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Distinct chemical factors in hydrolytic reactions catalyzed by metalloenzymes and metal complexes

Leonardo F. Serafim,  Vindi M. Jayasinghe-Arachchige, Lukun Wang, Parth Rathee, Jiawen Yang, Sreerag Moorkannur N.  and Rajeev Prabhakar 

The selective hydrolysis of the extremely stable phosphoester, peptide and ester bonds of molecules by bio-inspired metal-based catalysts (metallohydrolases) is required in a wide range of biological, biotechnological and industrial applications. Despite the impressive advances made in the field, the ultimate goal of designing efficient enzyme mimics for these reactions is still elusive. Its realization will require a deeper understanding of the diverse chemical factors that influence the activities of both natural and synthetic catalysts. They include catalyst–substrate complexation, non-covalent interactions and the electronic nature of the metal ion, ligand environment and nucleophile. Based on our computational studies, their roles are discussed for several mono- and binuclear metallohydrolases and their synthetic analogues. Hydrolysis by natural metallohydrolases is found to be promoted by a ligand environment with low basicity, a metal bound water and a heterobinuclear metal center (in binuclear enzymes). Additionally, peptide and phosphoester hydrolysis is dominated by two competing effects, *i.e.* nucleophilicity and Lewis acid activation, respectively. In synthetic analogues, hydrolysis is facilitated by the inclusion of a second metal center, hydrophobic effects, a biological metal (Zn, Cu and Co) and a terminal hydroxyl nucleophile. Due to the absence of the protein environment, hydrolysis by these small molecules is exclusively influenced by nucleophile activation. The results gleaned from these studies will enhance the understanding of fundamental principles of multiple hydrolytic reactions. They will also advance the development of computational methods as a predictive tool to design more efficient catalysts for hydrolysis, Diels–Alder reaction, Michael addition, epoxide opening and aldol condensation.

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Department of Chemistry, University of Miami, Coral Gables, FL 33146, USA. E-mail: rpr@miami.edu; Fax: +1 305-284-4571; Tel: +1 305-284-9372



Leonardo F. Serafim

focused on understanding the catalytic mechanism of DNA repairing proteins.

Dr Leonardo F. Serafim was born in Brazil, where he received his BSc in chemical engineering, from the Federal University of Ceara in 2013, and his MSc degree in chemistry in 2015, from the same university. In 2022, he obtained his PhD in chemistry from the University of Miami, under the supervision of Prof. Rajeev Prabhakar. Currently, he is a postdoctoral fellow in Prof. Ivaylo Ivanov's group at Georgia State University. His research is



Vindi M. Jayasinghe-Arachchige

involved with the popular gene editing tool CRISPR and the designing of novel CRISPR-Cas proteins.

Dr Vindi M. Jayasinghe-Arachchige received her BSc (Hons) in Chemistry from the University of Sri Jayewardenepura, Sri Lanka, in 2011. She obtained her PhD in chemistry from the University of Miami, Florida, under the supervision of Prof. Rajeev Prabhakar, in 2020. Currently, she is working as a postdoctoral fellow in Prof. Jin Liu's group at the University of North Texas Health Science Center, Texas. Her current research focuses on the studies of the mechanisms

I. Introduction

The design of small metal complexes that can efficiently mimic the activities of metalloenzymes has been one of the holy grails in chemistry.^{1–8} In the last few decades, a wide range of complexes have been designed to selectively catalyze diverse chemical reactions.^{9–38} They were by and large inspired by mono- and binuclear metal centers of enzymes.^{30,39–53} Despite the substantial progress made in the field, the existing metal complexes exhibit much slower activities and lower turnover numbers in comparison to natural enzymes. Therefore, there is intense interest in the development of the next generation of molecules with enhanced activities.^{4,5,7,29,30,54–62} Based on our research, this article is focused on the mechanisms of different metalloenzymes and their synthetic analogues that promote hydrolysis of peptide ($-(O=)C-NH-$), ester ($((R)(C=O)(OR))$) and phosphoester ($((O=)(RO)(RO)(P-O-R))$) bonds. Specifically, the

roles of distinct chemical factors such as metal ion(s), ligand environment, nature of substrates, coordination numbers and non-covalent interactions that control their functioning are elucidated. An improved understanding of these factors will help in the design of versatile catalysts not only for hydrolysis but also for many other reactions including epoxide opening, aldol condensation, Michael addition and Diels–Alder reactions.^{63–67}

The peptide, ester and phosphoester bonds are ubiquitous in a wide range of biologically, industrially and environmentally relevant molecules such as proteins, pharmaceuticals, deoxyribonucleic acid (DNA), pesticides, nerve agents and plastic products.^{12,14,68–77} As a result, their selective hydrolysis plays important roles in many critical applications like protein engineering, therapeutics, genomics, DNA repair and remediation of pesticides, nerve agents and plastics.^{78–84} As expected these bonds are extremely stable, and the half-life for the



Lukun Wang

Lukun Wang received his BSc in chemistry in 2018 from the University of Miami. Since 2019, he has been under the supervision of Prof. Rajeev Prabhakar in pursue of a PhD degree. His research focuses on investigating the mechanism of phosphoester hydrolysis and understanding the differences between the reaction pathways of natural enzymes and artificial catalysts by using various computational tools and techniques, including molecular dynamics simulations and quantum chemical calculations.



Parth Rathee

Parth Rathee obtained his BSc in Pharmaceutical Sciences from Birla Institute of Technology, in India, in 2019. Currently, he is pursuing his PhD under the supervision of Prof. Rajeev Prabhakar at the University of Miami. His research interests focus on investigating self-assembling peptide-based catalysts, organo-metallic catalysts, and natural enzymes. He is also interested in studying inhibition of enzymes by small peptidomimetics.



Jiawen Yang

Jiawen Yang received his BSc in chemistry from Shandong University, China, in 2018. He joined and accumulated lab experience from Prof. Dejun Sun's group from 2016 to late 2018 in Shandong University. Currently, he is a PhD student in Prof. Rajeev Prabhakar's group at the University of Miami. His research interests focus on the design of mononuclear metalloenzymes and di-nuclear peptidic synthetic analogues, and their small complex mimics, for phosphoester hydrolysis and superoxide dismutase.



Sreerag Moorkkannur N.

Sreerag N. Moorkkannur received his BSc in chemistry, in 2018, from Calicut University, in India, and he was awarded a MSc degree in chemistry, in 2020, from Pondicherry University, also in India. He worked as a Project Associate under Prof. E. D. Jemmis, at Indian Institute of Science, Bangalore, in 2021, studying the mechanistic aspects of organometallic reactions using various computational techniques. Since 2022 he has been under the supervision of Prof. Rajeev Prabhakar at the University of Miami, in pursue of a PhD degree. His research focuses on the mechanistic aspects of enzyme and organometallic complex catalyzed reactions.

hydrolysis of peptide, ester and phosphoester bonds is 350–600, 60–470 and $\sim 130\,000$ years, respectively, at room temperature and pH = 4–8.^{85,86} In nature, these bonds are hydrolyzed by highly specialized mono- and binuclear metal center containing enzymes, which depending on the nature of the scissile bond are categorized as proteases/peptidases, esterases and phosphatases/nucleases and in general known as metallohydrolases.^{12,14,69,70,87–93} These bonds can also be cleaved by a wide range of organic cofactor possessing enzymes.⁹⁴ For instance, serine proteases, cysteine proteases, threonine proteases, glutamic proteases, and aspartyl proteases utilize either a triad or dyad formed by specific amino acid residues to hydrolyze peptide bonds of their substrates.^{95,96} Metallohydrolases display significant structural diversity in terms of amino acid sequence, nature of metal ions and substrates, ligand environment and second coordination shell residues. Therefore, it is of fundamental importance to develop a deeper understanding of their reaction mechanisms. In these mechanisms, a multitude of chemical factors such as the nature of the metal center (mono- or binuclear), metal ions (di-, tri or tetravalent), ligand environment (symmetric or asymmetric), catalyst–substrate complexation (monodentate, bidentate or indirect), nucleophile (terminal or bridging) and non-covalent interactions play key roles. These distinct factors are productively utilized by these catalysts for their efficient functioning. However, their sources, extent and combinations are system dependent and it is not trivial to separate and elucidate them experimentally.^{29,97–101} The available experimental information provided an ideal platform to employ our theoretical and computational chemistry techniques to understand their roles in these reactions.

II. Mechanisms of natural metallohydrolases and their synthetic analogues

In this section, the aforementioned effects are systematically discussed for peptide, ester and phosphoester hydrolysis. These effects include substrate and water activation, nucleophilicity,

basicity and metal cooperativity and are discussed using the computed values of the scissile bond, O–H of water, metal–nucleophile, metal–ligand and metal–metal distances as parameters.

IIa. Peptide hydrolysis by metalloproteases

Here, the mechanisms of different mono- [insulin degrading enzyme (**IDE**), neprilysin (**NEP**) and matrix metalloproteinase (**MMP**)] and binuclear [bovine lens leucine aminopeptidase (**BILAP**) and *Streptomyces griseus* aminopeptidase (**SgAP**)] metalloproteases are discussed (Fig. 1). **IDE** contains a common Zn–N₂O [Zn(His, His and Glu)] catalytic core that is also possessed by other members of the family such as thermolysin (TLN) and carboxypeptidase A (Fig. 1).^{89,102–106} It catalyzes the degradation of several critical biomolecules like insulin, amyloid beta (A β), amylin and glucagon.⁸⁹ This enzyme plays a preventive role in the development of cancer, obesity, Alzheimer's disease (AD) and type-2 diabetes.^{107,108} It exhibits broad specificity and hydrolyzes a wide range of chemically diverse peptide bonds of its substrates like Val-His, His-Gln, Phe-Phe and Lys-Gly of A β .^{89,109,110} **NEP** is also a Zn–N₂O [Zn(His, His and Glu)] in Fig. 1] core possessing enzyme that hydrolyzes a variety of physiologically relevant molecules including A β , leucine⁵ or methionine⁵-enkephalin, bradykinin, atrial natriuretic factor (ANF) and substance P.^{111–113} It exhibits a preference for cleavage on the amino terminal side of hydrophobic residues. In comparison to **IDE** and **NEP**, **MMP**^{114,115} possesses a Zn–N₃ [Zn(His, His and His)] core in which a negatively charged Glu residue is substituted with a neutral His residue (Fig. 1). This core is also commonly found in other matrix metalloproteinases^{116,117} and carbonic anhydrase.^{118,119} **MMP** degrades collagen, elastin, gelatin, and other glycoproteins and proteoglycans.¹¹⁴ It is involved in cardiovascular diseases and many different types of cancers.^{120,121} Among binuclear metallohydrolases, the non-equivalent Zn1(O₃)-Zn2(NO₃) [Zn1(Asp, Glu, Asp)-Zn2(Lys, Glu, Asp, Asp)] core containing **BILAP** is extremely prevalent and found in humans, animals, bacteria, and plants (Fig. 1).^{9,70} It prefers to hydrolyze a leucine residue located at the N-terminus in a di- or tripeptide sequence, but is also capable of hydrolyzing other amino acids as well.⁴⁰ **BILAP** has been implicated in HIV, cancer, cataract, and cystic fibrosis.¹²² On the other hand, **SgAP** contains an almost equivalent Zn1(NO₂)-Zn2(NO₂) [Zn1(His, Asp and Asp)-Zn2(His, Glu, and Asp)] core and exhibits exceptional catalytic promiscuity by hydrolyzing both peptide and phosphoester bonds with remarkable efficiency (Fig. 1).^{123,124} It exhibits a preference for large hydrophobic N-terminus residues and can hydrolyze different amino acid (Gly, Met, Val, Ala, Lys)-pNA (*para*-nitro aniline) analogues.¹²⁴ Thus, due to their structural and catalytic properties, these enzymes serve as model systems to gain deeper understanding of peptide hydrolysis by metalloproteases.

All these enzymes follow the general acid/base mechanism(s) for the hydrolysis of different substrates (Fig. 2). In the first step of the mechanism for mononuclear enzymes (Fig. 3a), a water molecule is activated by a base to create the hydroxyl nucleophile. It could be generated by using either a metal bound or free water



Rajeev Prabhakar

Dr Rajeev Prabhakar is a professor of chemistry at the University of Miami, FL, USA. He received his PhD from Stockholm University (2003) and did his post-doctoral research at Emory University (2003–2006). His main research interests are applications of innovative theoretical and computational approaches to study the mechanisms of chemical and biochemical reactions and the design of catalysts, drugs and biomaterials.

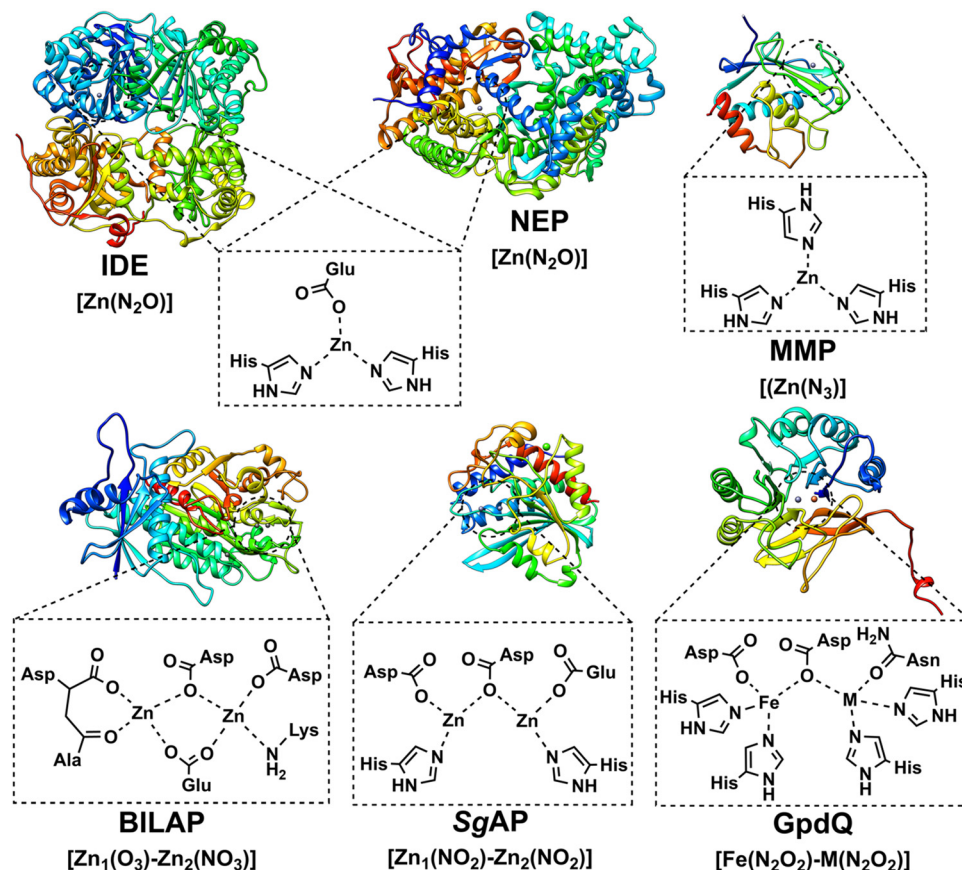


Fig. 1 Metal center(s) of mono- and binuclear metallohydrolases.

molecule and Glu, Asp or His-Asp dyad as a base in these systems. In the second step, the nucleophile generated in the previous step attacks the scissile peptide bond (C–N) of the substrate and creates a tetrahedral *gem*-diolate intermediate. In some cases, the first two steps could also occur synchronously in a single step. In the final step, the cleavage of the C–N bond through proton abstraction by the substrate collapses the intermediate. This process can also occur through distinct pathways in these enzymes. A vast majority of metalloproteases contain the Zn^{2+} ion due to its high Lewis acidity, a redox-inactive state, low ligand field stabilization energy, and flexible coordination number (3–5).¹²⁵ These properties facilitate activation of the substrate, creation of the nucleophile and release of the product. In the section below, the information concerning the roles of diverse chemical factors in these steps of the aforementioned metalloproteases derived from quantum chemical calculations is discussed. Almost all structures in DFT calculations discussed below were optimized using the hybrid B3LYP functional^{126,127} and the double zeta basis set. The energetically most feasible mechanisms were also investigated using other functionals such as MPW1PW91¹²⁸ and M06-2X.¹²⁹ The small energy differences (1.0–2.0 kcal mol^{−1}) between different functionals showed that energies were not very sensitive to the level of theory used in these calculations.

Ila1. Generation of the nucleophile and *gem*-diolate intermediate. Here, a metal bound water is commonly used to create the hydroxyl nucleophile (Fig. 3a). Its binding to a metal ion and hydrogen bonding by a neighboring negatively charged amino acid residue lower its pK_a value from ~ 14 to ~ 7 .^{130,131} This is a common strategy utilized by metallohydrolases to activate a water molecule. A vast majority of these enzymes possess Zn at their active sites.¹³² A low coordination number (3–4) of Zn^{2+} in these sites increases the acidity of the water molecule and promotes the formation of the hydroxyl nucleophile.¹²⁵ It is noteworthy that non-metallic proteases that contain only an organic cofactor activate a free water utilizing only non-covalent interactions.^{88,133,134} In the reactant of **IDE**, the Zn^{2+} metal ion is coordinated to three direct ligands (His, His and Glu), a nucleophile generating water molecule and the substrate through the Zn–carbonyl bond. In a concerted manner, a second coordination shell Glu residue functions as a base and triggers the nucleophilic attack on the substrate to generate the *gem*-diolate intermediate. Rather interestingly, the computed energetics predicts that this step depends on the nature of the substrate. For instance, it occurs with barriers of 14.3, 18.8 and 22.3 kcal mol^{−1} for three chemically distinct dipeptide Lys–Gly (polar–nonpolar), Phe–Phe (nonpolar–nonpolar), and His–Gln (polar–polar) substrates, respectively.¹³⁵ The mode of substrate binding and alterations in the metal–substrate

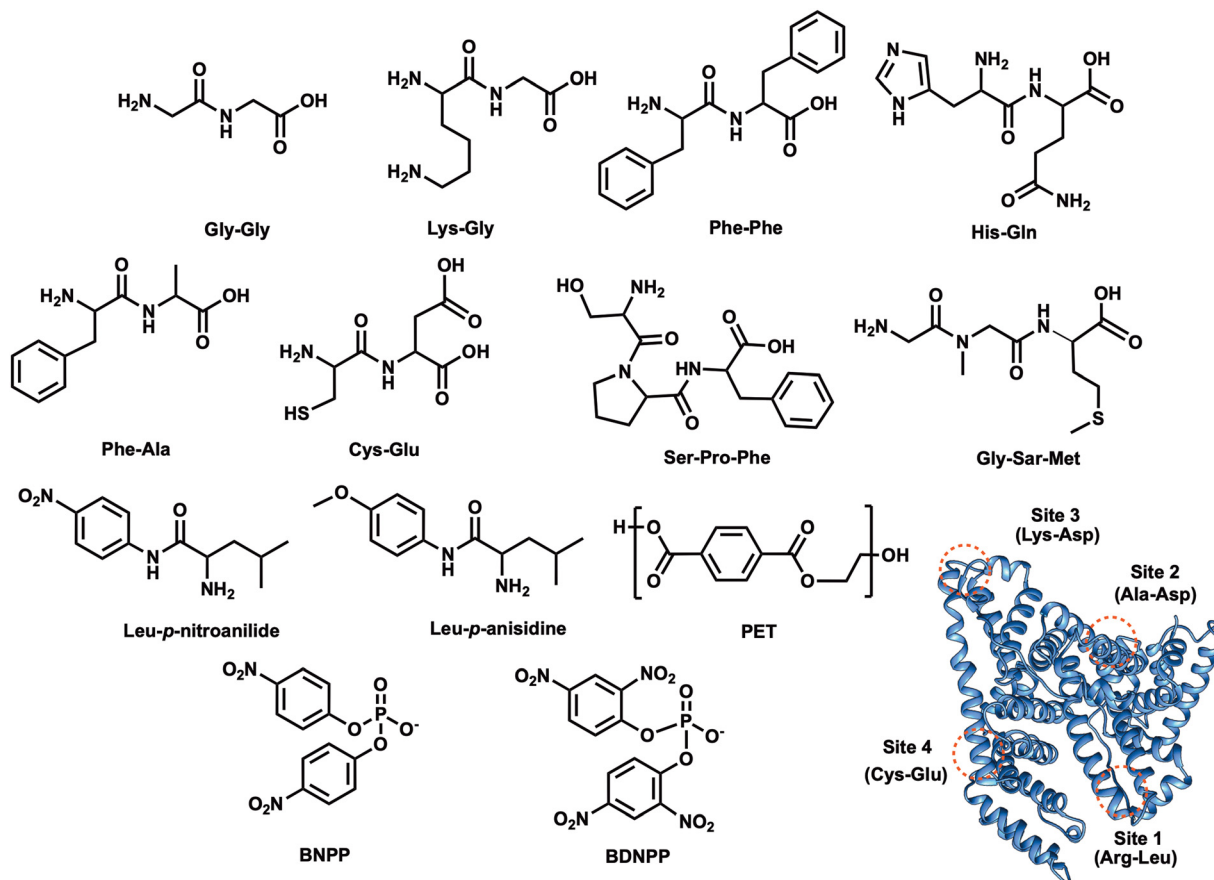


Fig. 2 Structures of the substrates used for peptide, ester and phosphoester hydrolysis.

and metal–water bonds contribute to the differences in computed barriers. These interactions provide measures of Lewis acid and nucleophile activation by the metal center. The computed barriers are in line with the measured value of $17.2 \text{ kcal mol}^{-1}$ for A β degradation by IDE.¹¹⁰ Additionally, they are comparable to the measured ($12.4\text{--}16.3 \text{ kcal mol}^{-1}$)¹³⁶ and calculated ($15.2 \text{ kcal mol}^{-1}$)¹³¹ barriers for thermolysin. Furthermore, this step is identified as the rate-limiting step of the entire mechanism for all three substrates. In the *gem*-diolate intermediate, the scissile peptide bond is significantly activated for all three substrates in comparison to the corresponding reactant.

NEP which also possesses the IDE like Zn–N₂O core interacts with the polyethylene terephthalate (PET) substrate (Fig. 2), an ester, through the metal–carbonyl bond. However, in comparison to IDE, this enzyme can utilize either a metal bound or free water for hydrolysis (Fig. 3a).¹³⁷ In the reactant of NEP, as discussed for IDE, the metal-bound water is significantly activated (1.04 \AA) due to the combined polarization by the Zn²⁺ ion and Glu base. It readily donates its proton to Glu and the hydroxyl nucleophile concomitantly attacks the substrate. The barrier of $9.1 \text{ kcal mol}^{-1}$ for this step is substantially lower than barriers computed for all three substrates of IDE due to the provision of a stronger nucleophile by the enzyme. However, in an alternative pathway, a non-metal bound water is activated by the His-Asp dyad base, instead of a Glu residue in the previous

pathway, to create the nucleophile. In the reactant, the nucleophile generating water interacts with the His residue of the dyad through a strong hydrogen bond. It is noteworthy that the Asp residue of the dyad facilitates proton abstraction by the His residue. Here, the water activation (0.99 \AA) is significantly less than the one obtained upon metal binding. However, the addition of a hydrogen bond to the nucleophile generating water by an external water further increases its acidity and elongates the O–H bond by an additional 0.01 \AA . It is still less activated than the water (1.04 \AA) upon metal binding. Here, the nucleophile attack takes place with a barrier of $13.9 \text{ kcal mol}^{-1}$ which is $4.8 \text{ kcal mol}^{-1}$ higher than the corresponding barrier for the attack by the metal-bound hydroxyl. This increase is attributed to the provision of a weaker nucleophile in the pathway. On the other hand, MMP utilizes a slightly different Zn–N₃ core formed by three neutral His ligands (Fig. 1).^{114,138} In the reactant of MMP, the metal–ligand bonds are longer than in the IDE case. Additionally, the metal–substrate interaction is weaker, while the metal–water coordination is stronger in comparison to the N₂O core containing IDE.¹³⁹ The computed barrier is $3.4 \text{ kcal mol}^{-1}$ lower than the IDE case, *i.e.* $15.4 \text{ kcal mol}^{-1}$ from the corresponding reactant for the same Phe–Phe substrate. However, unlike IDE, creation of the *gem*-diolate intermediate takes place in a stepwise manner for this enzyme. It shows that a single substitution in the metal center shifted the rate-determining step of the mechanism.

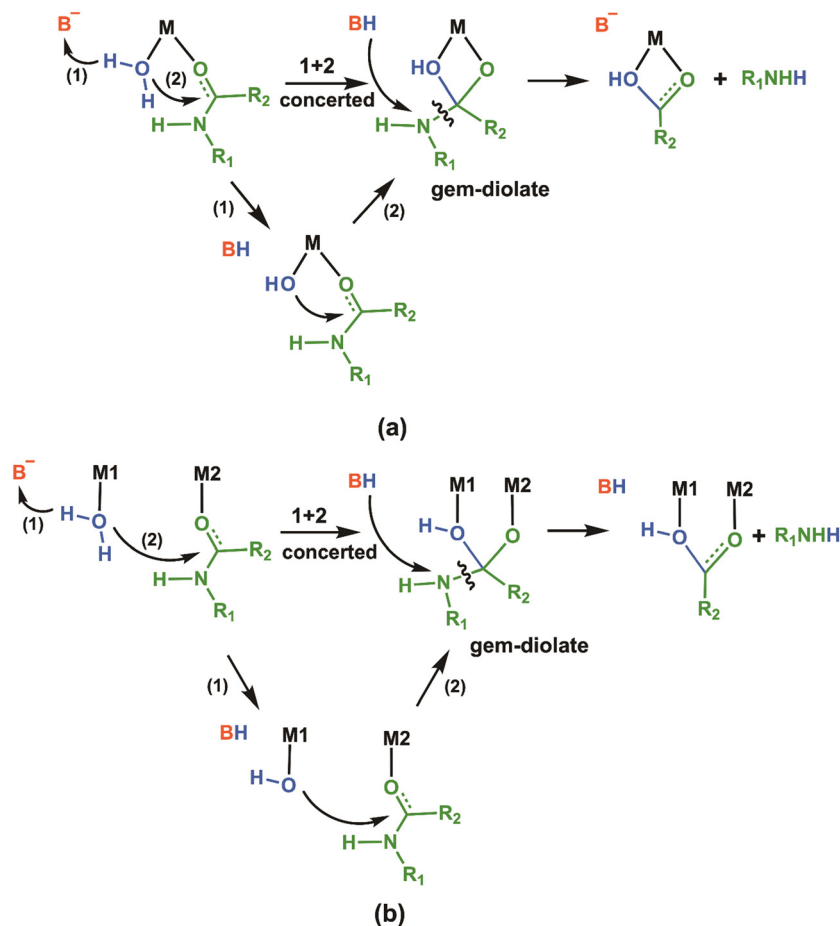


Fig. 3 General mechanisms of peptide hydrolysis for mononuclear (a) and binuclear (b) systems.

These results elucidate that the metal-bound water is more activated than a free water polarized by only non-covalent interactions. However, the inclusion of a hydrogen bond enhances its acidity. Due to the difference in the acidity of water, the basicity of Glu or the His-Asp dyad cannot be compared. The lower basicity of the ligand environment of **MMP** also influences the energetics of the mechanism.

In comparison to mononuclear metallopeptidases, their binuclear counterparts such as **BILAP** and **SgAP** follow a mechanism in which both nucleophile and *gem*-diolate intermediate generation occurs in a concerted manner (Fig. 3b). **BILAP** utilizes a non-equivalent binuclear core $\text{Zn1}(\text{O}_3)\text{-Zn2}(\text{NO}_3)$ for hydrolysis (Fig. 1). In the reactant, similar to mononuclear enzymes, the *L*-leucine-*p*-nitroanilide (Leu-*p*NA) substrate (Fig. 2) directly interacts with the Zn1 ion through the carbonyl group of the scissile peptide bond.¹⁴⁰ In contrast to them, the nucleophile generating water molecule bridges ($\mu\text{-OH}_2$) both metal ions and is more acidic. However, in the creation of the *gem*-diolate intermediate the nucleophile is provided by only one metal center. The greater nucleophilicity of a single metal bound hydroxyl group in comparison to the $\mu\text{-OH}_2$ mode is likely to be the reason for that. In this enzyme, there are two candidates to play the role of a base, a Zn2-bound Asp residue and a bicarbonate ion. The former has been proposed as a base in theoretical studies of other members of

the family such as *Aeromonas proteolytica* aminopeptidase (AAP),¹⁴¹ methionine aminopeptidase (MetAP),¹⁴² and prolidase¹⁴³ that lack the bicarbonate ion. In **BILAP**, the formation of the *gem*-diolate intermediate is found to take place through a similar barrier of $\sim 19.0 \text{ kcal mol}^{-1}$ using either metal bound Asp or the bicarbonate ion as the base. The substitution of *L*-leucine-*p*-nitroanilide possessing an electron withdrawing nitro group ($-\text{NO}_2$) with *L*-leucyl-*p*-anisidine (Fig. 2) that contains an electron donating methoxy group ($-\text{OCH}_3$) shortens both metal-nucleophile and scissile peptide bonds by 0.02 \AA . These changes weaken the nucleophile and strengthen the peptide bond. As a result, the barrier is increased by $7.5 \text{ kcal mol}^{-1}$ in comparison to that for the *L*-leucine-*p*-nitroanilide substrate. Furthermore, replacement of Zn1 and Zn2 with Mg and Co in the Mg1-Zn2 and Mg1-Co2 variants, on the basis of experiments,¹⁴⁴ reduces both metal-nucleophile and metal-substrate distances. The barrier for this step decreases slightly by $2.0 \text{ kcal mol}^{-1}$ for the Mg1-Zn2 enzyme and increases by $6.9 \text{ kcal mol}^{-1}$ for the Mg1-Co2 enzyme.

SgAP possesses an almost equivalent binuclear core, $\text{Zn1}(\text{NO}_2)\text{-Zn2}(\text{NO}_2)$ (Fig. 1), in comparison to a non-equivalent core of **BILAP**. The theoretical calculations propose a hybrid mechanism for the Leu-*p*NA (Fig. 2) hydrolysis catalyzed by the enzyme.¹⁴⁵ In the reactant, both functional groups (carbonyl and amine) of Leu-*p*NA

interact with the binuclear metal core, *i.e.* the amine group coordinates to Zn1, while the carbonyl group to Zn2. Additionally, similar to **BILAP**, the nucleophile generating water is symmetrically bound in a μ -OH manner to both metals. This binding mode was also in agreement with the fluoride inhibition experiments.¹⁴⁶ The hydrogen bonding of the water with two second coordination shell Glu residues leads to much greater activation ($\text{O-H} = 1.05 \text{ \AA}$) in comparison to its terminal bound form ($\text{O-H} = 1.02 \text{ \AA}$). The strengthening of the scissile peptide bond by 0.03 \AA in comparison to the corresponding bond in its free form suggests the complete absence of its Lewis acid activation. In the reactant, the bridging water form transforms into the terminal form by switching to the Zn1 site to adopt a more reactive conformation. The terminal form is $5.1 \text{ kcal mol}^{-1}$ endergonic from the reactant. From the terminal form, the nucleophile generation through proton transfer to the Glu base and simultaneous attack on the substrate occurs with a barrier of $14.1 \text{ kcal mol}^{-1}$ (Fig. 3b). A five-membered ring containing intermediate formed in the process is almost thermoneutral (exergonic by $1.4 \text{ kcal mol}^{-1}$). As proposed by the site-directed mutagenesis experiments,¹⁴⁷ this intermediate is stabilized by a Tyr residue.

These results predict that the water is significantly more activated in the bridging binding mode in comparison to the terminal binding mode. This process is predominantly controlled by the nucleophilicity of the hydroxyl ion. Additionally, the electronic nature of the substrate influences the energetics of the step, *i.e.* an electron donating group in *L*-leucine-*p*-nitroanilide is more amenable to hydrolysis by **BILAP**. The Mg-Zn variant of **BILAP** is more active than its wild-type form (Zn-Zn).

Ila2. Cleavage of the peptide bond. This process occurs through the collapse of the *gem*-diolate intermediate created in the previous step (Fig. 3a).

In **IDE**, Glu acts as both acid and base by abstracting the proton from the metal bound oxygen atom of the intermediate and donating its previously acquired proton to the peptide bond. This double proton transfer cleaves the peptide bond. Similar to the previous step, with the barriers of 9.2, 13.9 and $18.5 \text{ kcal mol}^{-1}$ a clear energetic preference is observed for the cleavage of the chemically distinct Lys-Gly, Phe-Phe, and His-Gln bonds, respectively. However, the scissile peptide bond lengths in the corresponding reactants are the same (1.37 \AA). The process occurs through the same pathway in the mechanism of PET hydrolysis by **NEP** (Fig. 3a). Here, the double proton transfer takes place with a barrier of $9.1 \text{ kcal mol}^{-1}$. It is worth mentioning that an ester bond is substantially more susceptible to hydrolysis than a peptide bond. Additionally, **MMP** with the rate-limiting barrier of $17.5 \text{ kcal mol}^{-1}$ for Phe-Phe hydrolysis is more active than **IDE**.

In the mechanism utilized by **BILAP**, the carbonic acid functions as an acid, similar to Glu in mononuclear metallo-peptidases, and cleaves the peptide bond through proton transfer (Fig. 3b).¹⁴⁰ This process occurs in the rate-limiting step with a barrier of $25.5 \text{ kcal mol}^{-1}$. The bicarbonate ion has been implicated in a similar acid/base role in the proposed mechanism of cyclopropane synthase.^{148,149} The barrier is in

line with the experimentally measured barrier of $18.7 \text{ kcal mol}^{-1}$.¹⁴⁴ In an alternative pathway, a cluster of three water molecules can play the role of the bicarbonate ion.¹⁵⁰ The barrier for such a pathway is $4.9 \text{ kcal mol}^{-1}$ higher than the one using the bicarbonate ion as the acid/base residue. However, **SgAP** accomplishes bond cleavage through a process similar to the one utilized by mononuclear metallopeptidases (Fig. 3b). The two-proton transfer process that this enzyme employs is supported by experiments.^{124,146} The overall barrier of $16.5 \text{ kcal mol}^{-1}$ for the mechanism is in agreement with the measured value of $13.9 \text{ kcal mol}^{-1}$.¹²⁴ The peptide bond cleavage step is the rate-limiting step of the mechanism.¹⁴⁵ The measured solvent kinetic isotope effects (KIEs) also suggest that the creation of the nucleophile in the first step and collapse of the *gem*-diolate intermediate in the second step is the rate-determining step at $\text{pH} = 6.5$ and < 8 , respectively.¹⁴⁶

The above-discussed results indicate that binuclear enzymes are more efficient in activating the water molecule to create a hydroxyl nucleophile due to its bridging binding mode. However, the nucleophilicity of the hydroxyl group is lower in this mode.

IIb. Peptide hydrolysis by synthetic analogues

In this section, the mechanisms of several mononuclear and binuclear analogues of metallohydrolases are discussed (Fig. 4). These complexes are mostly synthesized using non-biological metal ions and missing the effects of the catalytic acid/base residue and non-covalent interactions provided by the second coordination shell residues of natural metallohydrolases. Their activities can be switched from residue-selective to sequence-specific by changing the pH. For instance, the cleavage is residue-selective in acidic aqueous solutions, while sequence-specific in mildly acidic and neutral solutions.¹⁵¹ Thus, they are very useful models to understand the roles of metal ions, residue-selectivity and the sequence-specificity in peptide hydrolysis. Additionally, they can be used as hydrolytic agents in modern bioanalytical and bioengineering applications such as protein footprinting, proteomics and bioengineering of fusion proteins.^{152–154} A vast majority of currently available enzymes and synthetic agents are ill-suited for these applications.¹⁵⁵ For instance, enzymes exhibit broad specificities, function under narrow temperature and pH conditions and are expensive. On the other hand, synthetic reagents such as cyanogen bromide are toxic and provide limited selectivity and low yields. They also require harsh conditions and a larger quantity of the starting material. Thus, metal complexes discussed below provide useful insights into the mechanisms of natural enzymes and help with the design of the next generation of analogues. It is noteworthy that they also possess several shortcomings such as a lack of suitable metal ion(s), ligand environment, coordination number and second coordination shell residues. None the less, similar to enzymes, such molecules can utilize either a metal bound or a free water molecule for hydrolysis. However, these pathways cannot be readily distinguished by purely kinetic methods.

IIb1. Pd complexes. The $[\text{Pd}(\text{H}_2\text{O})_4]^{2+}$ (**I_b**) complex is one of the simplest mimics of mononuclear metallopeptidases (Fig. 4).

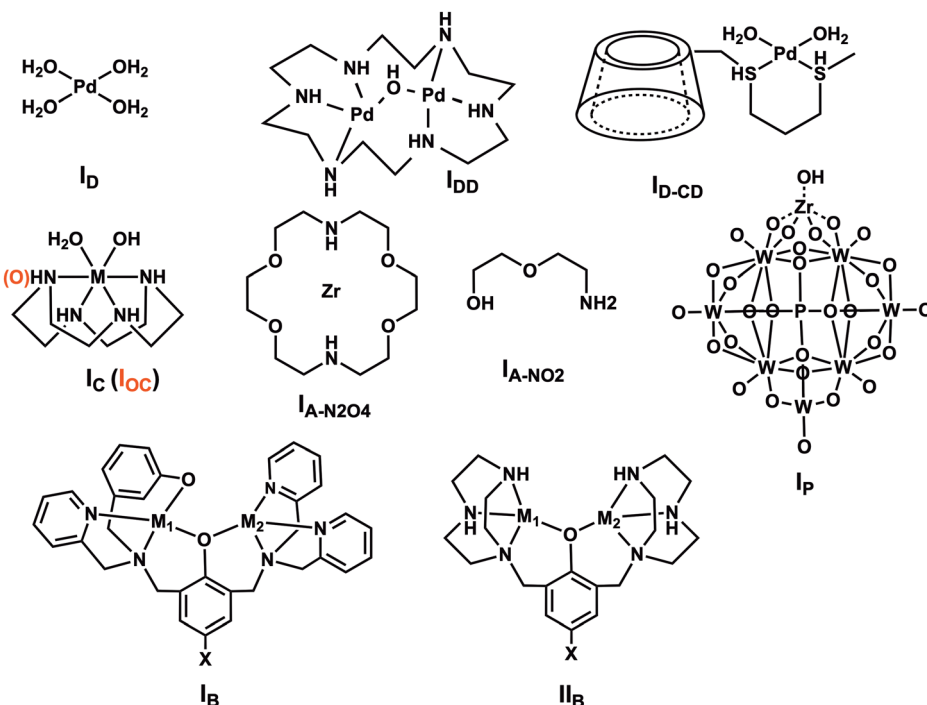


Fig. 4 Structures of the metal complexes used for peptide, ester and phosphoester hydrolysis.

It can hydrolyze the proximal X-Y (Gly-Gly, Gly-Pro, and Gly-Sar) peptide bond (Sar = sarcosine) in X-Y-Met and X-Y-His sequences in weakly acidic aqueous solutions (Fig. 2).¹⁵⁶ However, unlike natural enzymes, it anchors Met and His residues,

respectively, of the sequences and cleaves all X-Y peptide bonds irrespective of their chemical nature (Fig. 5a). Thus, the substrate provides a major portion of the ligand environment. It has been reported to hydrolyze the R-Gly-Pro-Met, R-Gly-Pro-His,

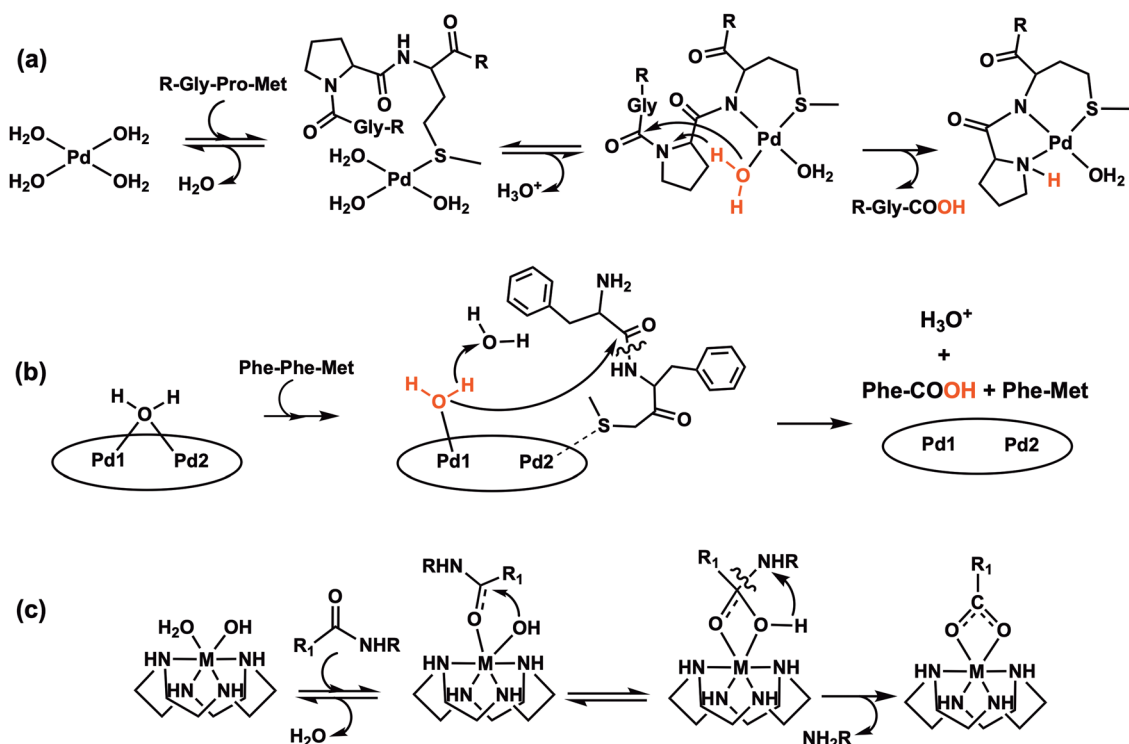


Fig. 5 Mechanisms of peptide hydrolysis employed by metal complexes (a) I_D, (b) I_{DD} and (c) I_C.

R-Gly-Sar-Met and R-Gly-Gly-Met peptide with the measured rate constants 6.0×10^{-2} , 9.4×10^{-2} , 1.4×10^{-2} and $2.8 \times 10^{-3} \text{ min}^{-1}$, respectively, at pH 2.0 and 60 °C that correspond to barriers of 24.0, 23.8, 25.0 and 26.1 kcal mol⁻¹ respectively.¹⁵⁶ The mechanisms for the cleavage of all four sequences catalyzed by **I_D** were investigated using DFT calculations.¹⁵⁷ According to the suggested mechanism, similar to mononuclear enzymes, a Pd bound water is utilized for hydrolysis (Fig. 5a). Here, the activation of both the substrate and water molecule by the metal cation and substrate is substantially weaker than in the mononuclear enzymes. Proton transfer from the Pd-bound water to the substrate with the simultaneous nucleophilic attack cleaves the peptide bond (Fig. 5a). The computed barriers of 38.3, 41.4, 39.8 and 39.2 kcal mol⁻¹ for the hydrolysis of the Gly-Pro-Met, Gly-Pro-His, Gly-Sar-Met and Gly-Gly-Met peptide, respectively, are in agreement with the measured rate constants at pH 2.0 and 60 °C.¹⁵⁶ The corresponding barriers using an external water molecule are much higher (> 50.0 kcal mol⁻¹) for all four sequences.

A second metal center is included in this complex, [Pd₂(μ-OH)([18]aneN₆)]⁴⁺ (where [18]aneN₆ is 1,4,7,10,13,16-hexaazacyclooctadecane), **I_{DD}**, to create a mimic of binuclear hydrolases (Fig. 4).¹³⁹ In **I_{DD}**, unlike **I_D** and natural enzymes, the Phe-Phe-Met substrate is not coordinated to a metal ion and associates indirectly through hydrogen bonding with the metal bound water. This complex, in contrast to **I_D**, utilizes an external water molecule trapped between the substrate and the metal-bound water molecule for hydrolysis (Fig. 5b). The external water abstracts a proton from the Pd1-coordinated water and forms a hydronium ion (H₃O)⁺. The subsequent nucleophilic attack to cleave the peptide bond occurs with a barrier of 31.0 kcal mol⁻¹. The inclusion of the second metal center lowers the barrier by 4.4 kcal mol⁻¹ in comparison to mononuclear **I_D**.¹³⁹ Evidently, **I_{DD}** employs a much different mechanism than **I_D** and natural enzymes. Nonetheless, the effect of the second metal center is similar to the one observed in the hydrolysis of phosphate esters, where binuclear model complexes are found to be more effective than the mononuclear ones.^{158–160} However, the barrier for **I_{DD}** is still substantially higher than that for both mono- and binuclear enzymes.

Furthermore, substrate specificity is incorporated in the complex by attaching the hydrophobic moiety of β-cyclodextrin (CD) to create the 6-S-2-(2-mercaptomethyl)-propane-6-deoxy-β-cyclodextrin diaqua palladium(II) complex, **I_{D-CD}** (Fig. 4).¹⁶¹ In **I_{D-CD}**, the hydrophobic enzyme like cavity of CD could enhance its activity because low entropy and conformational enthalpy are spent in approaching the transition state.¹⁶² This complex has been reported to sequence-specifically cleave the unactivated tertiary Ser-Pro peptide bond in the sequence Ser-Pro-Phe of the bradykinin substrate at pH 7.0 and 60 °C (Fig. 2).¹⁶¹ The hybrid quantum mechanics/molecular mechanics (QM/MM: B3LYP/Amber) calculations show that both the substrate and water molecule are directly coordinated to the Pd ion.¹⁶³ Again, Pd-bound water and not a free water is energetically more feasible for hydrolysis, as observed for natural enzymes and **I_D**. The barrier of the concerted

mechanism employed by the complex is 32.8 kcal mol⁻¹, which is similar (31.0 kcal mol⁻¹) to the one computed for the binuclear **I_{DD}** complex. The presence of CD increases the nucleophilicity of the hydroxyl group and moves it closer to the electrophile. Its removal from the model increases the barrier by 7.4 kcal mol⁻¹. However, this barrier is 9.0 kcal mol⁻¹ higher than the measured value.¹⁶¹ It could be partially attributed to the measurement of the rate constants at 60 °C, whereas the calculations were performed at 25 °C. Therefore, due to the temperature dependence of the pre-exponential constant in the Arrhenius equation, it is not possible to accurately estimate the measured barrier at 25 °C. However, the optimum location of the CD ring is not clear. The inclusion of two -CH₂ groups downstream from the S atom of the substrate increases its activity by as much as 3×10^5 times. Rather surprisingly, the addition of the second CD ring has only a small effect on the barrier. It should facilitate a rapid formation of the substrate-catalyst complex and accelerate the rate of reaction. However, in computational models the substrate is already coordinated to the complex. The substitution of Pd with biologically relevant Zn and Co increases the barrier by 3.1 kcal mol⁻¹ (35.9 kcal mol⁻¹) and decreases by 6.2 kcal mol⁻¹ (26.6 kcal mol⁻¹), respectively.

These results suggest that either the inclusion of the second metal center (**I_{DD}**) or the hydrophobic CD cavity (**I_{D-CD}**) enhances the activity of **I_D**. Their higher barriers can also be attributed to the cleavage of the hydrolysis resistant tertiary peptide bond formed by a Pro residue.

IIB2. Metal-cyclen complexes. In these complexes, a macrocycle ring of 1,4,7,10-tetraazacyclododecane (cyclen) and 1-oxa-4,7,10-triazacyclododecane (oxacyclen) is coordinated to a metal ion (Fig. 4). For instance, transition metal complexes of cyclen (**I_C**) and oxacyclen (**I_{OC}**), where M = Co(III) or Cu(II), have been reported to selectively hydrolyze a wide range of biomolecules such as lysozyme, albumin, myoglobin, and Aβ peptide.^{164–167} Furthermore, the inclusion of an organic group or an aromatic chain (pendant) provides bond specificity in a manner different from that due to the CD cavity of **I_{D-CD}** and enzyme active sites.¹⁶⁸ Based on its chemical structure, the pendant covalently links to a specific chemical group of the substrate and positions the metal center adjacent to the scissile bond. In the reactants of **Co-I_C** and **Cu-I_C**, the hydroxyl nucleophile is already attached to the metal ion unlike a water molecule in **I_D** (Fig. 5c).¹⁶⁹ The Cu(II)-nucleophile bond distance in **Cu-I_C** is 0.07 Å longer than the corresponding distance in the **Co-I_C** case. Additionally, all metal-ligand bond distances for **Cu-I_C** are longer than the ones for **Co-I_C**. However, the Phe-Ala substrate is bound directly only in the latter, *i.e.* the coordination number of the Cu(II) and Co(III) ions is 5 and 6, respectively. The scissile peptide bond in **Cu-I_C** is also 0.01 Å longer than the bond in **Co-I_C**. The nucleophilic attack on the substrate occurs with a barrier of 19.8 and 24.1 kcal mol⁻¹ for **Cu-I_C** and **Co-I_C**, respectively (Fig. 5c). This difference is attributed to the provision of a stronger nucleophile in the former. In the next step, proton transfer from the nucleophile to the N atom of the substrate leads to its cleavage (Fig. 5c). From the respective intermediates,

the barriers for this process are comparable for **Cu-I_C** and **Co-I_C**, *i.e.* 19.0 and 19.5 kcal mol⁻¹, respectively. The removal of the pendent from **Cu-I_C** and **Co-I_C** reduces the barrier by 3.0 and 9.3 kcal mol⁻¹, respectively. The barrier of 30.5 kcal mol⁻¹ for the **Co-I_C** complex without the pendent is in agreement with the measured barrier of 25.9 kcal mol⁻¹ for the hydrolysis of myoglobin at pH 9.0 and 50 °C.¹⁷⁰ Additionally, barriers of 39.8 kcal mol⁻¹ for **Co-I_C** and 40.1 kcal mol⁻¹ for its oxacyclen derivative **Co-I_{OC}** for the same mechanism are supported by the measured data that their activities differ by only four times.¹⁶⁴ Furthermore, Ni (in the triplet state) is the most feasible substitution among Ni(II), Zn(II), Cd(II), and Pd(II) complexes without the pendent and hydrolyzes the peptide bond with the lowest barrier of 27.2 kcal mol⁻¹.

The results discussed above suggest that the electronic state of the metal ion is critical in the activities of these complexes. In the mechanisms of **I_D** and **I_C** only the first step is different, *i.e.* the former created the hydroxyl nucleophile from a water molecule, while it is already present in the latter. It is found that with a barrier of 33.7 kcal mol⁻¹ **Cu-I_C** is more active than **Co-I_C** and **I_D** that hydrolyze the dipeptides with the barriers of 38–42 kcal mol⁻¹.

IIB3. Zr-azacrownether complexes. To specifically study the effects of the coordination number of the metal ion and charge of the ligand on the hydrolysis of the Gly-Gly (neutral) dipeptide (Fig. 2), the activities of 11 different N₂O₄, N₂O₃, and NO₂ core containing mononuclear Zr(IV) complexes (**I_A**), 4,13-diaza-18-crown-6 (**I_{A-N₂O₄}**), 1,4,10-trioxo-7,13-diazacyclopentadecane (**I_{A-N₂O₃}**) and 2-(2-methoxy)-ethanol (**I_{A-NO₂}**), respectively, and their analogues have been investigated (Fig. 4).¹⁷¹ Additionally, the effect of the charge of the substrate is investigated using Gly-Glu (negative) and Gly-Lys (positive) as the substrates. Due to the high coordination number of Zr(IV), the metal ion provides binding sites to the Gly-Gly substrate and hydroxide nucleophile.

Based on the experimental information, the protonation states (singly protonated, doubly protonated or doubly deprotonated)

and number of ligands were altered (Fig. 4). They all exist in the hepta-coordinated state and both substrate and hydroxyl nucleophile are directly coordinated to the Zr(IV) ion (Fig. 6a). Among the complexes with the N₂O₄ ligand, double deprotonation of the nitrogen atoms in **I_{A-DN₂O₄}** elongates the most metal-ligand distances and increases the Lewis acidity of the metal ion.¹⁷¹ The longest metal-nucleophile distance in the complex is also indicative of a stronger nucleophile.¹⁷² These factors render **I_{A-DN₂O₄}** as the most active complex in this ligand environment. In **I_{A-DN₂O₄}**, the nucleophile attack on the substrate occurs with a barrier of 13.9 kcal mol⁻¹ (Fig. 6a). The single and double protonation of ligands increase the barrier by 6–12 kcal mol⁻¹. It is apparently caused by a decrease in the nucleophilicity of the hydroxyl ion. The intermediate formed in this process is endothermic by 13.6 kcal mol⁻¹. It becomes more unstable by 7–23.0 kcal mol⁻¹ upon the single and double protonation of the ligands. The overall barrier of the mechanism increases by 10.0–17.0 kcal mol⁻¹ in the protonated complexes and becomes comparable to the barriers of **Co-I_{MC}** and **I_D** complexes.¹⁶⁹ On the other hand, the deprotonated form of a smaller NO₂ core with two hydroxyl ligands (**I_{A-DNO₂-2H}**) creates the most active form among all 11 complexes (Fig. 4). In **I_{A-DNO₂-2H}**, Zr(IV) is bound to the substrate, nucleophile and two additional hydroxyl groups. In the complex, the Zr-substrate and Zr-nucleophile distances are the longest and the peptide bond is most activated. Consequently, it is the most active complex with the lowest barrier (28.9 kcal mol⁻¹). In comparison to the Gly-Gly substrate, the barrier for Gly-Glu and Gly-Lys dipeptides is reduced by 1.4 kcal mol⁻¹ and increased by 3.1 kcal mol⁻¹, respectively. Similar to **IDE**, this simple complex also exhibits substrate preference. These results are supported by the measured level of hydrolysis for the substrates, *i.e.* Gly-Glu, pH 7.2, 60 °C, 20 h, yield 97% > Gly-Gly, pH 7.0, 60 °C, 20 h, yield 90% > Gly-Lys, pH 7.1, 60 °C, 20 h, yield 17%.¹⁷³

These results show that among the two competing effects, Lewis acidity and nucleophile activation, the latter dominates the activities of the complexes.

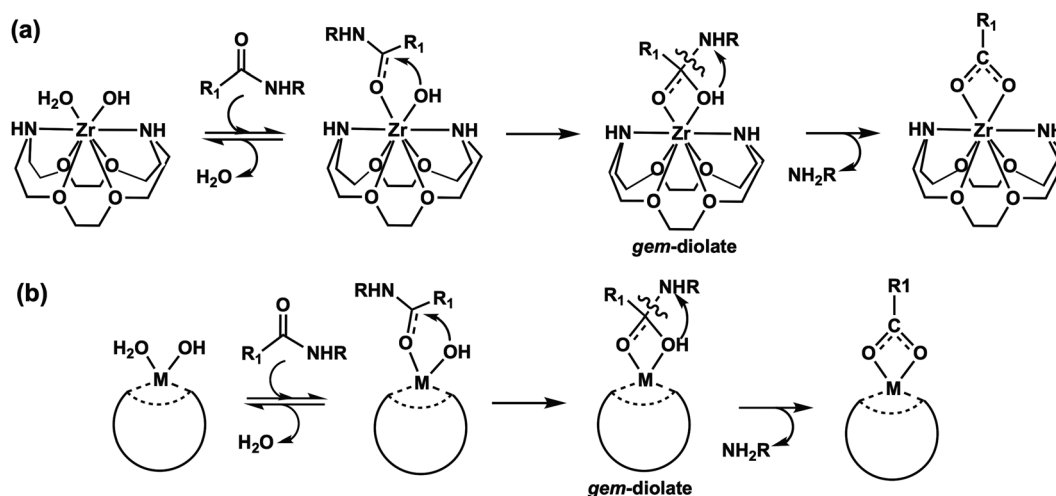


Fig. 6 Mechanisms of peptide hydrolysis employed by metal complexes (a) **I_A** and (b) **I_P**.

Iib4. Zr-polyoxometalates. Zr(IV)-substituted polyoxometalates (Zr-POMs, **I_P** in Fig. 4) are chemically more complex than the previous mononuclear metal complexes (**I_D**, **I_C** and **I_A**). They are metal-oxygen clusters that also hydrolyze multiple peptide bonds of many critical molecules such as human serum albumin (HSA), hen egg-white lysozyme (HEWL), myoglobin, insulin chain B and cytochrome *c*.^{174–178} The monomeric 1 : 1 Zr(IV) Keggin POM (**I_P**) has been reported to catalyze the hydrolysis of four distinct peptide bonds [Arg–Leu (site 1), Ala–Asp (site 2), Lys–Asp (site 3) and Cys–Glu (site 4)] of HSA (Fig. 2) at pH 7.4 and 60 °C.¹⁷⁸ Its lack of selectivity is similar to that of mononuclear peptidases that are also known to hydrolyze different peptide bonds of their substrates. **I_P** and drug molecules such as ibuprofen and diazepam have been proposed to predominantly interact at site 4 – known as Sudlow's drug site II (Fig. 2).^{179–182} The structures of the **I_P**-HSA complexes were built using molecular docking and MD simulations.¹⁸³ The binding free energy for this site computed using the lambda (λ) particle approach^{184–186} is found to be the most favorable (–21.8 kcal mol^{–1}) among the four binding sites.

The mechanism of HSA hydrolysis by **I_P** was investigated using the two-layer QM/MM ONIOM method utilizing the previous MD equilibrated structures (Fig. 6b).¹⁸⁷ In the reactant, similar to other complexes, the hydroxyl nucleophile is already coordinated to the Zr(IV) ion but unlike natural enzymes, there is no direct metal–substrate coordination. The substrate interacts indirectly through two hydrogen bonds with the metal-bound hydroxyl group. These interactions suggest that Lewis acidity of the Zr ion is not critical. The generation of the tetrahedral intermediate through the attack by the metal-coordinated hydroxyl group occurs with a barrier of 18.3 kcal mol^{–1}. This process is endothermic by 8.0 kcal mol^{–1}. The barrier for the process using an external water molecule is higher by 4.1 kcal mol^{–1}. In the next rate-limiting step, the cleavage of the peptide bond through proton transfer from the nucleophile occurs with an overall barrier of 27.5 kcal mol^{–1}. The high strain of a four-membered ring in the step contributes to its high energy barrier. This process is quite different in natural enzymes, where a base/acid residue facilitates the bond cleavage. The barrier, although significantly higher than that for natural metallohydrolases, is comparable to the measured values of 24.6–27.0 kcal mol^{–1} at pD 5.4 and 60 °C for the hydrolysis of 18 different dipeptides by its dimeric form.¹⁸⁸ Additionally, it is comparable to that of the most active forms of **I_{D-CD}** (Co-variant) and **I_C** (Ni-variant) with the barriers of 26.6 and 27.2 kcal mol^{–1}, respectively. Furthermore, the computed barrier for the peptide bond cleavage is 36.0, 31.0, 35.5 and 27.5 kcal mol^{–1} for the Arg–Leu (site 1), Ala–Asp (site 2), Lys–Asp (site 3) and Cys–Glu (site 4) sites, respectively, and exhibits a clear energetic preference for their hydrolysis.

Our results indicate that the Lewis acidity of the Zr ion and the nucleophilicity of the hydroxyl ions are different for these cleavage sites. This site preference is similar to that of **IDE** albeit several orders of magnitude slower due to the lack of the acid/base residue and protein surroundings.

Iic. Phosphoester hydrolysis

Glycerophosphodiesterase (**GpdQ**) from *Enterobacter aerogenes*^{189,190} exhibits extensive substrate promiscuity (Fig. 1). It is predominantly a diesterase but hydrolyzes a range of non-natural phosphomono-, phosphodi- and phosphotriester substrates such as 4-nitrophenyl phosphate (NPP), glycerol-3-phosphoethanolamine (GPE), bis(4-nitrophenyl) phosphate (BNPP), and diethyl 4-nitrophenyl phosphate (paraoxon) and several organophosphate pesticides and nerve agents such as paraoxon, demeton, sarin, soman and VX (Fig. 2).^{74,75,189–192} Consequently, it could be used in agricultural remediation and as an anti-warfare agent.^{73–75,193} This enzyme creates its catalytically active binuclear Fe(N₂O₂)–Zn(N₂O₂) [Fe(His, His, Asp, Asp)–Zn(His, His, Asp, Asn)] core only in the presence of the substrate (Fig. 1).^{192,194–197} After its generation, **GpdQ** utilizes its remarkable coordination flexibility, also known as “breathing of the active site cleft”, to hydrolyze diverse substrates with different sizes and chemical compositions. On the other hand, as discussed above, the Zn1(NO₂)–Zn2(NO₂) core containing **SgAP** (Fig. 1) is also capable of accelerating the first-order hydrolysis of the phosphodiester bis(4-nitrophenyl) phosphate (BNPP) (Fig. 2) by 10¹⁰-fold in comparison to the uncatalyzed reaction.^{123,198} This is one of the rare examples in which an aminopeptidase hydrolyzes its transition-state analogue, a phosphoester, at an enormous rate.

The phosphoester hydrolysis can occur through three distinct mechanisms shown in the More O'Ferrall-Jencks diagram (Fig. 7).¹⁹⁹ (a) Dissociative or elimination–addition mechanism: It involves the formation of a trigonal metaphosphate intermediate and release of the leaving group (R₁O) which precede the formation of the phosphate–nucleophile (P⁺–OH[–]) bond (Fig. 7a). (b) Dissociative–associative or synchronous mechanism: It occurs through the concomitant formation and cleavage of the phosphoester bonds (S_N2 type mechanism in Fig. 7b). (c) Associative or addition–elimination mechanism:

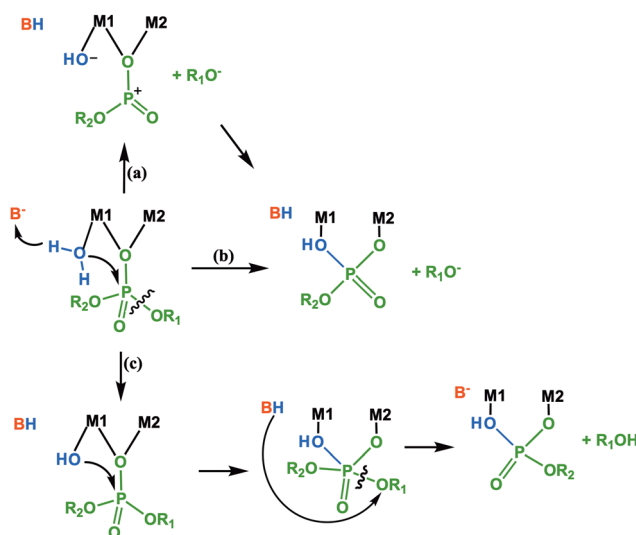


Fig. 7 General mechanisms of phosphoester hydrolysis employed by binuclear systems.

This mechanism involves the formation of a pentavalent phosphorane intermediate by the nucleophile attack (Fig. 7c). In general, binuclear metallohydrolases have been reported to utilize one of these mechanisms depending on the chemical nature of the substrate.^{12,200–209} However, the dissociative mechanism has not been observed for such substrates. Since the associative mechanism has been found to be the energetically most feasible mechanism,^{145,210} the activities of both **GpdQ** and **SgAP** are discussed using only this mechanism for BNPP hydrolysis (Fig. 1 and 2).

Iic1. Creation of the phosphorane intermediate. In the reactant of **GpdQ**, one of the nitrophenyl groups directly interacts with the active site, while the second group is exposed to the solvent.²¹¹ In this structure, the scissile P–O bond of BNPP is significantly elongated by 0.10 Å in comparison to its free form due to double Lewis acid activation.²¹⁰ The nucleophile generating water is also directly coordinated to the Fe ion (M1 in Fig. 7). These interactions lower its pK_a value significantly and increase its acidity (O–H = 1.02 Å). This strategy has also been utilized by other binuclear phosphoesterases like *E. coli* alkaline phosphatase²⁰⁸ and OpdA.²⁰² The pK_a of the water molecule for **GpdQ** is measured to be ~ 9.5 .¹⁹² In the mechanism, proton transfer from the Fe-bound water through a chain of three water molecules to the His-Asp dyad creates the hydroxyl nucleophile.²¹⁰ A histidine residue has been proposed to play a similar role in the mechanisms of PAP,²¹² RNaseZ²¹³ and phosphoprotein phosphatases.²¹⁴ Due to the high acidity of the water molecule and basicity of the dyad, this process occurs through a very low barrier of 1.9 kcal mol^{−1} and forms an intermediate that is endergonic by only 0.6 kcal mol^{−1}. In the intermediate, phosphorane species is not created and the hydroxyl nucleophile is still bound to the Fe ion (Fig. 7c).

However, in comparison to **GpdQ**, among the two almost equivalent metal sites (site 1 or 2) in **SgAP**, the actual location of the nucleophile generation is not known (Fig. 1).¹⁴⁵ Here, the generation of the nucleophile at site 2 is found to be slightly more favorable by 1.8 kcal mol^{−1}. In the reactant, in comparison to monodentate binding to Zn in **GpdQ**, BNPP coordinates in an asymmetric bridging mode to both metals but through a much shorter bond with Zn1. In this binding mode, similar to **GpdQ**, the scissile P–O bond of BNPP gets activated by 0.08 Å than the corresponding bond in its free form due to double Lewis acid activation. However, the activation is lower than the one (0.10 Å) observed for **GpdQ**. The nucleophile generating water in **SgAP** is more activated (O–H = 1.03 Å) than in the **GpdQ** case. In the first step of the mechanism employed by **SgAP**, in contrast to the mechanism utilized by **GpdQ**, abstraction of a proton from the Zn2 bound water by the Glu base occurs synchronously with the nucleophilic attack on BNPP (Fig. 7). The barrier for the concerted process is 16.8 kcal mol^{−1}. It is in excellent agreement with the measured value of 18.3 kcal mol^{−1}.¹²⁴ For **GpdQ**, the barrier for such a concerted pathway is significantly higher by ~ 10.0 kcal mol^{−1}.²¹⁰ In the intermediate, the nucleophile is attached to the substrate. Here, the P–O bond is significantly activated by 0.21 Å and stabilized through hydrogen bonding by two second coordination

shell residues. It is endergonic by 3.6 kcal mol^{−1}, whereas this process is almost thermoneutral in **GpdQ**.

Iic2. Cleavage of the phosphodiester bond. As noticed in the first step, **GpdQ** and **SgAP** utilize different pathways for the cleavage of the scissile P–O bond (Fig. 7). In **GpdQ**, an in-line (associative–dissociative) nucleophilic attack by the Fe-bound hydroxyl group cleaves the P–O bond. The process crosses over a barrier of 10.1 kcal mol^{−1} in this rate-determining step. In the final product, negatively charged *p*-nitrophenolate and *p*-nitrophenyl phosphate fragments are formed. However, in **SgAP**, donation of a proton by the neutral Glu to the leaving group of BNPP, instead of a nucleophile attack in **GpdQ**, cleaves the P–O bond. It takes place with a small barrier of 5.7 kcal mol^{−1} and the same *p*-nitrophenol and *p*-nitrophenyl phosphate fragments are formed. They are bound in the same fashion as the phosphate ion in the crystal structure.²¹⁵

These results show that the heterobinuclear **GpdQ** and homobinuclear **SgAP** utilize distinct mechanisms for BNPP hydrolysis. **GpdQ** bypasses the formation of the phosphorene intermediate and uses the S_N2 type associative–dissociative mechanism. However, **SgAP** utilizes the associative mechanism that involves the generation of the phosphorane intermediate. Additionally, the cleavage of the P–O bond is the rate-determining process for **GpdQ**, while the generation of the nucleophile is the rate-determining process for **SgAP**. Furthermore, **GpdQ** catalyzes this reaction with 6.7 kcal mol^{−1} lower barrier than the barrier computed for **SgAP**. Our results are in line with experimental observations that in general heterobinuclear centers are more active than their homobinuclear counterparts for hydrolysis.²⁷ Since **SgAP** catalyzes both peptide and phosphoester hydrolysis, it provides a unique opportunity to compare the controlling factors of these reactions. For instance, the first step of BNPP hydrolysis is dominated by its double Lewis acid activation, while for Leu-*p*NA hydrolysis by the stronger nucleophilicity of the metal bound hydroxyl group. In general, they are competing effects and the two reactions catalyzed by **SgAP** provide an ideal system to study their influences. Furthermore, in contrast to the second step in the Leu-*p*NA hydrolysis, the nucleophile generation is the rate-limiting step for the BNPP hydrolysis. These results suggest that the chemical nature of the substrate influences the energetics of the nucleophile generation and its attack on the electrophile.

IId. Synthetic analogues

IId1. Metal–cyclen complexes. The cyclen group containing complexes of divalent Zn and Cu (**Zn-I_C** and **Cu-I_C**) have also been reported to hydrolyze the phosphoester bond of BNPP and supercoiled DNA, respectively (Fig. 2 and 4).^{216,217} Additionally, several cyclen ring possessing complexes of tri- and tetravalent lanthanides (Eu, La, Zr and Ce) have also been used for this reaction.^{218–222} Therefore, BNPP hydrolysis by **I_C** is investigated using two types of metal ions, divalent [Zn(II), Cu(II) and Co(II)] and tetravalent [Ce(IV), Zr(IV) and Ti(IV)].²²³ In the energetically most feasible mechanism for the divalent metal–cyclen complexes, BNPP interacts with the metal ion in a

monodentate mode and due to its Lewis acidity the metal ion activates the scissile phosphoester bond by ~ 0.04 Å.²²³ The hydroxyl nucleophile is also bound to the metal ion. Its attack on the phosphorus center of BNPP generates a five-membered trigonal bipyramidal phosphorane intermediate (Fig. 8a). This process occurs with a barrier of 20.7 kcal mol⁻¹ and the intermediate is endergonic by 15.5 kcal mol⁻¹. In the intermediate, the scissile P-OR bond *trans* to the nucleophile is substantially elongated but not completely broken. In the next barrierless step, the intermediate collapses through the cleavage of the P-OR bond. The barriers of 22.0 and 23.4 kcal mol⁻¹ for **Cu(II)-I_C** and **Co(II)-I_C**, respectively, are slightly higher than the barrier (20.7 kcal mol⁻¹) for **Zn(II)-I_C**. This difference is caused by the provision of a stronger nucleophile by **Zn(II)-I_C**. Thus, both Lewis acidity and nucleophilicity of the metal center are critical in the activities of these complexes.

Due to the high coordination numbers (6–12) of tetravalent (Ce, Zr and Ti) metals, BNPP coordinates in a bidentate manner in their complexes. In contrast to Lewis acid activation of BNPP in the complexes of divalent ions, here the P-O bond actually becomes stronger by ~ 0.03 Å. The barriers (>30 kcal mol⁻¹) for all tetravalent metal complexes are substantially higher than that of their divalent counterparts. In comparison to the divalent metals, the lower activity of the complexes with different coordination numbers (7–9) is caused by the strengthening of the scissile phosphoester bond and weakening of the nucleophilicity of the hydroxyl nucleophile.

A comparison of peptide (29.7 – 31.9 kcal mol⁻¹) and phosphoester (20.7 – 23.4 kcal mol⁻¹) hydrolysis reveals that **I_C** is a more efficient phosphoesterase with divalent metals. That is due to the higher Lewis acidity of the metal ion in phosphoester hydrolysis.

IId2. Hetero- and homobinuclear complexes. The hetero-binuclear asymmetric Fe^{III}-Zn^{II} core containing complex

[I_B(OH)Fe(III)(μ-OH)Zn(II)]⁺ (FZ-I_B), where **I_B** (bpbmp) = 2-bis-[(2-pyridylmethyl)-aminomethyl]-6-[(2-hydroxybenzyl)-(2-pyridylmethyl)-aminomethyl]-4-methylphenol, contains catalytically active **[(OH)Fe(III)(μ-OH)Zn(II)(OH₂)]⁺** species in aqueous solution (Fig. 4).²²⁴ This complex is a true mimic of metallohydrolases and increases the rate of hydrolysis of bis(2,4-dinitrophenyl) phosphate (BDNPP) by 4.8×10^3 -fold ($k_{\text{cat}} = 9.13 \times 10^{-4}$ s⁻¹) at pH 6.5 (Fig. 2). In contrast, a homobinuclear Cu^{II}-Cu^{II} core-possessing symmetric complex **[II_BCu(II)(μ-OH)Cu(II)]²⁺ (CC-II_B)**, where **II_B** (bcmp) = 2,6-bis(1,4,7-triazacyclonon-1-ylmethyl)-4-methylphenol, hydrolyzes BDNPP with a second order rate constant of 0.047 M⁻¹ s⁻¹ at pH 8.0 and 40 °C (Fig. 4).^{24,225} **CC-II_B** is also able to hydrolyze DNA and induces cell death in pancreatic cancer cells. Its Zn variant (**ZZ-II_B**) also hydrolyzes BDNPP with an apparent second order rate constant of 0.028 M⁻¹ s⁻¹ under the same conditions (Fig. 4).²²⁶ In the reactant of **FZ-I_B**, both Fe(III) and Zn(II) ions are coordinated to 6 ligands in a distorted octahedral coordination environment (Fig. 8b).²²⁷ Here, Fe^{III} is located in the N₂O₂ site and Zn(II) in the N₃O site. BDNPP is coordinated to the Zn(II) ion in a monodentate fashion which is supported by experimental data.²²⁴ However, as noticed above for other metal complexes, no Lewis acid activation of the substrate is observed and the catalyst-substrate interaction actually strengthens the scissile P-O bond by 0.04 Å in comparison to the corresponding bond in its free form.²²⁷ That is in contrast to the activation of the scissile P-O bond of BNPP in the mechanisms utilized by **GpdQ**²¹⁰ and **SgAP**.¹⁴⁵ In an S_N2-type reaction, an in-line concerted attack of the Fe(III) bound hydroxyl nucleophile of **FZ-I_B** on the electrophilic P atom of BDNPP leads to the cleavage of the scissile P-O bond with a barrier of 15.8 kcal mol⁻¹. It is in good agreement with the measured barrier of 20.1 kcal mol⁻¹.²²⁴ It is, however, 5.7 kcal mol⁻¹ higher than the corresponding barrier computed for **GpdQ**,²¹⁰ demonstrating that **FZ-I_B**, formed by organic ligands, is substantially less active than natural

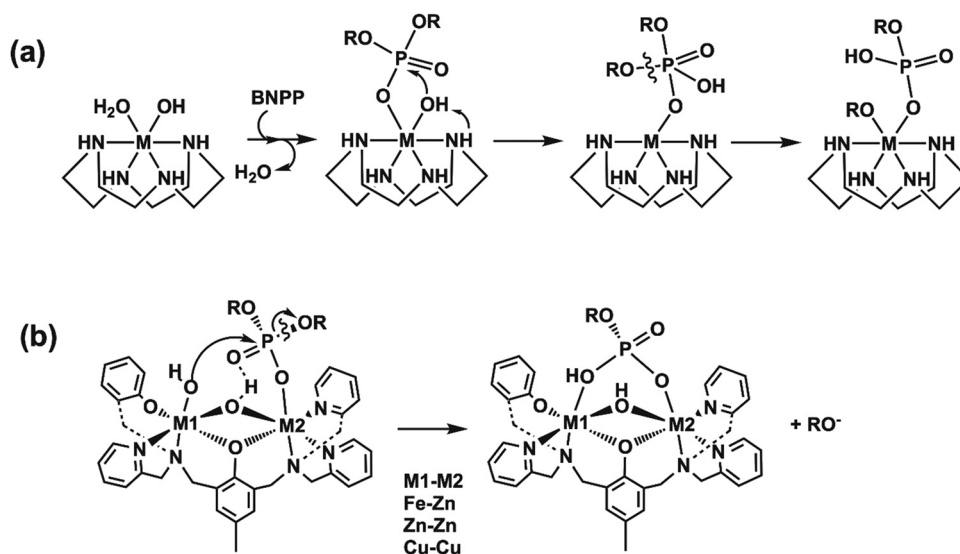


Fig. 8 Mechanisms of phosphoester hydrolysis employed by metal complexes (a) **I_C** and (b) **I_B**.

enzymes. In contrast to the use of double Lewis acid activation by the binuclear metal cores of natural hydrolases, this mechanism is dominated by the nucleophilicity of the Fe(III)-coordinated hydroxyl group.

In contrast to **FZ-I_B**, BDNPP in di-zinc core containing **ZZ-I_B** interacts through a strong hydrogen bond with the μ -OH group and through π - π interactions with the ligand of Zn1. In **ZZ-I_B**, the computed barrier for the cleavage of the P-O bond through the attack by the -OH nucleophile is only 6.3 kcal mol⁻¹, which is 9.5 kcal mol⁻¹ lower than the barrier in **FZ-I_B**. Due to the low ligand field stabilization energy of the Zn ion, both metal-nucleophile distances in **ZZ-I_B** are substantially longer than the Fe-nucleophile distance in **FZ-I_B**. The reason for the significantly lower barrier in **ZZ-I_B** when compared to **FZ-I_B** is the provision of a stronger nucleophile in the former. Similar to **ZZ-I_B**, BDNPP does not bind directly to the metal ions in the dicopper core-possessing **CC-I_B** (coordination numbers are 5 and 6 for Cu1 and Cu2, respectively). However, the substrate binding mode is quite different in the two systems. In this structure, the hydroxyl group is terminally bound to the Cu2 atom. Despite the lack of a direct bond between the metal and substrate, rather surprisingly, the P-O bond in **CC-I_B** is 0.03 Å longer than in **FZ-I_B** due to Lewis acid activation. For **CC-I_B**, the barrier for the cleavage of the P-O bond is 11.4 kcal mol⁻¹, 4.4 lower and 5.1 kcal mol⁻¹ higher than the corresponding barrier for **FZ-I_B** and **ZZ-I_B**, respectively.

The above results demonstrate that the ligand environments and substrate binding modes of the three distinct metal clusters in the same ligand environment are different. In the absence of Lewis acid activation the energetics of this process is dominated by the nucleophilicity of the hydroxyl ion. The identities of the metal ions determine the activities of their complexes, and **ZZ-I_B** is found to be the most active complex. Overall, complexes formed with the asymmetric ligand **I** are more reactive than their symmetric counterparts formed by ligand **II**. The terminal hydroxyl group is a stronger nucleophile than its bridging counterpart. An electron donating group (-CH₃) at the *para* position of the centered phenolate group of the ligand increases the nucleophilicity of the hydroxyl group.

III. Summary and outlook

The design of efficient synthetic metallohydrolases has been widely acknowledged as a formidable task. It is not surprising that in comparison to natural enzymes, hydrolytic reactions promoted by the existing metal complexes are substantially slower and occur with lower catalytic turnover. It could be due to the following reasons: (1) the absence of chemical properties of amino acid residues of enzyme active sites; (2) the presence of one to three more ligands than the metal centers of enzymes; (3) the lack of combined influence of non-covalent interactions; and (4) the absence of second coordination shell residues of enzymes. The design of analogues with improved activities requires a deep understanding of the roles of metal ions, ligands (direct and indirect) and the environment in the

functioning of natural and synthetic metallohydrolases and chemical modifications in the latter on the basis of those insights. However, realization of this goal is very challenging and requires a rigorous integration of experiments and theoretical calculations. The experimental mechanistic studies on natural enzymes are hindered by extremely fast rates of reactions, a lack of substrate bound structures, kinetic indistinguishability of pathways, inhibition of enzymatic activities upon metal and ligand substitutions and the silence of the diamagnetic metal centers to NMR, electronic, and ESR spectroscopy. On the other hand, despite the availability of considerable experimental data, the mechanistic and structural information regarding the activities of synthetic analogues and chemical alterations for their improvement are not consistently available. However, the current experimental information concerning both natural and synthetic metallohydrolases provides an ideal platform to employ theoretical and computational chemistry techniques to understand the “design” principles and to develop the next generation of synthetic analogues for these critical reactions.

Our computational studies are useful in either addressing or reconfirming the multiple issues regarding metallohydrolases [mono-(**IDE**, **NEP** and **MMP**) and binuclear (**BILAP** and **SgAP**) metalloproteases and non-enzymatic (**I_D**, **I_C**, **I_A** and **I_P**) systems for peptide hydrolysis and binuclear(**GpdQ** and **SgAP**) phosphoesterases and non-enzymatic (**I_C**, **I_B** and **II_B**) systems for phosphoester hydrolysis]. For the enzymatic systems the following issues are addressed: (1) a low basicity of the ligand environment in enzymes such as **MMP** enhances the Lewis acidity of the metal ion and promotes hydrolysis; (2) a metal bound water is more suitable than a free water for the creation of hydroxyl nucleophile; (3) the water is more acidic in the bridging form than in the terminal form in binuclear enzymes; (4) the electronic nature of the metal and substrate predominantly controls the energetics of hydrolysis; (5) the different metal centers such as hetero- and homobinuclear in **GpdQ** and **SgAP**, respectively, utilize distinct mechanisms for the hydrolysis of the same BNPP substrate; (6) heterobinuclear metal centers are generally more reactive than their homobinuclear counterparts; and (7) peptide hydrolysis is controlled by the nucleophilicity of the metal bound hydroxyl group, whereas phosphoester hydrolysis is controlled by double Lewis acid activation. For the non-enzymatic systems the following issues are addressed: (1) the inclusion of the second metal center (**I_{DD}**) and the hydrophobic cavity of CD (**I_{D-CD}**) exert similar effects in **I_D** complexes; (2) the electronic nature of the metal ion is critical in controlling the hydrolytic activities of metal complexes; (3) among Lewis acidity and nucleophile activation, the latter dominates the activities of metal complexes; (4) the mode of substrate binding influences the nucleophilicity of the hydroxyl ion; (5) the terminal hydroxyl group is a stronger nucleophile than its bridging counterpart; and (7) due to low ligand field stabilization energy, the biologically relevant divalent metal ions such as Zn, Cu and Co are more suitable for hydrolysis.

Quite clearly, these impressive metal complexes can hydrolyze extremely stable bonds at good rates. They were synthesized under

diverse conditions and their activities were measured using distinct substrates. In this aspect, our results elucidating the effects of the metal ions, ligands, metal cooperativity, non-covalent interactions, Lewis acidity and nucleophilicity in hydrolysis will be helpful in designing the next generation of synthetic analogues. Although the existing computational techniques can provide useful mechanistic insights utilizing experimental data, they still cannot make accurate predictions in cases where such information is unavailable. Their success as a predictive tool will depend on accurate computations of different chemical factors such as electronic, entropic, solvent and dynamical effects.^{228–230}

Author contributions

L. S., V. J.-A., and L. W. wrote the first draft of the manuscript and P. R., J. Y., S. M. and R. P. revised the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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