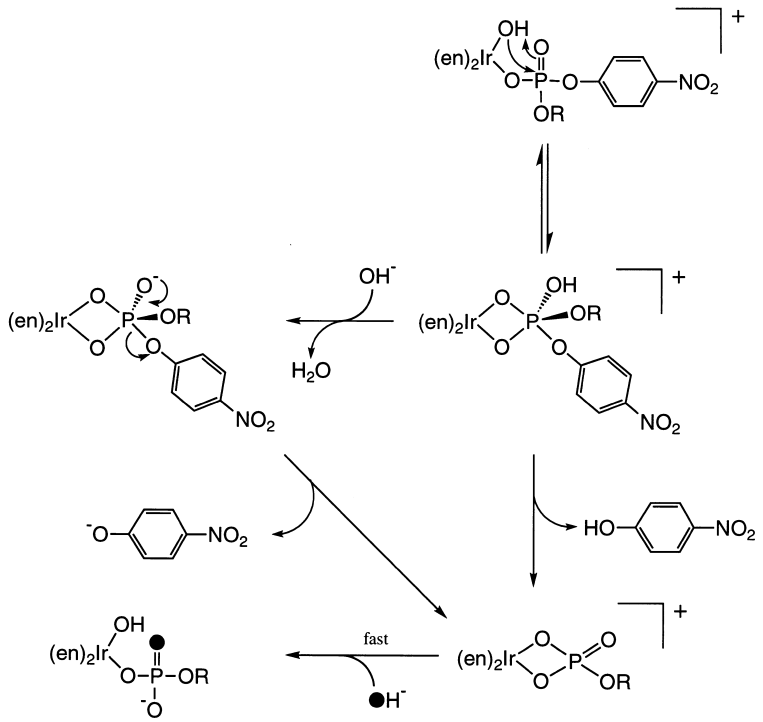


Fig. 6. Proposed mechanism for the Ir(III)-promoted hydrolysis of phosphodiesters [70].

coordinated water molecules [71,72]. Moreover, it was also found that the two open sites needed to be oriented *cis* to one another. These experiments supported the mechanism proposed by Hendry and Sargeson, in which, following substrate binding to the metal ion, the substrate is attacked by a metal-coordinated hydroxide. Recently, heavy-atom isotope studies were employed to study the mechanism of phosphomonoester hydrolysis by three different Co(III) complexes [73]. The magnitude of the isotope effects indicate that loss of the leaving group is involved in the rate-determining step and that the reaction proceeds via a concerted mechanism, i.e. nucleophilic attack by the Co(III)-bound hydroxide occurs simultaneously with bond cleavage.

The mechanism of metal-promoted amide hydrolysis is less certain. Substitutionally inert metal complexes of Co(III) have been used to demonstrate that amide hydrolysis can be promoted both through activation of the carbonyl [74] and intramolecular attack of a coordinated hydroxide [74,75]. In most of these studies, however, the complexes were designed such that substrate activation and intramolecular attack of a metal-bound hydroxide could not occur simultaneously. Sayre has suggested five different mechanisms by which amides may be hydrolyzed by metal complexes (Fig. 7) [76]. Three of these mechanisms resemble the one proposed for phosphodiester hydrolysis, in that the mechanisms entail substrate binding with activation of the carbonyl and simultaneous involvement of a metal-coordinated hydroxide. Support for these mechanisms comes from the hydrolysis of amides at various pHs using both tetra- and pentaamine Co(III) complexes [77]: the rates of hydrolysis were much faster with the complex containing two open coordination sites and were maximized at a pH above the pK_a of the coordinated water. Thus, it appears that the metal requirements for amide hydrolysis are similar to those of phosphodiester hydrolysis.

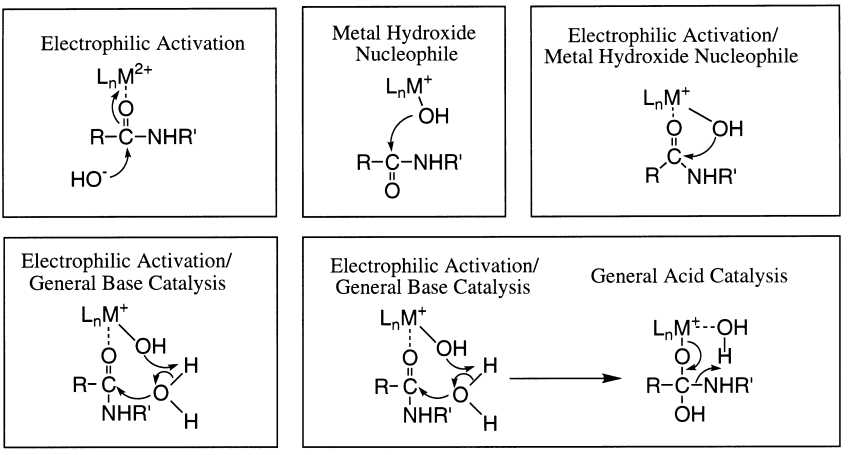


Fig. 7. Possible mechanisms by which metal complexes can promote the hydrolysis of amide bonds (adapted from Ref. [76]).

3.3. Conclusions based on natural hydrolases and exchange-inert systems

Based on the information gained from both natural hydrolases and exchange-inert model systems, a metal ion in the catalytically active site of a synthetic hydrolase should be able to (1) provide two *cis*-oriented labile sites to bind both the substrate and a water molecule; (2) reduce the pK_a of the coordinated water (i.e. provide a metal-bound hydroxide nucleophile at near neutral pH); (3) activate the substrate towards nucleophilic attack and/or stabilize the transition state; and (4) release the products at a reasonable rate.

Recently, a number of metal ions and complexes have been demonstrated to hydrolyze phosphodiester or amide bonds, and more infrequently, RNA, DNA, or proteins. In many instances, however, it has been difficult to draw any definitive conclusions about the role of the metal ions, either because the coordination environment of the metal is not known or because the necessary experiments were not performed. Those systems for which information on the role of the metal ion can be obtained almost invariably involve well-defined metal complexes (often mononuclear) and the hydrolysis of model substrates. The rest of this review will focus on recently reported synthetic nucleases and peptidases utilizing labile metal ions for which the coordination geometry is known and detailed kinetic and/or mechanistic information is available. Examples of the hydrolysis of RNA, DNA, and proteins will also be discussed.

4. Labile metal ions often used in synthetic hydrolases

Two metal ions which meet the criteria of being both substitutionally labile and strong Lewis acids (based both upon their high charge density and ionization potential) are Cu^{2+} and Zn^{2+} (Table 1). The Lewis acidity of these cations is such that not only can they serve to activate phosphodiester bonds towards nucleophilic attack via charge neutralization and perhaps polarization of the bond, but they also significantly lower the pK_a of a coordinated water, thereby providing metal-bound hydroxide at near-neutral pH [78]. In fact, if $k^*_{on}I_2$ (rate constant for water release times the ionization potential of the dication) is used as a measure of the potential for Lewis acid catalysis, then Cu^{2+} and Zn^{2+} are better than any other biologically relevant divalent metal ions [68]. Another advantage of using Cu^{2+} and Zn^{2+} in synthetic nucleases is that their coordination chemistry is well defined and Cu(II) complexes are spectroscopically rich and are therefore easily probed. While these facts will not make copper- or zinc-containing synthetic hydrolases any more efficient, they can help produce a system where the reaction mechanism can be studied in great detail. Recently, complexes utilizing lanthanide ions, which also contain both a high charge density and ligand substitution rates (Table 1), have shown promise as synthetic hydrolases. Although determining mechanisms have been hampered due to the relatively poorly understood coordination environment of lanthanide ions and the difficulty in obtaining stable complexes [79–82], some significant advances are being made.

5. Mechanistic studies on labile metal-promoted phosphodiester hydrolysis[6]

5.1. Copper complexes

The metal complex we have studied most thoroughly is copper(II) 1,4,7-triazacyclononane dichloride, $Cu([9]aneN_3)Cl_2$ (Fig. 8). This complex catalytically hydrolyzes the activated phosphodiesters bis(4-nitrophenyl) phosphate (BNPP) and ethyl 4-nitrophenyl phosphate (ENPP) [85,86]. Significantly, this complex is a true catalyst, demonstrating both turnover and rate enhancement; the rate law determined for the hydrolysis of BNPP and ENPP by $Cu([9]aneN_3)Cl_2$ is shown in Eq. (1). The observed half-order dependence on the metal complex

$$\text{rate} = k[\text{substrate}]^1[Cu([9]aneN_3)Cl_2]^{0.5} \quad (1)$$

concentration indicates that in solution, $Cu([9]aneN_3)Cl_2$ is in a monomer–dimer equilibrium with the monomer being the precursor to the active catalyst. The kinetically determined dimer formation constant (K_f) is $1220\ M^{-1}$ at pH 7.3. Therefore, although the monomer is the catalytically active species, it is the dimer which is strongly favored at near-neutral pH. Moreover, a pH versus rate profile exhibits a kinetic pK_a (7.3) which is identical to the thermodynamic pK_a, implying that a metal-coordinated hydroxide may be important in the reaction pathway. It should be noted, however, that because of the monomer–dimer equilibrium, the pK_a determined via titration is not simply the pK_a of the coordinated water (Fig. 9): it is, however, the kinetically relevant pK_a. Substrate saturation studies with ENPP indicated that the reaction follows Michaelis–Menten kinetics, implying the formation of a complex–substrate intermediate. These results enabled us to propose a mechanism for the $Cu([9]aneN_3)Cl_2$-catalyzed hydrolysis of activated phosphodiesters (Fig. 10) [86].

An aspect of the reaction we wanted to probe in more detail was the rate-limiting hydrolytic step (k_3) and the nature of the transition state [87]. Solvent deuterium isotope effects were used to distinguish between a general base mechanism and a mechanism involving direct nucleophilic attack by a metal-coordinated hydroxide. The value obtained ($^Dk = 1.14$) indicated that no proton is in flight during the rate-

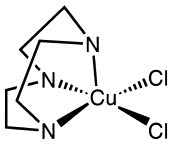


Fig. 8. Structure of copper(II) 1,4,7-triazacyclononane dichloride, $Cu([9]aneN_3)Cl_2$. The chloride ligands are hydrolyzed when the complex is dissolved in water.

[6] It should be noted that there are some mechanistic similarities between the metal-promoted hydrolysis of phosphate esters and phosphoric acid anhydrides such as triphosphates. For extensive reviews on the hydrolysis of triphosphates, see Refs. [83,84].

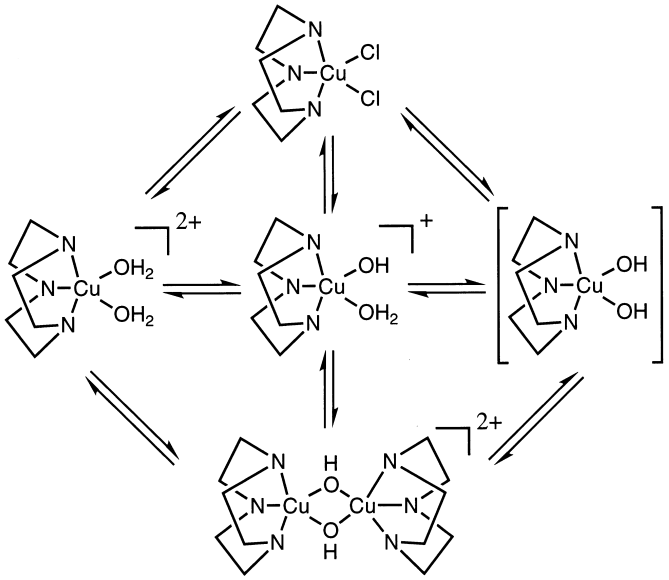


Fig. 9. Proton equilibria for Cu([9]aneN$_3$)Cl$_2$ dissolved in water. The neutral structure in brackets does not exist in an appreciable concentration in the pH range at which experiments were performed (pH = 7–8).

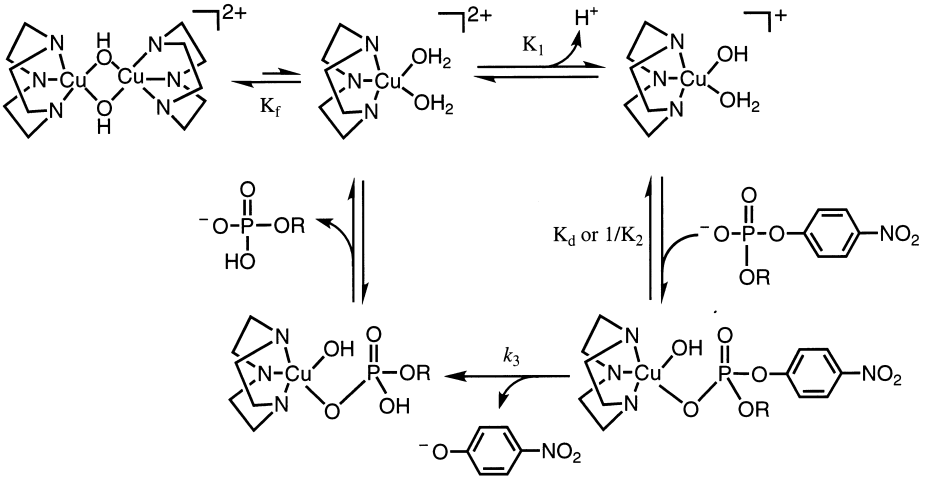


Fig. 10. Mechanism of Cu([9]aneN$_3$)Cl$_2$-catalyzed hydrolysis of activated phosphodiesters.

limiting step, and is consistent with an intramolecular attack by a metal-coordinated hydroxide.

Intramolecular attack by a metal-coordinated hydroxide can either lead to a pentavalent phosphorane intermediate or the reaction can proceed via a concerted

mechanism through a phosphorane-like transition state. Secondary ^{15}N isotope effects were used to probe these two possibilities [73,87]. Because heavy atoms prefer to be more stiffly bonded and N–O bonds are stiffer than N–C bonds in terms of vibrational frequency, the heavy atom will prefer the neutral starting material in which no quinoid resonance structure is available (Fig. 11). As a result, an isotope effect of greater than unity should be observed (i.e. the product nitrophenolate should be enriched in ^{14}N, relative to the starting material). The extent to which this isotope effect is observed, however, depends on the extent to which loss of 4-nitrophenolate is rate-limiting. If the reaction involves the rate-limiting formation of a phosphorane intermediate followed by the rapid release of 4-nitrophenolate, then no isotope effect will be observed. The results obtained from these experiments ($^{15}k = 1.0013 \pm 0.0002$) indicate that the mechanism proceeds via a concerted pathway with approximately 50% bond formation and 50% bond cleavage in the transition state. The results from these isotope studies allowed us to present a more detailed description of the hydrolytic step (Fig. 12) [87].

A variety of other copper(II) complexes are also known to promote model phosphodiester hydrolysis, and the reactivities of these complexes further support the metal-hydroxide mechanism. Catalytic hydrolysis of phosphodiesters was observed with $Cu(bipy)^{2+}$ (bipy = 2,2′-bipyridine) and the rate of hydrolysis increased dramatically with pH, consistent with the participation of a Cu-OH in the hydrolytic mechanism [88]. Evidence that the substrate phosphodiester was bound directly to the metal ion in the transition state was provided by the observation of saturation kinetics. Together, these observations support an intramolecular nucleophilic attack by a metal-coordinated hydroxide ion in a highly labile system; an essentially identical mechanism to that of $Cu[9]aneN_3^{2+}$. In an interesting elaboration, a modified bipyridine ligand with two quaternary amine functional groups was synthesized and used with Cu^{2+} to promote hydrolysis [89]. The presence of the additional cationic groups, positioned appropriately to provide electrostatic inter-

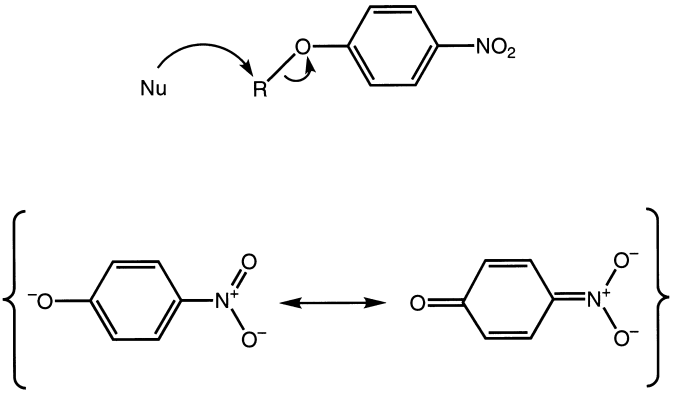


Fig. 11. Resonance structures of the product nitrophenolate which lead to the observed ^{15}N isotope effect. Because heavy atoms prefer to be more stiffly bonded and N–O bonds are stiffer than N–C bonds, the product will be enriched in ^{14}N in competition experiments.

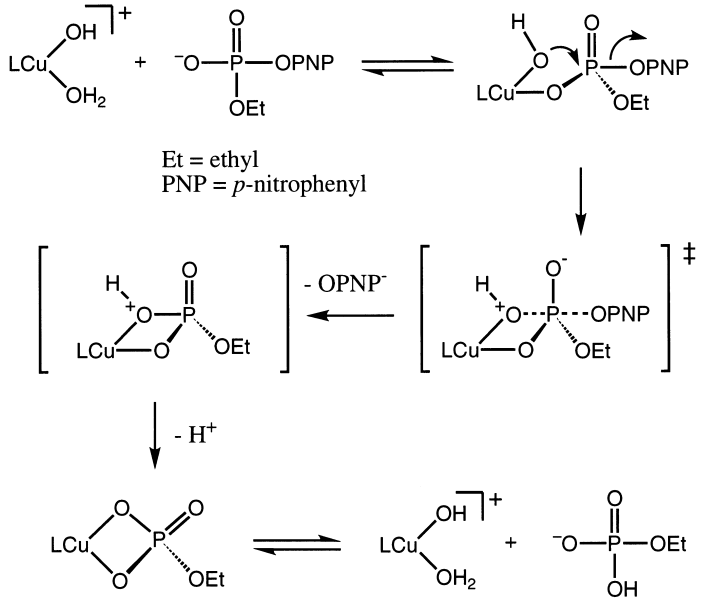


Fig. 12. A detailed description of the transition state and intermediates formed in the $Cu([9]aneN_3)Cl_2$-catalyzed hydrolysis of activated phosphodiesters.

action with the phosphodiester, greatly accelerated the rate of hydrolysis. This observation supports the idea that neutralizing the negative charge on the substrate substantially increases the rate of hydrolysis.

Ligand structure and the ability to stabilize the four-membered ring transition state has also been shown to be important in controlling the rate of phosphodiester hydrolysis reactions [90]. The reactivity of three related copper(II) complexes (Fig. 13) toward hydrolysis and transesterification of bis(4-dinitrophenyl) phosphate (BDNPP) were compared [91] and both the rate of hydrolysis and the mechanism were dependent on the structure of the complex. Interestingly, although complex B contained a potential alkoxide nucleophile, it did not react via transesterification, presumably because the alkoxide was prevented from attacking the phosphorous atom by the short carbon linker.

5.2. Zinc and lanthanide complexes

Zinc complexes also promote hydrolysis of phosphodiesters, and these complexes serve as useful functional models for zinc-dependent phosphohydrolase enzymes. In a detailed set of experiments, Koike and Kimura [92] demonstrated that the complexes $Zn[12]aneN_3^{2+}$ and $Zn[12]aneN_4^{2+}$ (Fig. 14) effectively promoted the hydrolysis of phosphodiesters, and the pH dependence of the reactions was consistent with a metal-hydroxide mechanism [92]. The tridentate complex, $Zn[12]aneN_3^{2+}$, was substantially more effective than the tetradentate complex; this observation

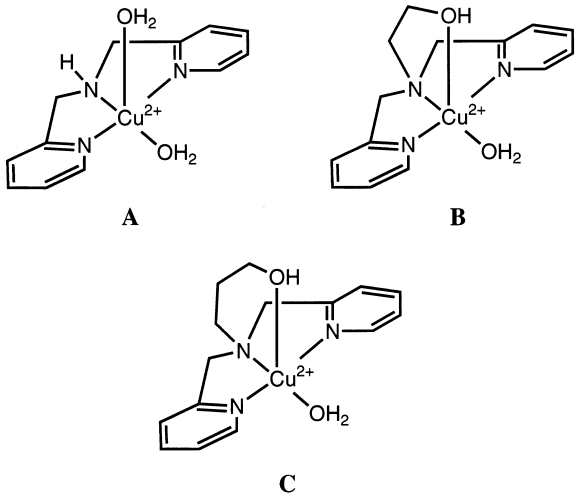


Fig. 13. Model complexes used to probe the difference in the rate between the hydrolysis and the transesterification of activated phosphodiester bonds [91]. Complex A utilizes a hydrolytic mechanism, whereas complexes B and C cleave phosphodiesters via transesterification.

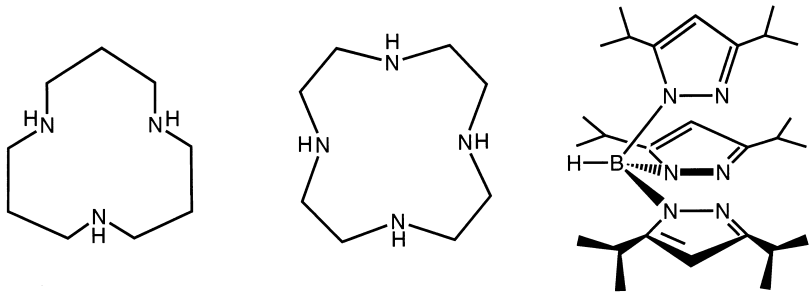


Fig. 14. Structures of some tridentate and tetradentate ligands utilized in the study of zinc-promoted phosphodiester hydrolysis: 1,5,9-triazacyclododecane ([12]aneN$_3$); 1,4,7,10-tetraazacyclododecane ([12]aneN$_4$); and hydrotris(3,5-diisopropyl-1-pyrazolyl)borate (HB(3,5-*i*Pr$_2$pz)$_3$).

correlates with the lower pK_a for the coordinated water in Zn[12]aneN$_3^{2+}$ and with the expected greater Lewis acidity of the zinc in the complex with fewer nitrogen donors. Interestingly, when Kitajima and coworkers [93] reacted Zn(HB(3,5-*i*Pr$_2$pz)$_3$)(OH) (Fig. 14) with the activated phosphodiester bis(4-nitrophenyl) phosphate in non-aqueous solvent, only one nitrophenolate was released. Reaction of the stable product, the zinc-complexed phosphomonoester, with an additional equivalent of Zn(HB(3,5-*i*Pr$_2$pz)$_3$)(OH) gave a binuclear complex in which the phosphate monoester asymmetrically bridged the two zinc ions. The absence of further hydrolysis in the presence of excess Zn(HB(3,5-*i*Pr$_2$pz)$_3$)(OH)

revealed the importance of intramolecular nucleophilic attack in this sterically restricted system [93].

Zinc complexes have also been shown to act cooperatively with other functional groups in the hydrolysis of phosphodiesters. For example, Breslow et al. [94] demonstrated that a zinc ion and an imidazole functioned cooperatively in the hydrolysis of an activated RNA model. In this reaction it is proposed that the zinc serves to activate the phosphodiester, while the imidazole acts as a general base catalyst to deprotonate the pendant alcohol. Surprisingly, when the two ions were incorporated into the same molecule using an imidazole-based ligand with an additional pendant imidazole group, only a small increase in reactivity was observed [95]. In an elegant model of the chemistry of alkaline phosphatase, Kimura et al. [96] demonstrated that when an alkoxy group was attached to the tetraamine ligand, [12]aneN_3, the resulting pentacoordinate zinc complex was an effective hydrolase. The alkoxide served as a highly potent nucleophile and in non-aqueous solvents, the transesterification reaction was 125 times faster than direct hydrolysis by coordinated hydroxide [96].

Lanthanide ions have proven extraordinarily effective at promoting transesterification of phosphodiesters, as occurs in the cleavage of RNA. However, elucidating the detailed mechanisms by which the metal ions promote this reaction is challenging. Lanthanide metal complexes are highly labile, and the precise coordination number and geometry are difficult to determine [79–82]. Morrow and co-workers [97–99] have recently demonstrated the importance of two open coordination sites in the transesterification of the 4-nitrophenyl ester of propylene glycol. In these studies, chelating macrocycles (Fig. 15) which form remarkably inert complexes with a variety of lanthanide ions were used; typical dissociation half-lives varied from 10 to 100 days [99]. The integrity of the complexes is important in these studies because the free lanthanide ions are substantially more effective transesterification catalysts than the complexed ions, presumably due to their greater Lewis acidity. Kinetic analysis of transesterification by these inert complexes revealed pH versus rate profiles consistent with a catalytic requirement for formation of metal-bound hydroxide [99]. Interestingly, the transesterification rate did not necessarily increase with increasing Lewis acidity of the lanthanide complexes (as reflected in the pK_a of the coordinated water), suggesting additional requirements for effective catalysis. Further studies revealed that highly Lewis-acidic La^{3+} and Eu^{3+} complexes bind to phosphate diesters with binding constants of approximately the same order of magnitude as those measured for transition metal complexes [98] and binding of the phosphodiester was necessary for transesterification to occur. Laser-induced luminescence studies were used to demonstrate that catalytically active complexes possess two bound water molecules [97,98]. Transesterification activity correlated with deprotonation of one bound water and binding of the phosphate diester, presumably with displacement of the second water molecule. Thus, the mechanism for lanthanide-promoted transesterification appears substantially similar to transition-metal-promoted phosphodiester hydrolysis.

The mechanism of lanthanide-promoted hydrolysis of phosphodiesters is perhaps less well-studied than the corresponding transesterification reaction, despite the fact

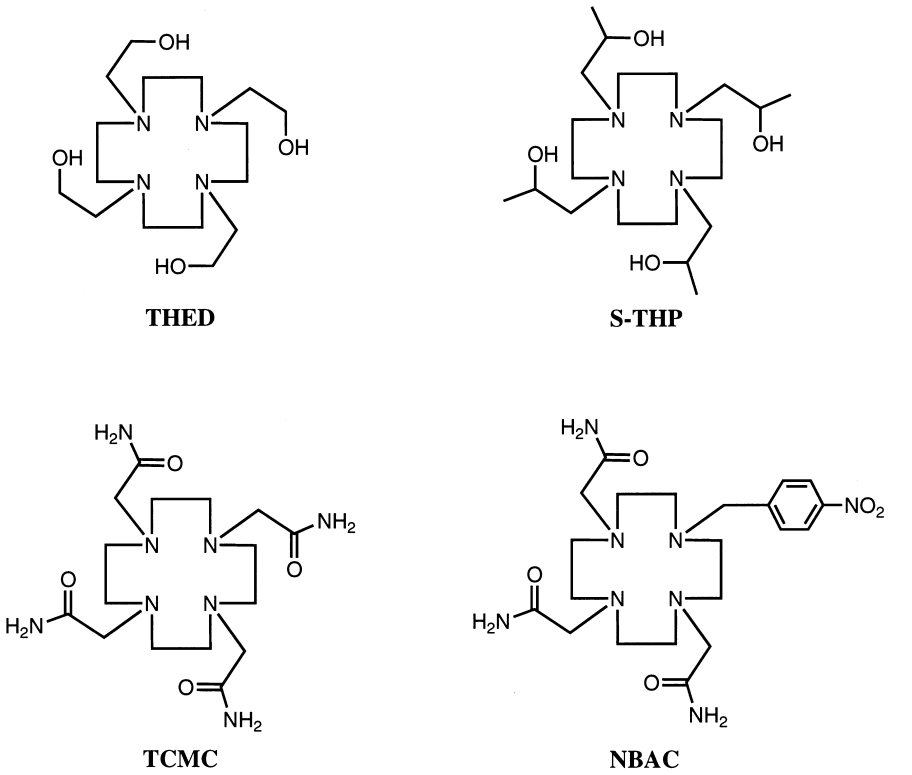


Fig. 15. Structures of a family of polydentate ligands that form relatively stable complexes with various lanthanide ions: 1,4,7,10-tetrakis(2-hydroxyethyl)-1,4,7,10-tetraazacyclododecane (THED); (1S,4S,7S,10S)-1,4,7,10-tetrakis(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane (S-THP); 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane (TCMC); and 1-(4-nitrobenzyl)-4,7,10-tris(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane (NBAC).

that a number of ions [100,101] and complexes [102–104] have been utilized to affect hydrolysis. In some instances, hydrogen peroxide was shown to significantly enhance the rate of the reaction [100,101,103]; the observed second order dependence in both lanthanide ions and H_2O_2 has led to the proposal that a bis(μ-η^2:η^2) peroxo species is the active core when free ions are used to promote the reaction (Fig. 16)

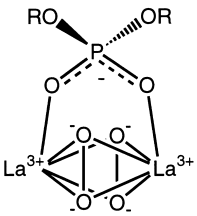


Fig. 16. Proposed active species in the hydrolysis of phosphodiesters by lanthanide ions plus H_2O_2.

[101]. When lanthanide complexes are utilized, however, the reaction is proposed to proceed in a manner analogous to transition metal complexes in which the phosphodiester binds to the metal, followed by an intramolecular attack from a coordinated hydroxide [103,104]. Evidence for this proposed mechanism is provided by time-resolved luminescence spectroscopy which indicates the presence of coordinated water molecules and by the pH versus rate profiles which exhibit a kinetic pK in the region of the pK of a lanthanide-coordinated water [103].

5.3. Comparison of different synthetic nucleases

We have compared the hydrolytic efficiency of a number of well characterized metal complexes in order to evaluate possible factors contributing to the rate enhancement (Table 2) [86]. Because of the wide variety of experimental conditions reported in the literature, it is often difficult to compare complexes under a standard set of conditions. Through the use of measured activation parameters for $Cu([9]aneN_3)^{2+}$-catalyzed hydrolysis and the previously determined rate law, we have calculated the rate constants for $Cu([9]aneN_3)^{2+}$-catalyzed hydrolysis under the conditions used in a number of other studies. In making the comparisons, we were limited to those complexes for which published pH-rate profiles allowed manipulation of the published rate data to a constant pH. The rate constants and conditions under which they were calculated are listed in Table 2. One of the more interesting comparisons is between $Cu([9]aneN_3)^{2+}$ and $Zn([12]aneN_3)^{2+}$, as these two complexes have the same pK_a (7.3) and yet differ by an order of magnitude in the hydrolytic rate constant. In as much as the pK_a of the coordinated water is indicative of the Lewis acidity of the metal and the nucleophilicity of the coordinated hydroxide, one might expect these two species to exhibit similar rates. The fact that they do not clearly implies that the metal ion is influencing the reaction in ways which are

Table 2
Calculated rate constants for the hydrolysis of activated phosphate esters by various complexes

Nucleophile[a]	Substrate[a]	k (pH = 7.2)[b]	T (°C)	Ref.
OH-	BNPP	4×10^{-7} s^{-1}	100	[c]
[9]Cu-OH$^+$	BNPP	9×10^{-4} s^{-1}	100	[81]
(bpy)Cu-OH$^+$	ENPP	1×10^{-2} M^{-1}s^{-1}	75	[83]
[9]Cu-OH$^+$	BNPP	5×10^{-2} M^{-1}s^{-1}	75	[81]
$(en)_2$CO-OH^{2+}	BNPP	3×10^{-5} s^{-1}	50	[69]
[9]Cu-OH$^+$	BNPP	9×10^{-6} s^{-1}	50	[81]
[12]Zn-OH$^+$	BNPP	6×10^{-5} M^{-1}s^{-1}	35	[87]
[9]Cu-OH$^+$	BNPP	8×10^{-4} M^{-1}s^{-1}	35	[81]
$(en)_2$Ir-OH^{2+}	ENPP	3×10^{-5} s^{-1}	25	[67]
[9]Cu-OH$^+$	BNPP	5×10^{-7} s^{-1}	25	[81]

[a]Abbreviations: [9] = 1,4,7-triazacyclononane; [12] = 1,5,9-triazacyclododecane; bpy = 2,2′-bipyridine; en = ethylenediamine; BNPP = bis(4-nitophenyl) phosphate; and ENPP = ethyl 4-nitophenyl phosphate.
[b]Rate constants at pH = 7.2 were derived from the published pH versus rate profiles and are approximate.
[c]See Ref. [105].

as yet poorly understood. Another interesting comparison is between $Cu([9]aneN_3)^{2+}$ and the complexes of the trivalent ions Co^{3+} and Ir^{3+}. The hydrolysis reaction is so slow that ligand exchange on Co^{3+} never becomes rate limiting and the greater charge density of Co^{3+} relative to Cu^{2+} appears to account for the increased rate of hydrolysis with Co^{3+}. In the case of the exchange-inert Ir^{3+} complex, the preorganization of the nucleophile and the substrate within the metal coordination sphere reveals that there is a substantial rate enhancement attributable to the elimination of the substrate binding equilibrium.

6. Examples of metal-based synthetic nucleases

6.1. *Cu([9]aneN$_3$)Cl$_2$*

A unique feature of $Cu([9]aneN_3)Cl_2$ (Fig. 8) is that not only is a great deal known about the mechanism by which it catalyzes activated phosphodiester hydrolysis, but it is also able to carry out hydrolysis reactions on the biologically relevant substrates RNA and DNA. Chin et al. [106] established that $Cu([9]aneN_3)Cl_2$ hydrolyzes the RNA dinucleotide adenylyl(3′→5′)adenosine (ApA). Product analysis revealed that the only fragments produced were consistent with a hydrolytic mechanism. Moreover, the presence of both adenosine 2′-monophosphate (2′-AMP) and adenosine 3′-monophosphate (3′-AMP) as products, directly implicates attack by the 2′-OH resulting in a 2′,3′-cyclic adenosine monophosphate (2′,3′-cAMP) intermediate. Recently, using RNA hairpin oligomers, we established that $Cu([9]aneN_3)Cl_2$ is also able to hydrolyze both single-stranded and double-stranded RNA [107]. $Cu([9]aneN_3)Cl_2$ cleaved the RNA at all phosphodiester bonds, indicating that specific base–complex interactions are not important in the reaction mechanism. Moreover, the ability to hydrolyze double-stranded RNA is notable because double-stranded RNA is generally a poorer substrate for hydrolysis [108,109].

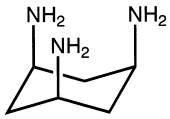
In addition to being able to hydrolyze RNA, $Cu([9]aneN_3)Cl_2$ is also capable of cleaving the more challenging substrate DNA [110]. When $Cu([9]aneN_3)Cl_2$ was incubated with circular single-stranded M13 DNA under near-physiological conditions, the DNA was non-specifically degraded. This is consistent with the results obtained for the hydrolysis of RNA, in which no specific base-complex interactions were observed [107]. Double-stranded DNA is also a substrate for $Cu([9]aneN_3)Cl_2$; supercoiled DNA (Form I) was first cut with a single-stranded nick to relax circular DNA (Form II) before being further degraded to linear DNA (Form III). In addition, a related complex, $[Cu(i\text{-}Pr_3[9]aneN_3)(OH_2)(CF_3SO_3)](CF_3SO_3)$, is also capable of cleaving double-stranded DNA [110]. This complex was tested because we had previously determined that it is much more reactive than $Cu([9]aneN_3)Cl_2$ in hydrolyzing activated phosphodiesters [110]. Significantly, the increase in reactivity observed by $[Cu(i\text{-}Pr_3[9]aneN_3)(OH_2)(CF_3SO_3)](CF_3SO_3)$ in hydrolyzing activated phosphodiesters, correlated with an increase in reactivity with DNA as a substrate.

An unfortunate aspect of the chemistry of $Cu([9]aneN_3)Cl_2$ is that in the presence of reducing agents, $Cu([9]aneN_3)Cl_2$ is able to perform oxidative chemistry [110]. One of the rationales behind choosing triazacyclononane as a ligand is that, due to geometric constraints, this facially chelating ligand should inhibit formation of the tetrahedral Cu^+ oxidation state (it is the Cu^+ oxidation state which reacts with O_2 in oxidative cleavage mechanisms). We determined, however, that $Cu([9]aneN_3)Cl_2$ cleaves DNA by at least two different mechanisms: an O_2-independent mechanism (presumably hydrolysis) and an O_2-dependent mechanism [110]. Although the nature of the O_2-dependent pathway (which accounts for approximately 30% of the cleavage under aerobic conditions) is not known, it is presumably an oxidative pathway initiated by adventitious reducing agents. Nonetheless, $Cu([9]aneN_3)Cl_2$ is one of only a few metal complexes shown to cleave DNA in the absence of O_2 or some other oxidant.

6.2. Other synthetic mononuclear nucleases

A number of other metal complexes are known which are also capable of hydrolyzing RNA. For example, Cu(II) complexes of 2,2′-bipyridine (bpy) and 2,2′,2″-terpyridine (trpy) are able to hydrolyze both ApA [111] and $poly(a)_{12-18}$ oligomers [112,113]. Also, two complexes closely related to $Cu([9]aneN_3)Cl_2$, $Zn([9]aneN_3)(NO_3)_2$ and $Zn([12]aneN_3)(NO_3)_2$ (Fig. 14), catalytically hydrolyze the RNA dinucleotide ApUp [114]. The mechanism proposed for this reaction is analogous to the one proposed for the hydrolysis of ApA by $Cu([9]aneN_3)Cl_2$; following binding of the phosphate, a metal-coordinated hydroxide is proposed to assist, via general base catalysis, in the attack of the 2′-OH, resulting in a 2′,3′-cyclic intermediate. Perhaps the complexes which have shown the greatest promise in hydrolyzing RNA, however, are lanthanide complexes. As mentioned previously, many of the characteristics which make Cu(II) and Zn(II) complexes desirable as hydrolases, such as strong Lewis acidity and fast ligand substitution rates (Table 1), can also be found in lanthanide complexes. Thus, despite the difficulties in working with lanthanide systems [79–82], lanthanide complexes with hexadentate and octadentate ligands have been utilized and they are quite effective at RNA transesterification/hydrolysis [81,99,108,115]. In the two cases tested, however, the complexes were unable to utilize either double-stranded RNA [115] or an RNA–DNA hybrid duplex region [108] as a substrate.

Given the extreme resistance of DNA towards hydrolysis, it is not surprising that there are many fewer examples of well-defined metal complexes which are able to hydrolyze DNA. The Cu(II) complex, $[(TACH)Cu(OH)(OH)_2]^+$ [116], as well as three different Co(III) complexes, $[(cyclen)Co(OH)(OH_2)]^{2+}$, $[(tamen)Co(OH)(OH_2)]^{2+}$, and $[(trpn)Co(OH)(OH_2)]^{2+}$ (Fig. 17) [117,118], have recently been reported to hydrolyze supercoiled DNA. In an elegant experiment, Hettich and Schneider [118] demonstrated that the introduction of alkylammonium substituents onto the cobalt ligands increased the rate of hydrolysis of the supercoiled DNA substantially. Using saturation kinetics, they determined that while the rate constant of the cleavage step, k_{cat}, remained fairly constant, the affinity of the



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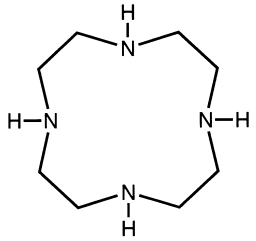

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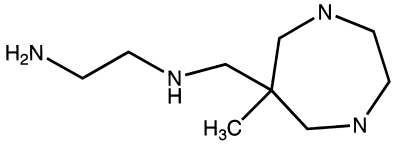
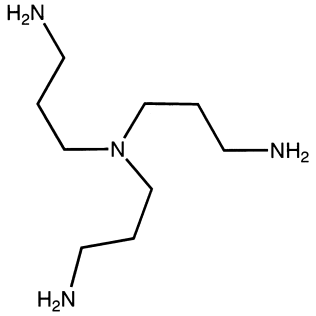


tamen



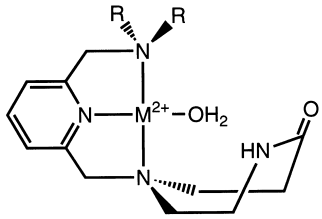
trpn

Fig. 17. Structures of some polyamine ligands: *cis*,*cis*-1,3,5-triaminocyclohexane (tach), 1,4,7,10-tetraazacyclododecane (cyclen), 6-(4-amino-2-azabutyl)-6-methyl-1,4-diazacycloheptane (tamen), tris(3-aminopropyl)amine (trpn).

complex for the DNA, K_m, increased considerably with increasing charge. Lanthanide complexes have also been used to effect hydrolysis of DNA. Macrocyclic complexes of La(III), Eu(III), Yb(III) and Ce(III) have been used to hydrolyze supercoiled DNA and both single-stranded and double-stranded DNA oligomers [115,119,120]. Interestingly, when Ce(III) salts were used to promote the hydrolysis of DNA, the reactions required O_2, presumably because Ce(IV) is the active species [121]. In some cases it was observed that the addition of two different ions promoted the hydrolysis to a much greater extent than either ion individually, suggesting a cooperativity between the two ions [122,123]. It should be noted, however, that in all of these examples of lanthanide-promoted hydrolysis of DNA, the metal complexes were not independently characterized. As a result, great care must be exercised when extracting mechanistic information.

7. Mechanistic studies on labile metal-promoted amide hydrolysis

The hydrolysis of amides by labile metal complexes is not as well studied as the corresponding reaction with phosphodiester bonds and, as a result, there is much



M = Cu, Zn

R = -Me or -CH_2COO^-

Fig. 18. Preorganized metal complexes exhibiting facile amide bond cleavage.

less mechanistic information available on labile metal-promoted amide hydrolysis. Moreover, much of the mechanistic information which is available was obtained with complexes which were generated in situ and, therefore, the coordination environment of the metal ions is not completely certain. Groves and Chambers [124] demonstrated that Cu(II) and Zn(II) efficiently hydrolyze unactivated amide bonds when the metal binding site anchors the metal above the plane of the amide bond and that the catalytically active species is a metal–hydroxide complex (Fig. 18). Divalent transition metal ions have also been used to hydrolyze various picolinamides [125,126] and the results lend support to the involvement of a metal-bound hydroxide [125]. Moreover, if amide NH deprotonation was prevented, the rate enhancements increased significantly, since NH deprotonation inhibits loss of the amine leaving group [126]. One example of amide hydrolysis by a well-characterized labile metal complex is the hydrolysis of DMF by a Cu(II)-bipyridine complex derivative [127]. Interestingly, no hydrolysis could be observed by $Cu(tpy)^{2+}$ (tpy = 2,2′,2″-terpyridine) which has only one vacant coordination site. These results lend credence to a mechanism consisting of substrate binding via the carbonyl, followed by the involvement of a metal-bound hydroxide, either through direct nucleophilic attack or general base catalysis.

8. Examples of metal-based synthetic peptidases

8.1. Cu([9]aneN$_3$)Cl$_2$

We discovered that the synthetic nuclease Cu([9]aneN$_3$)Cl$_2$ is also a synthetic peptidase [128]. When the unactivated dipeptide glycyl–glycine (gly–gly) was incubated with Cu([9]aneN$_3$)Cl$_2$, gly–gly was hydrolyzed, with glycine as the only product. Although the reaction was slow, Cu([9]aneN$_3$)Cl$_2$ acted as a true catalyst, exhibiting both rate enhancement and turnover. The protein bovine serum albumin (BSA) was also found to be a substrate for hydrolysis by Cu([9]aneN$_3$)Cl$_2$ [128]. Remarkably, in HEPES buffer, Cu([9]aneN$_3$)Cl$_2$ exhibited specificity, cleaving BSA

in one general location. The fact that the termini were susceptible to Edman degradation, and that performing the reaction anaerobically gave no change in either the extent of cleavage or the products observed, provided direct evidence for a hydrolytic cleavage mechanism. Both reactions exhibited a large buffer effect; gly–gly and BSA were hydrolyzed more rapidly in sodium bicarbonate buffer than in HEPES buffer. This result suggests a mechanism where the bifunctional sodium bicarbonate buffer acts as a proton transfer agent and facilitates breakdown of the tetrahedral intermediate. $Cu([9]aneN_3)Cl_2$ is the first metal complex shown to hydrolyze both proteins and nucleic acids.

8.2. Other metal complexes

Although the hydrolysis of peptide bonds by metal ions is well known, there are very few examples of peptide hydrolysis using well-defined metal complexes. One example is a Ce(IV)-cyclodextrin complex, which has been shown to hydrolyze various di- and tripeptides [129]. Various Pt(II) diamine complexes, as well as $[PtCl_4]^{2-}$, have also been used to effect hydrolysis of peptide bonds under acidic conditions [130]. In these reactions, the metal binds to the sulfur of a cysteine or methionine and selectively hydrolyzes the peptide bond involving the carboxyl group of the amino acid to which the complex is bound (Fig. 19). Following coordination of the Pt^{2+} ion to the sulfur atom on the substrate, the reaction proceeds through a rate-determining unimolecular step; this step is believed to be the displacement of a Pt^{2+} ligand by the amide oxygen. This in turn activates the carbonyl for nucleophilic attack by an external water molecule. It should be noted, however, that direct attack by a metal-coordinated water cannot be ruled out by the kinetic data [130].

Because Pt^{2+} is essentially inert with respect to ligand substitution, Pd(II) complexes, which are much more substitutionally labile, were tested for activity [131,132]. $[PdCl_4]^{2-}$ and various Pd(II) complexes also promote the regioselective hydrolysis of Cys- and Met-containing polypeptides (Fig. 19), although the reactions must be performed under rather acidic conditions to prevent the formation of inactive hydroxo-bridged oligomers. In these reactions, the active catalyst has been shown to be a Pd(II)-substrate dimer, in which the sulfur atoms from two polypeptides bridge the two Pd atoms, forming a four membered ring (Fig. 20) [131,132]. Because of the *trans* labilizing effect of the sulfur atoms and the acidic conditions under which these experiments are performed, the amine chelating ligands are quite labile and are replaced by water. Thus, the active catalyst is the same

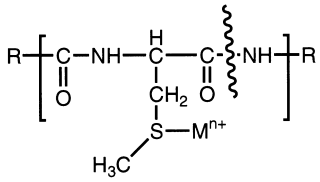


Fig. 19. Regioselective hydrolysis of sulfur-containing peptides by Pt(II) and Pd(II) complexes.

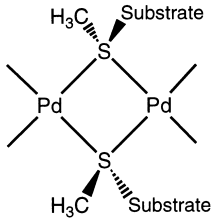


Fig. 20. Proposed active species in the hydrolysis of sulfur-containing peptides by Pd(II) complexes.

regardless of whether the starting material is $[PdCl_4]^{2-}$, $[Pd(en)(H_2O)_2]^{2+}$, or $[Pd(H_2O)_3(OH)]^{+}$ [131]. The hydrolysis of the ligand-Pd(II) bond can be prevented, however, simply by replacing the ligand nitrogen atoms with sulfur atoms, which are not displaced [133]. Interestingly, the rate of peptide hydrolysis decreases as the number of sulfur atoms on the ligand increases from zero to one to two, indicating the importance of the coordinated water molecules and suggesting that, in Pd(II) complexes, peptide hydrolysis proceeds via attack of a metal-coordinated water molecule [133].

More recently, *cis*-$[Pd(en)(H_2O)_2]^{2+}$ was shown to hydrolyze dipeptides His-X, where X is any one of a number of non-sulfur-containing amino acids [19]. Significantly, this reaction is catalytic. Hydrolysis is initiated by binding of the complex to histidine; the same regioselectivity is observed as described above (i.e. X-His is not a substrate for hydrolysis) and the rate of hydrolysis is partially dependent on the steric bulk of the residue to the carboxy terminus of the cut site [19]. In contrast to the Pd(II)-promoted hydrolysis of peptide bonds containing sulfur residues, however, the active species in this reaction is a monomeric substrate-Pd(II) complex in which the amine ligands remain coordinated. This reaction has been studied in great detail and the complex set of substrate-catalyst equilibria determined [19,134].

The site-specific hydrolysis of the protein cytochrome, *c*, has also been realized using Pd(II) complexes, indicating the versatility of these complexes [135]. The major cut site was between His_{18} and Thr_{19} as determined via Edman degradation, although there were other minor sites not identified. Despite the presence of the histidine residue at the cut site, it was hypothesized that it was Cys_{17} which was responsible for the specificity. This notion is supported by the fact that the tripeptide Cys–His–Ala is hydrolyzed much more rapidly than His–Ala [19]. Thus, it appears as if His is a ligand for the Pd(II) complexes only in the absence of a suitable sulfur-containing residue.

9. Future directions

The rationale for designing synthetic hydrolases is two-fold: to create a complex which has utility in biochemistry and molecular biology, and to more fully understand the function of metal ions in naturally occurring hydrolases. These two goals

are not mutually exclusive and although the recent advances towards the development of metal-based synthetic hydrolases is impressive, there is much work still to do. The rate at which current metal complexes hydrolyze RNA, DNA, or proteins is still far below the enzymatic rate and the exact role of metal ions in naturally occurring hydrolases is far from certain. In fact, much of the insight we have today comes from crystal structures of enzymes, which strictly speaking reveal nothing about the mechanisms.

Based in part on the results and observations described in this review, the effort to design more efficient synthetic hydrolases has, to a large extent, moved away from traditional d-block transition metal complexes and a number of new directions are being explored. As this review demonstrates, one area of active research is in the use of lanthanide complexes as synthetic hydrolases. Another approach towards designing more efficient hydrolytic agents has been to utilize redox active transition metal complexes in the presence of O_2 [136] or H_2O_2 [137,138]. Although these are conditions which are typically used to effect oxidative cleavage, the products observed in the cleavage of amides [136], proteins [138], and DNA [137] were consistent with hydrolysis, presumably due to the formation of nucleophilic peroxides [137,139].

Recent advances have also led to the hydrolysis of DNA using metal ions in the presence of polydentate ligands which are covalently attached to DNA binding agents. While this approach has been used previously with great success in the oxidative cleavage of both proteins [140,141] and DNA [142–144], it has only recently been employed in hydrolytic reactions. The most common technique has been to covalently attach a lanthanide binding site to a DNA oligomer to affect site-specific hydrolysis of complementary single-stranded RNA and DNA [15,145,146]. Using a similar approach with a transition metal ion, Barton and coworkers [147] linked a DNA intercalator to a small, α-helical peptide which was specially designed to contain a zinc binding site. This latter system is especially noteworthy because it is able to hydrolyze both supercoiled DNA and linear double-stranded DNA oligomers using relatively low concentrations of complex [147].

Finally, polynuclear metal complexes have also shown promise as metallohydrolases. Recently, dinuclear lanthanide complexes were used to hydrolyze both activated phosphodiesters and simple dinucleotides, as well as double-stranded DNA [123,148]. Significantly, the dinuclear complexes are five times more reactive than the corresponding mononuclear complexes and kinetic analysis has demonstrated that both metals are involved in the mechanism [148]. Dinuclear and trinuclear transition metal complexes have also been synthesized and provide not only increased hydrolytic activity, but also well-defined coordination environments from which mechanistic information can be obtained [106,118,149–156]. At the present time, however, this area of research is still relatively new and the mechanism through which polynuclear complexes promote hydrolysis is not fully understood. It is clear, however, that the dinuclear complexes are more reactive than the corresponding mononuclear ones and that the distance between the two metal atoms is important [149,150], suggesting cooperativity between the two ions. Whether this cooperativity is the result of perturbation in a monomer–dimer equilibrium, double Lewis activation, stabilization of the leaving group, or a combination of these effects is not

currently known. However, future experiments will continue to increase our understanding of these reactions and provide further insight into the role of metal ions in naturally occurring hydrolases.

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