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Zirconium(IV)-Assisted Peptide Hydrolysis

by

Miki Kassai

Under the Direction of Dr. Kathryn B. Grant

ABSTRACT

The development of new reagents to efficiently cleave peptides and proteins has become increasingly important for protein structural studies and other applications. However, this has proved to be a very challenging task due to the extreme stability of the peptide amide bond. Transition metal complexes cleave proteins and peptides through either oxidative or hydrolytic pathways. However, hydrolytic cleavage is preferred over oxidative cleavage, because the latter process produces irreversibly modified peptide fragments. Metal-assisted peptide hydrolysis is introduced in Chapter I. The metals Ce(IV), Co(II), Co(III), Cu(II), Fe(III), Mo(IV), Ni(II), Pd(II), Pt(II), Zn(II), and Zr(IV) are described as promising non-enzymatic hydrolysis reagents. In Chapter II, Zr(IV)-assisted hydrolysis of the dipeptide Gly-Gly and of its *N*- and *C*- blocked analogs is described. The highest levels of cleavage were observed at pH values ranging from 4.4 to 4.7. When the pH was raised to ~ 7.0, hydrolysis yields were decreased and amounts of zirconium precipitation were increased proportionately. Zirconium(IV)-assisted peptide hydrolysis in the presence of 4,13-diaza-18-crown-6 is reported in Chapter III. The goal of this work was to use an azacrown ether to reduce Zr(IV) precipitation and enhance levels of hydrolysis at neutral pH. An experiment in which 16 glycine containing dipeptides were hydrolyzed by Zr(IV) and by Zr(IV)/4,13-diaza-18-crown-6 indicated that 4,13-diaza-18-crown-6 markedly enhanced the reactivity of Zr(IV) under near physiological conditions. Because

Zr(IV) precipitation was not reduced in these reactions, we proposed that hydrolysis of peptides by Zr(IV)/4,13-diaza-18-crown-6 might be heterogeneous in nature. In Chapter IV, seventeen macrocyclic and open-chain Zr(IV) ligands were compared in order to gain mechanistic insights that would enable hydrolysis yields at neutral pH to be further improved. While the macrocyclic ligands 4,13-diaza-18-crown-6 and 4,10-trioxa-7,13-diazacyclopentadecane tended to produce higher levels of Zr(IV)-assisted dipeptide cleavage, it was not necessary to have a ring structure to enhance Zr(IV) reactivity. With respect to the open-chain ligands, the potential ability to form multiple chelate rings appeared to coincide with reduced levels of Zr(IV) precipitation as well as with reduced levels of dipeptide hydrolysis. In Chapter V, a summary of our results and conclusions is presented.

INDEX WORDS: Cleavage, Blocked peptides, Dabsyl chloride, 4,13-Diaza-18-crown-6, Dipeptides, Fluorescamine, Hydrolysis, Metals, Zirconium

ZIRCONIUM(IV)-ASSISTED PEPTIDE HYDROLYSIS

by

Miki Kassai

A Dissertation Submitted in Partial Fulfillment of Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

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Zirconium(IV)-Assisted Peptide Hydrolysis

by

Miki Kassai

Chair:	Dr. Kathryn B. Grant
Committee:	Dr. Alfons L. Baumstark
	Dr. Dabney W. Dixon

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List of Abbreviations

Ac	=	acetyl
Ala	=	alanine
Arg	=	arginine
Asn	=	asparagine
Asp	=	aspartate
BSA	=	bovine serum albumin
Cp	=	η^5-cyclopentadienyl
cyclen	=	1,4,7,10-tetraazacyclododecane
Cys	=	cysteine
CD	=	cyclodextrin
DABS-Cl	=	dabsyl chloride, dimethylaminoazobenzenesulfonyl chloride
DMF	=	*N*,*N*-dimethylformamide
DNA	=	deoxyribonucleic acid
Dtco-OH	=	dithiacyclooctan-3-ol
EDT	=	1,2-ethanedithiol
EDTA	=	ethylenediaminetetraacetate
en	=	ethylenediamine
ESI	=	electrospray ionization
Et	=	ethyl
Gln	=	glutamine

Glu	=	glutamate
Gly	=	glycine
Gly-Gly	=	glycylglycine
GSH	=	glutathione
half-life	=	$t_{1/2}$
HEPES	=	*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid
His	=	histidine
HPLC	=	high performance liquid chromatography
Ile	=	isoleucine
Leu	=	leucine
Lys	=	lysine
MALDI-TOF	=	matrix-assisted laser desorption/ionization time-of-flight
M	=	metal
Me	=	methyl
Met	=	methionine
MS	=	mass spectrometry
N_4	=	four nitrogen donor atoms
OMe	=	methyl ester
PDF	=	peptide deformylase
Phe	=	phenylalanine
PNA	=	peptide nucleic acid

Pro	=	proline
PS	=	polystyrene
py	=	pyridine
RFI	=	relative fluorescence intensity
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	serine
sol	=	coordinated solvent
TI	=	tetrahedral intermediate
TFA	=	trifluoroacetic acid
tren	=	tris(2-aminoethyl)amine
trien	=	triethylenetetramine
Tris	=	tris(hydroxymethyl)aminomethane
Thr	=	threonine
Trp	=	tryptophan
Tyr	=	tyrosine
Val	=	valine
Xaa	=	any amino acid
[9]aneN_3	=	1,4,7-triazacyclononane

CHAPTER I

Introduction

Major Advances in the Hydrolysis of Peptides and Proteins by Metal Ions and Complexes

(This chapter is verbatim as it appears in Grant, K. B.; Kassai, M; *Current Organic Chemistry* **2006**, *10*, 1035-1049. The manuscript was written by Prof. Kathryn B. Grant with assistant from Miki Kassai. Following the chapter is an unpublished epilogue written by Miki Kassai. The epilogue is a review of all pertinent literature published after the *Current Organic Chemistry* manuscript.)

Abstract

Metal ions and complexes that hydrolyze peptides and proteins have become increasingly important in recent years. These reagents have shown great promise for use in a variety of applications including protein sequencing and proteomics. When metal-assisted hydrolytic cleavage is accomplished under nondenaturing conditions of temperature and pH, their use can be extended to include the study of protein function and solution structure, the generation of semi-synthetic proteins, the proteolytic cleavage of bioengineered fusion proteins, and therapeutics. Yet, because of the extreme stability of the peptide amide bond, hydrolytically active metals are limited in number and there is now great interest in the development of new, more efficient reagents. In this review, we provide a description of relevant, early work with metal ions and complexes that have been used to hydrolyze unactivated peptide amide bonds in peptides and proteins. More importantly, we present an overview of recent contributions that have been made toward the development of synthetic metalloproteases that catalyze hydrolysis under near physiological conditions of temperature and pH.

Introduction

Reagents which mediate the selective cleavage of peptides and proteins have become increasingly important to a number of applications in the fields of chemistry and biology. Examples include the study of protein function and solution structure [1-8], the analysis of protein folding [9], and the mapping of enzyme active sites [10-20], metal and [14,16,18,19] ligand binding sites [21-25], and ligand induced conformational changes in protein structure [20,26-28]. Chemical protein footprinting is a new and promising application of this chemistry in which oxidizing iron complexes are used for the identification of solvent accessible amino acid residues in folded proteins [4,5,8,25-27,29]. Data from these protein cleavage studies provide crucial information regarding structural changes under equilibrium solution conditions and are therefore complementary to data obtained from x-ray crystallography [26]. These solution methods are essential for the study of proteins which are intrinsically difficult to crystallize (e.g., membrane proteins) for which little structural information is available. Cleaving agents are also indispensable for use in protein sequencing, and in newer applications such as proteomics [30], the generation of semi-synthetic proteins [31], and in proteolytic cleavage of bioengineered fusion proteins [32,33]. An important, long term goal is to develop new therapeutic agent designed to target disease [34-36].

Endopeptidases possess impressive catalytic power, but the broad substrate specificities exhibited by many of these enzymes (e.g., pepsin, chymotrypsin, thermolysin, pronase) make them inconvenient for use in sequencing experiments. (Groups of small peptide fragments are produced and tedious, limited proteolysis procedures must be employed.) In proteomics, highly complex protein mixtures must first be cleaved into smaller peptides. Electrospray ionization (ESI) MS/MS sequencing is then used to identify the original protein components in the mixtures.

Unfortunately, the peptides generated by non-specific endopeptidases are often too small to be matched to the proteins from which they were generated. Out of the more regioselective enzymes, the most affordable is trypsin. Hydrolysis occurs only at the *C*-terminal ends of lysine and arginine residues. As a result, the peptide fragments are intermediate in size and are usually able to provide an adequate amount of sequence information. However, other specific proteases such as Lys-C and Asp-N are costly and tend to generate very large peptides that fragment poorly in ESI MS/MS sequencing. The proteomic analysis of insoluble proteins (e.g., membrane proteins) is especially problematic. Fluorinated solvents such as hexafluoro-2-propanol are often needed, but the majority of endopeptidases do not work well under these conditions.

A second approach is the use of chemicals that mediate protein cleavage. The half-life for hydrolysis of unactivated amide bonds ranges from *ca.* 150 to 600 years at approximately neutral pH [37-39]. Thus, the extreme stability of amides has placed severe limits on the number of reagents available. Notwithstanding, most of the existing chemical agents require harsh conditions, must be used in high molar excess, and can produce low cleavage yields [40,41]. Cyanogen bromide is the most widely used, but the reaction is highly toxic, and is limited to cleavage at the carboxyl termini of methionine residues [42,43]. In the study of protein solution structure, the forcing conditions required (70% formic acid) would denature many folded proteins, resulting in a loss of relevant structural information. Additionally, peptides generated by cyanogen bromide are generally too large to fragment well in ESI-MS/MS sequencing experiments. The development of alternative cleavage strategies would therefore greatly facilitate the study of protein structure and function.

Metal ions and complexes are used to effect cleavage of the polypeptide backbone under nondenaturing conditions of temperature and pH [1-29,34-36,41,44-52]. Because mild conditions

can be employed, these reagents show great promise for use in the analysis of protein solution structure, in protein engineering (e.g., the generation of semi-synthetic proteins, the proteolytic cleavage of bioengineered fusion proteins), and in therapeutics. Thus, the design and synthesis of protein cleaving metal complexes has become an area of intensive study in recent years. Cleavage occurs by either an oxidative or a hydrolytic pathway and frequently requires precomplexation of the metal with the polypeptide.

Redox active metal ions and/or complexes of Cu^{II} [3,7,17,21,44], Cr^{III} [45], Cr^{V} [46], Fe^{III} [1,2,4-6,8,9,11,13,14,16,18,19,21,22,24-28], Ni^{II} [23], Rh^{III} [11], and V^{V} [10,12,15,20] have been used to promote the oxidative cleavage of peptide amide bonds in folded proteins under nondenaturing conditions. In the majority of cases (e.g., Cu^{II}, Cr^{III}, Cr^{V}, Fe^{III}, and V^{V}), cleavage is thought to arise from abstraction of peptide backbone α-carbon hydrogen atoms by hydroxyl radicals and other metal-generated, reactive oxygen species [45,53-56]. Subsequent degradation of the NH-Cα and Cα-C(O) bonds yields multiple, fragmented peptide products [57,58] (Fig. 1.1).

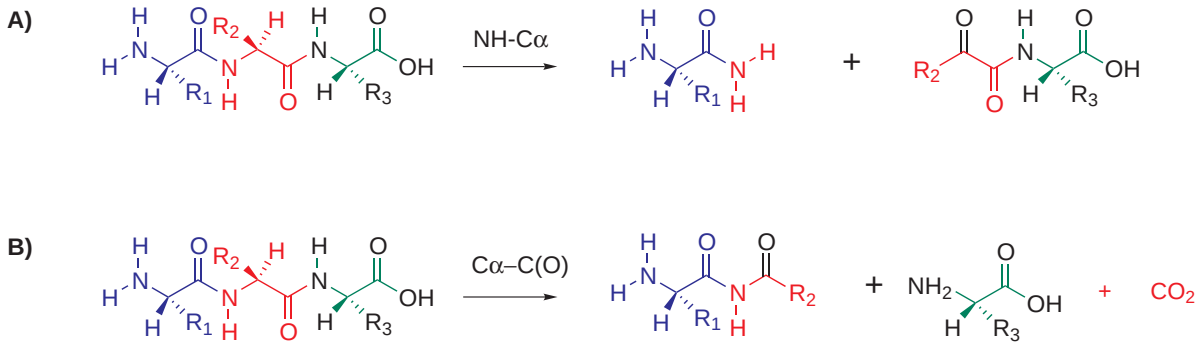


Fig. 1.1. Some typical reaction products produced by α-carbon hydrogen atom abstraction and subsequent fragmentation of the **A)** NH-Cα [57] and **B)** Cα-C(O) peptide bonds [58].

In contrast to oxidative cleavage, hydrolysis of peptides and proteins involves the addition of water across the amide bond in the peptide backbone (Fig. 1.2). This generates native

N-terminal amino and *C*-terminal carboxylate groups that can be easily reattached to form new peptide amide bonds, making the hydrolytic approach particularly attractive for use in protein sequencing, proteomics, and protein engineering applications.

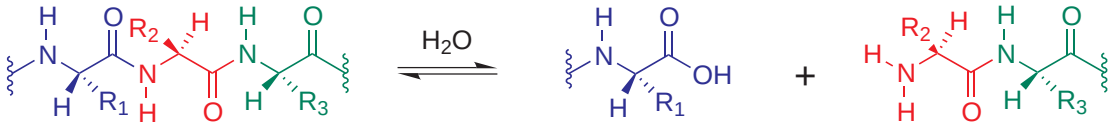


Fig. 1.2. Peptide amide bond hydrolysis.

Three basic mechanisms have been used to account for metal-promoted peptide hydrolysis [59,60]. In the first, the metal acts as a Lewis acid by activating an amide carbonyl carbon toward nucleophilic attack by a hydroxide (or water) molecule from the solvent (Fig. 1.3(A)). In the second case, the metal activates a coordinated hydroxide (or water) (Fig. 1.3(B)). The third explanation is a combined mechanism in which the metal activates the carbonyl carbon while delivering a hydroxide (or water) molecule to the amide bond (Fig. 1.3(C)). In common to the three pathways is the formation of a tetrahedral intermediate (TI) (Fig. 1.3). The extreme stability of the unactivated peptide amide bond at neutral pH [37-39] can be attributed to not only to amide resonance, but also to the poor nature of the tetrahedral intermediate "RNH^-" leaving group [59,61]. In fact, it is the departure of this leaving group from the TI that is the rate-determining step in the hydrolysis of unactivated amide bonds at neutral pH. Sayre has proposed that expulsion of the "RNH^-" group is facilitated by coordination of the metal ion to an alkoxide oxygen in the TI. In this way, the metal reduces the basicity of the alkoxide oxygen without decreasing its nucleophilicity [59]. According to Sayre, TI breakdown at neutral pH can also be accelerated by a fourth mechanism [59] in which a metal-bound water serves as a general acid catalyst that protonates "RNH^-" (Fig. 1.3(D)). Because RNH-M is a poor leaving group in

comparison to RNH_2, direct interaction between the metal and the leaving nitrogen results in the formation of a hydrolytically inactive peptide complex.

In the majority of cases, metal ions coordinate more strongly to individual amino acids than to peptides. As a result, metals often shift reaction equilibria in the direction of peptide hydrolysis [62]. Notwithstanding, in order to promote peptide amide bond hydrolysis under physiologically relevant conditions of temperature and pH, a candidate metal ion (or complex) should possess one or more of the following characteristics. In addition to being a strong Lewis acid, the metal center should be redox inactive to avoid side reactions that contribute to the production of protein damaging, reactive oxygen species. If the metal is oxophilic and readily forms complexes with high coordination numbers, it can more easily interact with an amide carbonyl oxygen in the peptide backbone (activating the carbon toward nucleophilic attack), while simultaneously delivering a hydroxide nucleophile to the scissile amide bond (Fig. 1.3(C)).

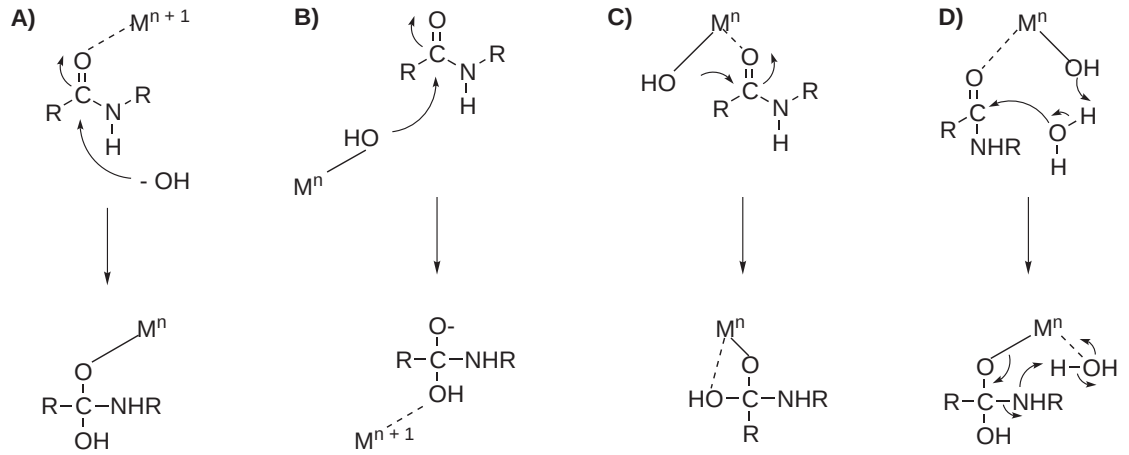


Fig. 1.3. Peptide amide bond hydrolysis promoted by metals: four alternative pathways lead to the formation of a tetrahedral intermediate [59,60]. The superscript n indicates the charge on the metal (M).

Fast-ligand exchange kinetics are also desirable as catalytic turnover can be achieved if the metal promotes facile release of the hydroxide nucleophile at the scissile amide bond as well as release

of any coordinated peptide hydrolysis products. While the metal should lower the pK_a value of water (≈ 15.7) to enable metal bound hydroxide nucleophile to exist in neutral media, it should not reduce the pK_a of the amide nitrogen hydrogen atom (≈ 15) to a markedly low value. (Metals bind strongly to deprotonated amide nitrogens and are much less polarizing than protons. As a result, substitution of an amide nitrogen hydrogen atom for metal greatly reduces the susceptibility of the amide carbonyl carbon atom towards nucleophilic attack [62].) Finally, sequence specific peptide hydrolysis can be achieved if the metal is capable of coordinating to anchoring side chains in amino acid residues such as cysteine (Cys), aspartate (Asp), histidine (His), and methionine (Met).

Metal ions and/or complexes of Ce^{IV}, Co^{II}, Co^{III}, Cu^{II}, Mo^{IV}, Ni^{II}, Pd^{II}, Pt^{II}, Zn^{II}, and Zr^{IV} have been used to effect hydrolytic cleavage of unactivated amide bonds in peptides [35,41,47-49,51,52,63-88]. In the case of Cu^{II}, Co^{III}, Ni^{II}, Pd^{II}, and Pt^{II}, intact proteins have been cleaved [32-34,36,49,50,74,75,82,86,89-98]. Although hydrolysis is sometimes accomplished at ~pH 7.0 and 37 °C [34-36,41,47-52], either extreme pH, and/or elevated temperatures are occasionally employed [32,33,63-78,82-86,88-90,92-94,96-98]. Significant progress has been made, but efficient hydrolytically active metals are limited in number. There is now great interest in the development of new metal-based reagents that target diverse amino acid sequences under mild conditions of temperature and pH.

In this review, we will present a description of relevant, early work with metal ions and complexes that have been utilized to hydrolyze unactivated peptide amide bonds in peptides and proteins. Additional, detailed information can be found in a number of excellent and comprehensive review articles written on this subject [60-62,99-111]. More importantly, this paper focuses on recent developments contributing to the design of synthetic metalloproteases

that catalyze the hydrolysis of peptide and protein substrates under near physiological conditions of temperature and pH. These reagents may serve as versatile biochemical tools for use in the study of protein function and solution structure, protein engineering, therapeutics, and other important applications in chemistry and biology.

Hydrolysis of Peptides and Proteins by Metal Ions and Complexes

Lanthanide Metals

Lanthanides are attractive candidates for use in peptide hydrolysis experiments. Because they are hard acids that interact preferentially with hard bases such as oxygen, hydrolytically inactive coordination involving amide nitrogens in the peptide backbone can be avoided [72]. Furthermore, in contrast to most transition metal complexes in which d-orbital bonding produces well defined coordination geometries, lanthanide ions form complexes with non-rigid, flexible geometries and high coordination numbers [72].

The first, published reports of lanthanide-assisted peptide hydrolysis were in 1956 and 1958 [64,65]. In these early, pioneering studies, Bamann and coworkers treated a series of dipeptides with Ce^{IV}, Ce^{III}, and La^{III}. Optimal results were obtained with Ce^{IV}, which was used to hydrolyze Gly-Leu in over 70% yield after 24 h at pH 8.6 and 70 °C. Cerium(IV) is the only lanthanide with an accessible +4 oxidation state and has enhanced Lewis acid strength imparted by its high positive charge density. Thus, it is not surprising that peptide hydrolysis by Ce^{IV} has consistently been found to be superior in comparison to other lanthanide ions (e.g., La^{III}, Ce^{III}, Pr^{III}, Nd^{III}, Sm^{III}, Eu^{III}, Gd^{III}, Tb^{III}, Dy^{III}, Ho^{III}, Er^{III}, Tm^{III}, Yb^{III}) [64,65,72,80]. This result can be accounted for by the exceptional ability of Ce^{IV} to activate the peptide amide carbonyl towards nucleophilic attack. Komiyama has proposed that Ce^{IV} withdraws electrons more efficiently than other lanthanides metals due to the stability of the trivalent state of cerium. Because the divalent

states of the lanthanides are relatively unstable, trivalent lanthanides are unable to withdraw electrons as efficiently and as a result, peptide hydrolysis yields are low [80,107,110]. It is also noteworthy that Ce^{IV} promotes facile, hydrolytic cleavage of deoxyribonucleic acids (DNA) [112-115].

In the peptide hydrolysis reactions described by Bamann and coworkers, the active hydrolytic species were heterogeneous lanthanide hydroxide gels [64,65]. Komiyama employed γ-cyclodextrin to solubilize Ce^{IV}, and was able to achieve homogeneous hydrolysis of the dipeptide Gly-Phe in 39% yield after 24 h at pH 8.0 and 60 °C [72]. When other dipeptides were investigated, Ce^{IV} was found to be relatively non-selective with respect to hydrolysis of specific amino acid sequences [72]. In a more extensive study of dipeptides, oligopeptides, and blocked peptides analogs, the same research group made a number of other important observations [80]. Cerium(IV) was shown to promote modest catalytic turnover and preferential hydrolysis of *N*-terminal peptide amide bonds. In addition, free *N*-terminal amino and *C*-terminal carboxylate peptide groups, which coordinated directly to the Ce^{IV} analog Pr^{III} in ^{1}H NMR experiments, were found to be required for efficient Ce^{IV} hydrolysis of dipeptides with no metal-coordinating side chains. (The presence of amino acids with metal-coordinating side chains in free *N*-terminal and *C*-terminal peptides reduced hydrolysis yields.) Interestingly, the dipeptide amide Asp-Phe-NH_2 was efficiently hydrolyzed by Ce^{IV}, indicating that the carboxylate side chain of aspartate interacts with Ce^{IV} in place of the *C*-terminal carboxylate group. This significant finding suggests that Ce^{IV} should be capable of promoting sequence-specific hydrolysis adjacent to Asp residues in large peptides and proteins. Using 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer, Komiyama and coworkers obtained optimal results with the dipeptide Gly-Phe, which was hydrolyzed by $[Ce(NH_4)_2(NO_3)_6]$ in ~ 90% yield after 24 h at pH 8.0 and 80 °C [80].

Divalent Zinc

Zinc(II) possesses a number of properties that have been shown to play an important role in promoting peptide hydrolysis. Because of its stable d^{10} electron configuration, Zn^{II} is redox inactive and able to avoid undesirable side reactions that contribute to oxidative peptide cleavage [116]. A second advantage arising from its filled d-shell is that there is no gain in ligand field stabilization energy associated with complex formation. Consequently, Zn^{II} is able to assume a variety of different ligand geometries without any energy costs. With respect to catalysis, Zn^{II} is kinetically labile and exchanges ligands rapidly [103,116]. Finally, Zn^{II} is a strong Lewis acid because of its high charge density. Thus, it is not surprising that Zn^{II} is a cofactor in the active sites of the majority proteases found in nature (e.g., carboxypeptidase A, carboxypeptidase B, thermolysin) [103].

One of the earliest examples of Zn^{II}–assisted peptide hydrolysis was published by Bamann and coworkers in 1961 [66]. In this study, the dipeptide Gly-Leu was hydrolyzed at low levels after treatment with Zn^{II} for 24 h at pH 8.6 and 70 °C [66]. More recently, Yashiro's group reacted $ZnCl_2$ with an extensive series of dipeptides [84]. They discovered that levels of hydrolysis were significantly higher in sequences containing either a serine (or threonine) at the *C*-terminal position. For example, upon treatment with $ZnCl_2$ for 24 h at 70 °C and pH 7.0, hydrolysis yields were 91%, 83%, 4% and 6%, for Phe-Ser, Gly-Ser, Ser-Gly, and Gly-Gly respectively [84]. Yashiro proposed that the hydroxyl group of the serine (or threonine) side chain was able to facilitate peptide amide bond hydrolysis by promoting an N→O acyl rearrangement. In Xaa-Ser(Thr) sequences, intramolecular attack of the side chain hydroxyl on the amide carbonyl carbon produces a favorable five-membered ring transition state (Fig. 1.4). However, in the case of Ser(Thr)-Xaa sequences (e.g., Ser-Gly), the authors pointed out that

hydrolysis yields are low because an unfavorable four-membered ring transition state is formed. In a survey of twelve metal salts conducted at pH 7.0 and 70 °C, Yashiro demonstrated that Gly-

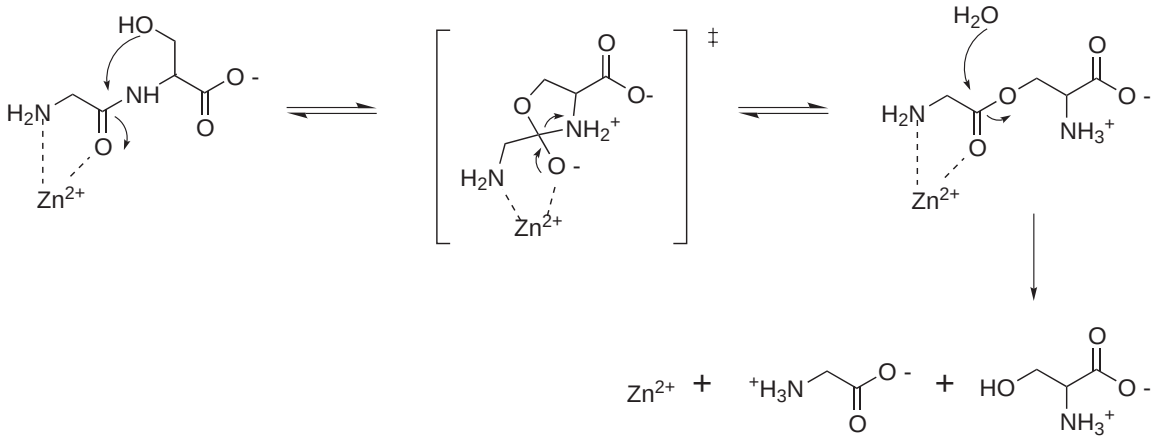


Fig. 1.4. In Zn^{II}–assisted hydrolysis of Xaa-Ser(Thr) sequences, the side chain hydroxyl group of Ser(Thr) participates in an N→O acyl rearrangement that involves the formation of a five-membered ring transition state [84].

Ser was most efficiently hydrolyzed by $ZnCl_2$ (in 83% yield), followed by the remaining eleven metals ($PrCl_3$ > $EuCl_3$ > $NiCl_2$ (52% yield) > $CeCl_3$ > $CdCl_2$ > $ErCl_3$ > $LaCl_3$ > $LuCl_3$ > $MgCl_2$ > $CaCl_2$ = $CuCl_2$ (6% yield)) [84]. In the case of $CuCl_2$, the authors attributed the extremely low levels of hydrolysis to hydrolytically inactive metal coordination involving the amide nitrogen in the peptide backbone. (For example, the pK_a value of the peptide amide nitrogen is lowered from ~ 15 to 8 and 4 by Ni^{II} and Cu^{II}, respectively [62]. Therefore, Cu^{II} would be expected to form a stronger metal-amide nitrogen bond at neutral pH.) Yashiro and coworkers also proposed that coordination to the free *N*-terminal amino group is important only in assisting in the coordination of Zn^{II} to the peptide backbone carbonyl oxygen (Fig. 1.4). By using an anchoring amino acid side chain to substitute for the *N*-terminal group, Zn^{II} should be capable of promoting hydrolysis of internal Ser(Thr)-Xaa sequences in intact proteins. In the case of Ni^{II} [49], Pd^{II} [89,91,92,96], Pt^{II} [96], and even Cu^{II} [32,33,75,90,94,95,98], hydrolytic cleavage of Ser(Thr)-Xaa protein sequences has already been reported in the literature.

Cobalt Ions and Complexes

In 1956, Meriwether and Westheimer reacted cobalt(II) ions with the blocked dipeptide Phe-Gly-NH_2 [63]. At pH values greater than 5.0, low levels of peptide hydrolysis could be detected after 72 h of treatment at 75° C. Similar results were obtained by Bamann and coworkers, who in 1961 showed that Co^{II} ions could be used to hydrolyze Gly-Leu at detectable levels after 24 h at pH 8.6 and 70 °C [66]. Yet Co^{II}, which is kinetically labile, is readily oxidized by atmospheric oxygen to inert Co^{III}. Because the above experiments appear to have been conducted under aerobic conditions, interpretation of these data might have been partially confounded if one-electron oxidation of Co^{II} had occurred.

Soon after these early Co^{II} studies were published, significant advances were made by Buckingham and coworkers, who developed a series of hydrolytically active Co^{III} complexes corresponding to the formula $[CoN_4(OH)(OH_2)]^{2+}$ (where N_4 = four nitrogen donor atoms, e.g., $(en)_2$, trien, and tren) [68, 69,101,102,106]. The best, overall results were consistently obtained with *cis*-β-$[Co(trien)(OH)(OH_2)]^{2+}$. At pH 8.0 and 45 °C, Bentley and Creaser showed that the β-trien complex was at least 50 times more effective in hydrolyzing Gly-Gly, compared to the α-trien, tren, and $(en)_2$ systems [47]. Buckingham used *cis*-β-$[Co(trien)(OH)(OH_2)]^{2+}$ to achieve quantitative hydrolysis of Gly-Gly and other dipeptides after only 30 min at pH 7.5 and 60 °C–65 °C [69,106]. In addition to dipeptides, the *N*-terminal amino acids of a number of oligopeptides and proteins were hydrolyzed in quantitative yields [47].

A necessary feature for efficient peptide hydrolysis by $[CoN_4(OH)(OH_2)]^{2+}$ complexes is the availability of two relatively labile *cis*-β sites in the octahedral coordination sphere of cobalt(III), with the remaining sites being occupied by inert nitrogen ligands [101]. The complex

$[Co(trien)(OH)(OH_2)]^{2+}$ exists in geometrical *cis*-α, *cis*-β, and *trans* configurations (Fig. 1.5). The *trans* geometry does not allow for the required *cis* chelation of the peptide substrate, so direct hydrolysis does not occur. (Unlike the trien complex, systems such as $[Co(en)_2(OH)(OH_2)]^{2+}$ undergo facile *cis-trans* isomerization, and as a result react more slowly and produce complex product mixtures [68,69,106].)

Although Co^{III} is a strong Lewis acid, $[CoN_4(OH)(OH_2)]^{2+}$ systems preferentially react with *N*-terminal amino acids to form inert complexes. Therefore, peptide hydrolysis is stoichiometric rather than catalytic [47,68,69,101,102,106]. Yet, the reduced lability of Co^{III} gave way to a number of informative mechanistic studies. For example, when *cis*-β-$[Co(trien)(OH_2)_2]^{3+}$ was reacted with Gly-GlyOEt at pH 7.5 - 8.0 for 1 h at 25 °C, the intermediate shown in Fig. 1.5(D) was isolated [106,117]. Its direct participation in peptide hydrolysis was subsequently confirmed in ^{18}O-labelling studies [106]. Taken together, these

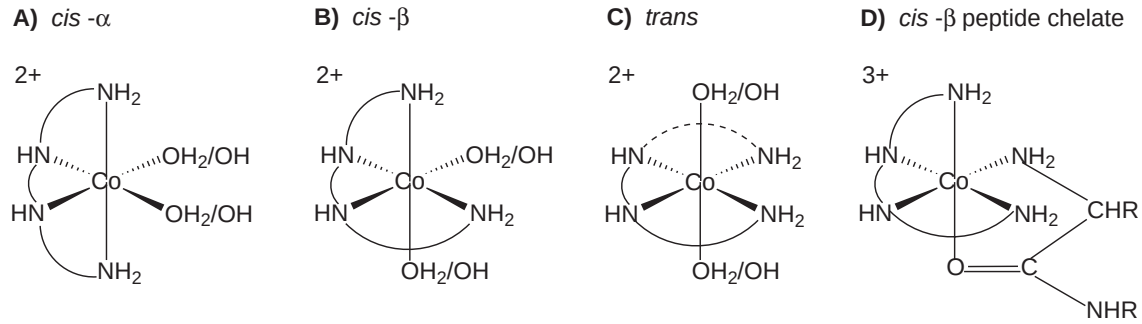


Fig. 1.5. A schematic diagram of the **A)** *cis*-α, **B)** *cis*-β, and **C)** *trans* isomers of $[Co(trien)(OH)(OH_2)]^{2+}$ [101]. **D)** Reaction intermediate isolated after *cis*-β-$[Co(trien)(OH_2)_2]^{3+}$ was treated with Gly-GlyOEt at pH 7.5-8.0 for 1 h at 25 °C [106,117].

results support the conclusion that peptide hydrolysis by *cis*-β-$[Co(trien)(OH_2)_2]^{3+}$ proceeds through a pathway similar to the carbonyl activation mechanism shown in Fig. 1.3(A).

Prior to 2000, Co^{III} complexes had been used to hydrolyze only the *N*-terminal amide

bonds in peptides and proteins. Cleavage of internal peptide amide bonds had not been achieved because the hydrolysis mechanism was shown to involve the coordination of Co^{III} to the free *N*-terminal peptide or protein amino group (Fig. 1.5(D)) [47,68,69,101,102,106]. This limitation was overcome by Kumar and coworkers, who used the simple Co^{III} complexes $[Co(H_2O)(NH_3)_5]^{3+}$ and $[Co(H_2O)_2(NH_3)_4]^{3+}$ to hydrolyze hen egg lysozyme at a single internal Ala-Trp bond under extremely mild conditions [50]. After 26 h of treatment at pH 7.0 and 37 °C, the yields obtained with hexaamminecobalt(III) chloride, pentaammineaquacobalt(III) chloride, and tetraammindiaquacobalt(III) chloride were 0%, 35%, and 45%, respectively, indicating that the number of open coordination sites was important in the protein hydrolysis reaction. Although precise mechanistic details (e.g., the isomer geometry of the $[Co(H_2O)_2(NH_3)_4]^{3+}$ complex) were not specified, ^1H NMR spectra of lysozyme revealed that Co^{III} induced significant chemical shift changes in specific resonances corresponding to Trp 108 [50]. Because this amino acid residue is accessible to solvent in folded lysozyme, Kumar and coworkers suggested that its role might be to anchor Co^{III} in the proximity of the scissile Ala110-Trp111 peptide amide bond. Finally, the *cis* and *trans* isomers of the complex $[Co(en)_2(OH_2)_2]Br_3$ were shown to be inactive towards hydrolysis. Based on this result, Kumar concluded that the large ligand size of "en" might have prevented the metal center of the complex from accessing the peptide backbone of lysozyme [50].

The next major advance was made by Suh and coworkers in 2003, when they presented the first evidence of catalytic protein hydrolysis by a Co^{III} complex [34]. Extensive screening of 1,4,7,10-tetraazacyclododecane (cyclen)-peptide nucleic acid (PNA) combinatorial libraries led to the design of a highly reactive conjugate (**1** in Fig. 1.6), consisting of a cyclen ligand covalently attached to a PNA oligomer that selectively binds to myoglobin. Using the Co^{III} complex of **1**, myoglobin was hydrolyzed at internal Leu72-Gly73 and Leu89-Ala90 sequences

under extremely mild conditions. After 30 h at pH 7.5 and 37 °C, hydrolysis yields were ~ 50% and more importantly, despite the kinetically inert nature Co^{III}, catalytic turnover was achieved (k_{cat} = 0.022 h^{-1}) [34]. When other metals were used to substitute for Co^{III}, Cu^{II} was found to be less reactive, while Ce^{IV}, Fe^{III}, Hf^{IV}, Pd^{II}, Pt^{IV}, and Zr^{IV} failed to display any activity. In addition, no hydrolysis occurred when myoglobin was treated with Co^{III}-cyclen (without PNA) and when the Co^{III}-cyclen-PNA conjugate was reacted with other proteins such as albumin, γ-globulin, elongation factor P, gelatin A, and gelatin B. Because biogenic ligands readily sequester labile metals such as Cu^{II}, the authors insightfully pointed out that the use of relatively inert Co^{III} might be advantageous in medical applications [34].

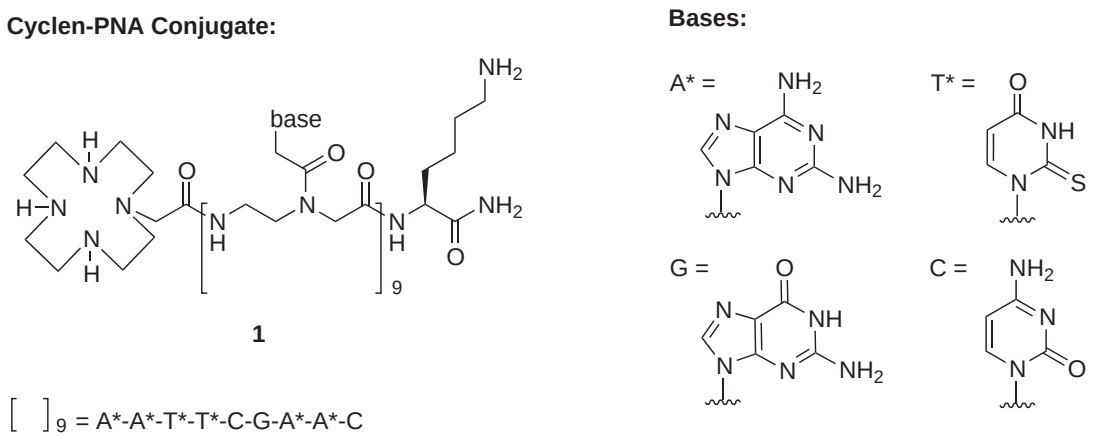
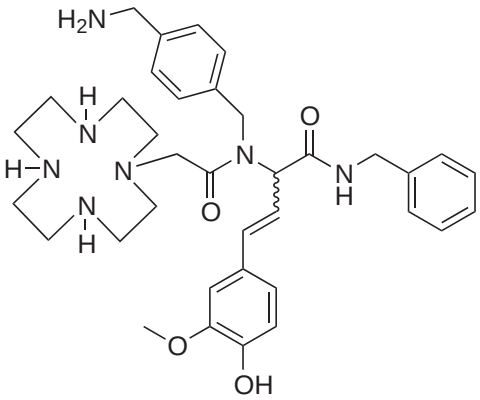


Fig. 1.6. A schematic diagram of cyclen-PNA conjugate **1** (compound **1** in [34]).

In 2005, Suh and coworkers published the results of a related study in which a combinatorial library consisting of ~15,000 modified cyclen-*N*-acylamino acid amide conjugates was screened against the enzyme peptide deformylase (PDF) [36]. This was done in an effort to identify a moderately sized, artificial metalloprotease capable of catalyzing the hydrolysis of a disease-related protein. (The molecular weights of **1** and other cyclen-PNA conjugates approach 3000 $gmol^{-}$ [34]. As a general rule, low molecular weight drugs are more effective as therapeutic

agents due to their ability to rapidly move across biological membranes.) PDF catalyzes deformylation of *N*-formylated proteins produced during prokaryotic translation. It is therefore an ideal target for antibiotic drugs: it is expressed in all Eubacteria (e.g., *Mycobacterium tuberculosis* which causes tuberculosis), but is absent from Archaea, animals, and fungi. Using the combinatorial approach described above, the authors identified the Co^{III} complex of cyclen conjugate **2** (Fig. 1.7).

Cyclen-*N*-Acylamino Acid Amide Conjugate:



2

Fig. 1.7. A schematic diagram of cyclen-*N*-acylamino acid amide conjugate **2** [36].

Cleavage of PDF was catalyzed at a single bond (Gln152-Arg153) in approximately 50% yield after 72 h of treatment at pH 7.5 and 37 °C ($k_{cat} \approx 0.05$ h^{-1}). Furthermore, the relatively low molecular weight of the Co^{III} complex (644 $gmol^{-1}$) enabled Suh and coworkers to conduct docking and molecular dynamics simulations. These studies predicted preferential interaction of PDF with the S enantiomer of the complex, as well as a number of hydrogen-bonding interactions and van der Waals contacts between the complex and specific amino acid residues near the scissile Gln152-Arg153 peptide amide bond [36].

Zirconium(IV)

Our research group has most thoroughly studied the early transition metal zirconium(IV). Our interest in using this metal to promote peptide hydrolysis was motivated by a number of factors. Zirconium(IV) is redox inactive and has enhanced Lewis acid strength imparted by its +4 oxidation state, enabling Zr^{IV} ions to efficiently hydrolyze DNA and activated phosphodiester bonds [118-120]. Because Zr^{IV} is oxophilic and forms complexes with high coordination numbers [121], we envisioned that this metal center should be capable of activating an amide carbonyl oxygen in the peptide backbone, while simultaneously delivering a hydroxide nucleophile to the scissile amide bond (Fig. 1.3(C)). (The pK_a values of Zr^{IV} bound water molecules are $\leq$ 0.6, enabling Zr-OH to readily exist in neutral media [122].) We also reasoned that the rapid-ligand exchange kinetics associated with Zr^{IV} [123] should help to promote catalytic turnover. Finally, the preference of Zr^{IV} for oxygen should avoid the formation of hydrolytically inactive peptide amide nitrogen complexes at neutral pH [124]. In spite of the numerous advantages of Zr^{IV}, Komiyama and coworkers had reported that Zr^{IV} effected only very low levels of peptide hydrolysis in 0.1 M Tris (**3** in Fig. 1.8) buffer pH 7.0 (~ 25% conversion of Gly-Phe after 24 h at 80 °C [80]).

In aqueous solutions of $[H^+] \leq 0.5$ M, Zr^{IV} ions form an octanuclear $[Zr_8(OH)_{20}(H_2O)_{24}]^{12+}$ species [123], while at pH values above 5.0, the formation of insoluble gels and precipitates [125] is thought to be responsible for the reduction in phosphodiester hydrolysis that occurs in this pH range [119]. In order to avoid the same complication with peptides (i.e., suppression of Zr^{IV}-assisted hydrolysis at neutral pH), we employed the azacrown ether 4,13-diaza-18-crown-6 (**4** in Fig. 1.8).

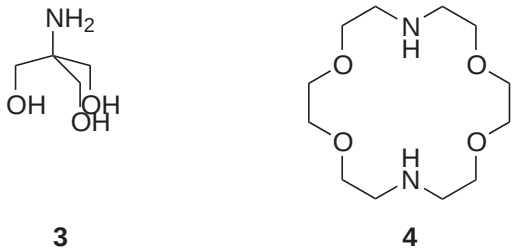


Fig. 1.8. Two reagents affecting Zr^{IV}-assisted peptide hydrolysis.

This ligand was of interest to us because it readily undergoes complexation with lanthanide metals [126] and because it lacks carboxylate groups which when present (e.g., in ethylenediaminetetraacetate (EDTA)) form hydrolytically inactive complexes with Zr^{IV} and lanthanide metals [35,119,127]. Our research led to the discovery that azacrown ether **4** is capable of accelerating Zr^{IV}-assisted peptide hydrolysis at pH 7.0 - 7.3 (37 - 60 °C) [35]. The following is a summary of our major findings.

Our first experiment involved 16 dipeptides corresponding to the sequences Xaa-Gly and Gly-Xaa [35]. A total of 2 mM of each dipeptide was reacted at ~ neutral pH and 60 °C in either 10 mM $ZrCl_4$, 19 to 22 mM 4,13-diaza-18-crown-6, or 10 mM $ZrCl_4$ with 19 to 22 mM 4,13-diaza-18-crown-6. While zirconium(IV)-assisted cleavage was always very low in the absence of 4,13-diaza-18-crown-6, zirconium hydrolysis of all neutral and negatively charged dipeptides was increased by 190% up to 1775% upon addition of **4**. Hydrolysis yields ranged from 35% for Gly-Leu up to 97% for Gly-Glu. Overall, our data showed that Zr^{IV}/4,13-diaza-18-crown-6 displayed a marked preference for efficient hydrolysis of peptides containing glycine and amino acids with oxygen rich side chains. The position of glycine with respect to the remaining amino acid in the dipeptide did not appear to have an effect on hydrolysis yields. While insoluble Zr^{IV} precipitates were formed in all of the $ZrCl_4$ reactions, to our surprise, the addition of 4,13-diaza-

18-crown-6 did not decrease the extent of Zr^{IV} precipitation.

At pH values > 5.0, Tris (**3** in Fig. 1.8) buffer reduces the formation of zirconium precipitates while increasing levels of $ZrCl_4$ phosphodiester hydrolysis [119,120]. Accordingly, we used Tris to substitute for the azacrown ether in an attempt to further improve hydrolysis yields. Interestingly, while **3** helped to reduce Zr^{IV} precipitation, peptide hydrolysis by Zr^{IV} was decreased when Tris was added [35]. This observation led us to speculate that hydrolysis of peptides by Zr^{IV}/4,13-diaza-18-crown-6 might have a heterogeneous component similar to peptide hydrolysis by lanthanide hydroxide gels [64-66,80].

The blocked peptide analog AcGly-GlyOMe was studied next (pH 7.0-7.2, 60 °C, 2 mM peptide, 10 mM $ZrCl_4$, 20 h) [35]. Although Zr^{IV}/4,13-diaza-18-crown-6 hydrolysis yields were 90% and 26% for Gly-Gly and AcGly-GlyOMe, respectively, Zr^{IV}-hydrolysis of AcGly-GlyOMe was increased by 2500% by the azacrown ether. The ability of Zr^{IV}/4,13-diaza-18-crown-6 to hydrolyze Gly-Gly irrespective of the presence of free and/or blocked *N*- and *C*-terminal groups is significant in light of the fact that, in most biochemical applications, protein hydrolysis involves cleavage of internal peptide amide bonds.

In order to evaluate Zr^{IV} activity under physiologically relevant conditions, Gly-Glu was reacted at 37 °C and pH 7.3 [35]. After 20 h, a total of 39% of the dipeptide was hydrolysed in the presence of Zr^{IV}/4,13-diaza-18-crown-6 and the azacrown ether increased levels of Zr^{IV}-hydrolysis by 550%. Time course experiments were then conducted at 37 °C and 60 °C. At 37 °C and pH 7.3, the $t_{1/2}$ for Zr^{IV}/4,13-diaza-18-crown-6 hydrolysis of Gly-Glu was 36.6$\pm$2.7 h. This represents a significant rate enhancement in comparison to the average half-life of ~ 200 years estimated for spontaneous hydrolysis of unactivated peptide amide bonds under nearly identical conditions (pH 6.8-7.0, 37 °C) [38,39]. At pH 7.1 and 60 °C, the $t_{1/2}$ was 69.3$\pm$5.5 h and 5.3$\pm$0.1

h for Zr^{IV}-hydrolysis of Gly-Glu without and with 4,13-diaza-18-crown-6, respectively. To test for catalytic turnover, 10 mM Gly-Glu, 5 mM $ZrCl_4$, and 15 mM of 4,13-diaza-18-crown-6 were reacted at pH 7.0 and 60 °C. Yields of free glycine were 56%, 75%, and 83% after 45 h, 94 h, and 138 h respectively. (When Zr^{IV} was omitted, there was 0% glycine at 138 h.) Thus, the greater than stoichiometric levels of hydrolysis indicated modest levels of catalytic activity.

Although 4,13-diaza-18-crown-6 forms stable Zr^{IV} complexes in organic solvents [128], interactions between Zr^{IV} and the azacrown ether are likely to be complicated in aqueous solutions. This is due to the propensity of Zr^{IV} to form polynuclear polyhydroxo species, insoluble gels, and precipitates [123,125]. Indeed, precipitation was observed in all of our peptide hydrolysis experiments. Thus, elucidating the precise mechanism through which Zr^{IV} promotes peptide hydrolysis will be a challenging task. Nevertheless, we employed ^{1}H NMR to obtain preliminary evidence of complex formation between Zr^{IV} and 4,13-diaza-18-crown-6 in D_2O [35].

In summary, 4,13-diaza-18-crown-6 (**4** in Fig**.** 1.8) enhances the rate of zirconium-assisted peptide hydrolysis in neutral solutions (pH 7.0 - 7.3; 37 - 60 °C). We found that Zr^{IV}/4,13-diaza-18-crown-6 displays a preference for cleavage of neutral and negatively charged peptides containing glycine and amino acids with oxygen rich side chains. The reaction is moderately catalytic and does not have an absolute requirement for the presence of free and/or blocked *N*- and *C*-terminal groups. Our current research in this area is focused on continued mechanistic studies to precisely define the nature of hydrolytically active zirconium species. We are also interested in design of second generation ligands that will further enhance the reactivity of zirconium(IV).

Copper(II) and Nickel (II) Ions

One of the first studies in which the kinetically labile ions Cu^{II} and Ni^{II} were used to promote peptide hydrolysis was published by Meriwether and Westheimer in 1956. At 75 °C, Phe-Gly-NH_2 was hydrolyzed at detectable levels by Cu^{II} at pH 5.0, and then by Cu^{II} and Ni^{II} at higher pH values [63]. (It was evident that Ni^{II} became more efficient in promoting hydrolysis as reaction pH was raised above 5.0.) At pH 8.6 and 70 °C, Bamann and coworkers were able to detect hydrolysis of Gly-Leu after 24 h and 48 h with Cu^{II} and Ni^{II}, respectively [66]. It can be inferred from their results that Cu^{II} is more effective than Ni^{II} in promoting peptide hydrolysis at this pH. Then, in 1965, Grant and Hay conducted a pH profile in which Cu^{II}-assisted hydrolysis of Gly-Gly was quantitated after 100 h of treatment at 85 °C [67]. Copper(II) displayed maximum reactivity at pH 4.2, where it promoted hydrolysis of Gly-Gly in ~ 55% yield. Because Cu^{II} lowers the pK_a value of the peptide amide nitrogen from ~15 to ~4 [62], the notable decrease in rate observed at pH values higher than 4.2 was attributed to metal-induced deprotonation of the amide nitrogen followed by the formation of a hydrolytically inactive Cu^{II} complex (**5** in Fig. 1.9). It is important to note that the pK_a of the peptide amide nitrogen is lowered from ~ 15 to 8 by Ni^{II} [62]. Therefore, in comparison to Cu^{II}, the efficiency of Ni^{II} ion-assisted peptide hydrolysis would be expected to begin to decrease at basic rather than at acidic pH values.

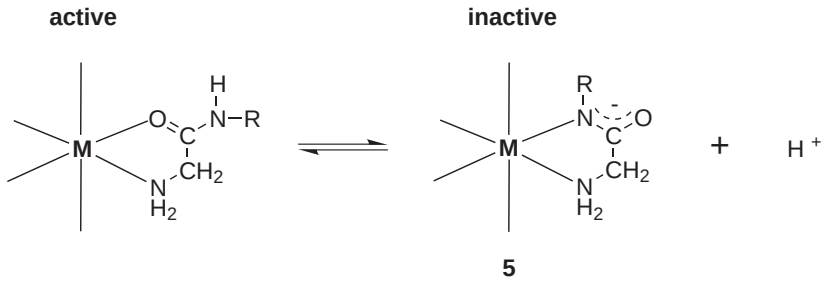


Fig. 1.9. Metal-induced deprotonation of a peptide amide nitrogen [67,101].

Copper(II) and nickel(II) ions have also been used to hydrolyze amide bonds in larger peptides [48,49,75,87]. Hydrolysis by $CuCl_2$ is specific for the Xaa-Ser(Thr) bond in oligopeptides containing Xaa-Ser(Thr)-His sequences (e.g., a yield of ~ 75% was obtained for AcPhe-Asp-**Lys-Ser-His-**Thr-Tyr-NH_2 at 62 °C and pH 8 after 24 h [75]). The driving force of the copper(II) reaction is thought to be the formation of an extremely stable Cu^{II} Ser(Thr)-His-Xaa complex (**6** in Fig. 1.10) [75]. The metal salt $NiCl_2$ hydrolyzes Xaa-Ser bonds in peptides containing a Thr-Xaa-Ser-Xaa-His-Lys motif [87] (e.g., in ~ 10% yield for AcLeu-Leu-Gly-Lys-Val-Thr-Ile-Ala-Gln-Gly-Gly-Val-Leu-Pro-Asn-Ile-Gln-Ala-Val-Leu-Leu-Pro-Lys-Lys-**Thr-Glu-Ser-His-His-Lys-**Ala-Lys-Gly-Lys at 37 °C and pH 7.4 after 24 h [49]). Here, the driving force is the formation of stable Ni^{II} square planar complexes involving cleavage products containing the sequence Ser-Xaa-His-Lys [48,49,129]). In the case of copper(II) and nickel(II), the hydroxyl group of the serine (or threonine) side chain is thought to facilitate peptide amide bond hydrolysis by promoting an N→O acyl rearrangement [48,75,104,105]. As discussed in an earlier section of this review article, Yashiro reacted a series of twelve metals salts with the dipeptide Gly-Ser and found that $CuCl_2$ produced extremely low levels of hydrolysis in comparison to ten of the other metals tested [84]. Because the reaction was run at pH 7.0, the authors attributed their results to hydrolytically inactive copper coordination involving the amide nitrogen in the peptide backbone of Gly-Ser. To account for efficient Cu^{II}-promoted hydrolysis of Xaa-Ser(Thr)-His sequences, Allen proposed an alternative mechanism in which strong binding of Cu^{II} to the released amino group prevents a reverse hydrolysis reaction, which would be thermodynamically favored in the absence of metal (Fig. 1.10) [105].

Work with small peptides has led to the use of Cu^{II} and Ni^{II} ions to cleave intact proteins [32,33,49,90,95,130]. Horse heart myoglobin was hydrolyzed by $CuCl_2$ at Gln91-Ser92 and

Ala94-Thr95 in the sequence **Gln91-Ser92-His93**-Ala94-Thr95-Lys96 (in 27% yield at 50 °C, pH 7.0, 3 days [95]). Hydrolysis of denatured IgG antibody has been accomplished with $CuCl_2$ at a single Lys-Thr bond in the hinge-region sequence Asp-**Lys-Thr-His**-Thr (in 73% yield at 62 °C, pH 8.2, 24 h [90]). Humphreys and coworkers then utilized $CuCl_2$ to successfully cleave a bioengineered γ1 Fab' fusion protein at the Lys-Ser bond in the sequence Asp-**Lys-Ser-His** (in ~ 85% yield at 62 °C, pH 8.0, 15 h [33]). In the case of the metal salt $NiCl_2$, bovine histone 2A was hydrolyzed at the Glu-Ser bond in the sequence **Thr-Glu-Ser-His-His-Lys** (in low yield at 37 °C, pH 7.4, 2 days [49]). Kasprzak and coworkers demonstrated that the same hydrolysis reaction occurs in histone 2A within living Chinese hamster ovary cells exposed to nickel(II) acetate for 3 to 7 days. This experiment is significant in that it presents direct evidence that Ni^{II}-promoted peptide hydrolysis may contribute to nickel-induced toxicity *in vivo* [130]. In protein hydrolysis by Cu^{II} and Ni^{II}, there is an apparent, strong preference for cleavage of Xaa-Ser and Xaa-Thr bonds. The mechanism(s) underlying this chemistry may be related to Cu^{II} and Ni^{II} peptide hydrolysis reactions in which the hydroxyl group of the serine (or threonine) side chain promotes an N→O acyl rearrangement [48,75,104,105].

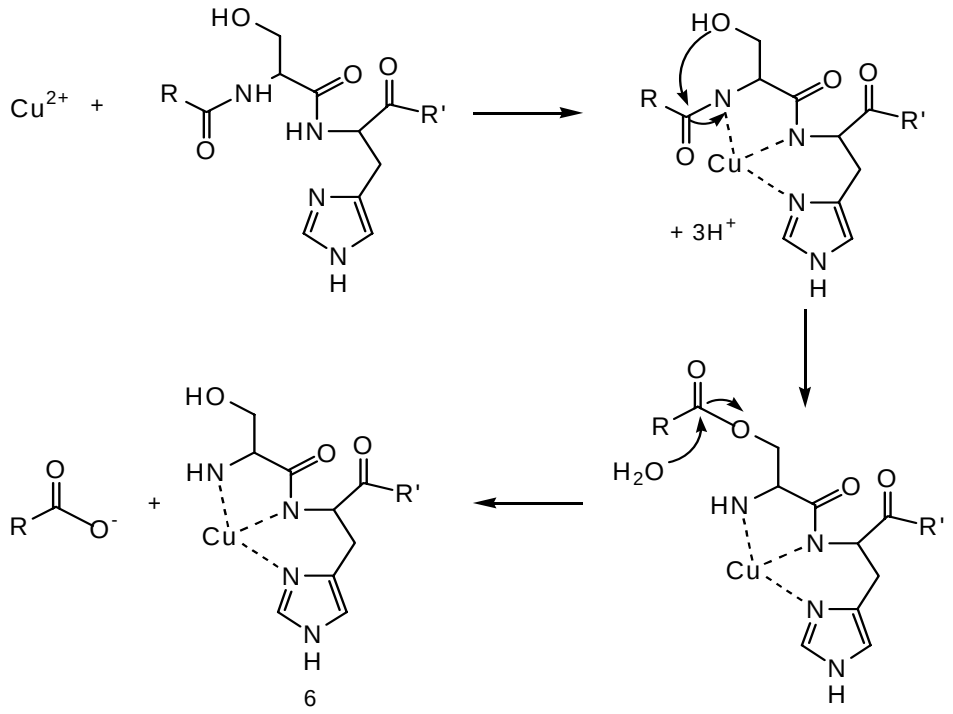


Fig. 1.10. In Cu^{II}–assisted hydrolysis of Xaa-Ser(Thr)-His sequences, the side chain hydroxyl group of Ser(Thr) participates in an N→O acyl rearrangement. Proton transfer from the hydroxyl to the deprotonated, copper-bound amide nitrogen could take place [75,105].

Protein Hydrolysis by Copper(II) Complexes

While Cu^{II} ions promote hydrolysis of amide bonds in peptides and proteins, a variety of ligands have been employed in an effort to enhance the reactivity of this metal, especially at pH values above 4.2 [61,111]. In many cases, the goal has been to design reagents capable of facilitating protein hydrolysis under mild conditions. In 1995, Hegg and Burstyn published the first example in which a small copper complex was utilized to hydrolyze an intact, folded protein. In this study, the macrocycle Cu([9]aneN_3)Cl_2 (**7** in Fig. 1.11) was reacted with bovine serum albumin (BSA) in 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. The Cys243-Cys244 and Gln219-Lys220 bonds in BSA were hydrolyzed in ~ 15% overall yield after 13 days at pH 7.8 and 50 °C [74]. Alternatively, when the bifunctional buffer sodium

bicarbonate was utilized as a substitute for HEPES, the reaction rate was increased dramatically. Burstyn hypothesized that sodium bicarbonate acts as a proton transfer agent that speeds up the rate determining breakdown of the hydrolytic tetrahedral intermediate (Fig. 1.3). Although protein fragments corresponding to cleavage at the Cys243-Cys244 and Gln219-Lys220 bonds were initially generated in the presence of sodium bicarbonate, they were subsequently degraded in a non-sequence specific fashion into a large number of products.

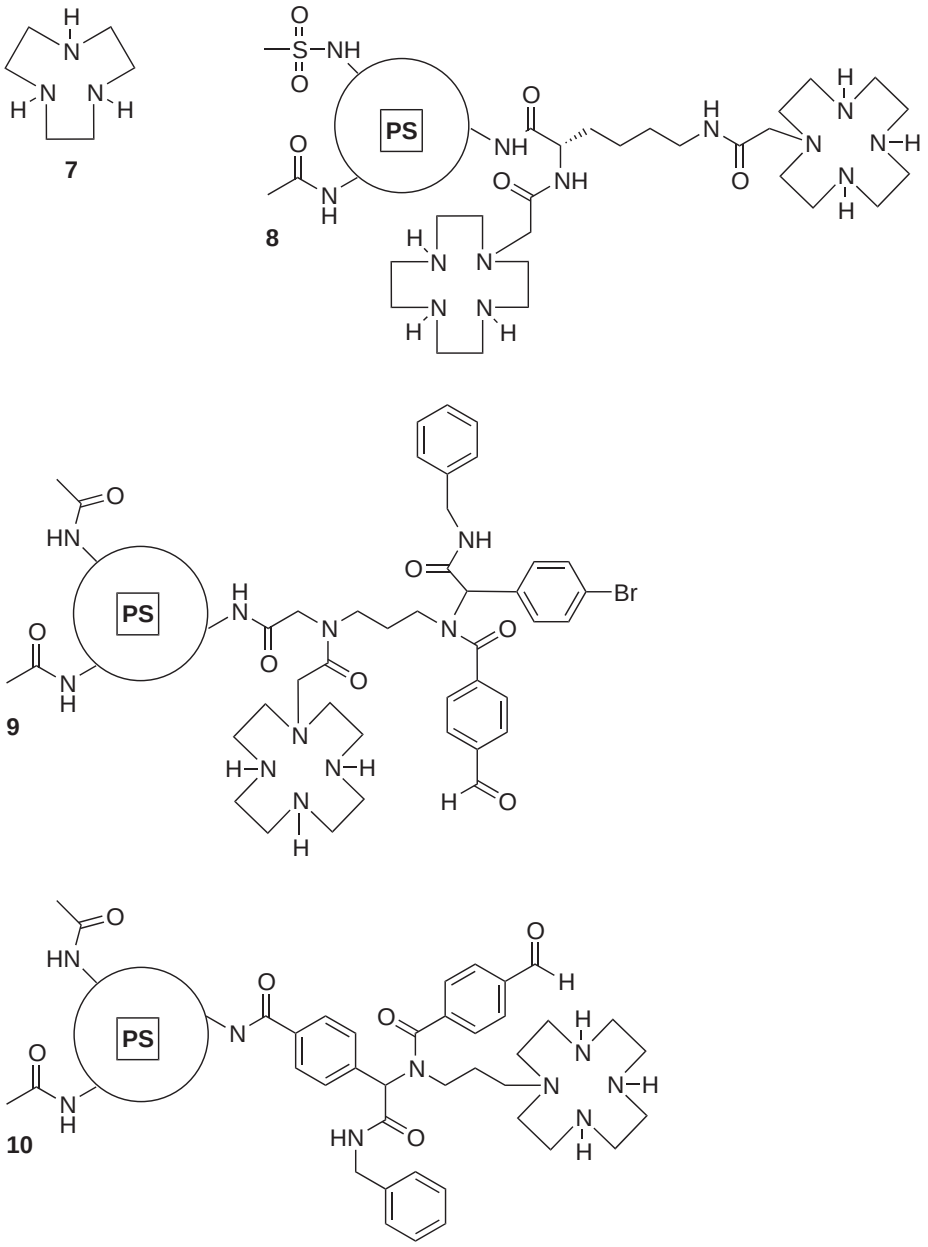


Fig. 1.11. Copper(II) ligands used in the hydrolysis of intact proteins [74.94,98].

In a creative attempt to simulate active sites found within natural metalloproteases, Suh and coworkers developed a design strategy in which Cu^{II} complexes of cyclen are randomly attached to hydrophobic, cross-linked polystyrene (PS) [111]. (Hydrophobic microenvironments within proteins can lead to transition state stabilization by enhancing polar interactions in enzyme-substrate complexes.) As an example, Suh's group synthesized cyclen-polystyrene conjugate **8** shown in Fig. 1.11 [94]. Hydrolysis reactions were conducted in which horse heart myoglobin, bovine serum albumin, and bovine serum γ-globulin were treated with the Cu^{II} complex of **8** in boric acid buffer at 50 °C and pH 9.0 [94]. While bovine serum albumin and γ-globulin were not appreciably degraded, considerable sequence-specific hydrolysis of myoglobin was observed after only 4 h. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and Edman degradation analyses indicated that the primary hydrolysis sites were at Gln91-Ser92 and Ala94-Thr95, the same bonds hydrolyzed by treatment of horse heart myoglobin with $CuCl_2$ [95]. Upon additional incubation in the presence of the Cu^{II} complex of **8**, the myoglobin peptide fragments produced upon initial hydrolysis were further degraded, resulting in the formation of multiple, unidentified products [94]. The overall rate of protein degradation was then determined by monitoring the disappearance of myoglobin electrophoretic bands resolved on SDS-PAGE gels. This analysis showed that the overall degradation of myoglobin by the Cu^{II} complex of **8** is catalytic (k_{cat} = 0.34 h^{-1}) with a $t_{1/2}$ of only 2 h at 50 °C and pH 9.0. When cross-linked polystyrene was not utilized as a reaction scaffold, hydrolysis of myoglobin could not be detected, even after 3 days of treatment with the free Cu^{II} cyclen complex at 50 °C and pH 7.0 to pH 9.0.

In an effort to design a catalyst that could be used to hydrolyze a broad range of protein substrates, Suh and coworkers synthesized two cyclen-polystyrene conjugates (**9** and **10** in Fig.

1.11) in which the central catalytic unit of each is attached to an aldehyde group [98]. Suh reasoned that the aldehyde would increase binding of the two conjugates to various proteins through the formation of reversible imine bonds with the ε-amino groups of surface lysine residues. The Cu^{II} complexes of cyclen-polystyrene conjugates **9** and **10** were then reacted with horse heart myoglobin, bovine serum albumin, human serum albumin, and chicken egg white lysozyme at 50 °C and pH 9.0 to pH 9.5. MALDI-TOF mass spectra revealed that each of the four proteins was hydrolyzed at multiple, initial sites. Of the peptide fragments that were produced, some were stable, but others were rapidly degraded to unidentified products. (In the case of myoglobin, the primary, initial hydrolysis sites were again at Gln91-Ser92 and Ala94-Thr95.) Overall degradation rates were then monitored by quantitating the decrease in intensity of the SDS-PAGE bands of the four protein substrates. These analyses revealed that protein degradation by the Cu^{II} complexes of cyclen-polystyrene conjugates **9** and **10** was extremely rapid: $t_{1/2}$ values were on the order of minutes and k_{cat} values ranged from (6.0 to 18.0) x 10^{-4} s^{-1}. Copper-assisted cleavage of the protein substrates was much slower in control reactions in which a hydrogen atom was used to substitute for the aldehyde groups of **9** and **10**. Suh also showed that cyclen-polystyrene conjugates **9** and **10** could be recovered and then reused to degrade bovine serum albumin. The recovered polymers were able to cleave BSA with nearly identical efficiencies in comparison to the freshly prepared polymers.

Molybdocene Dichloride

The first report of successful molybdenum-promoted peptide hydrolysis was published by Erxleben in 2005 [52]. (Bamann and coworkers had attempted to react Gly-Leu with MoO_4^{2-} at pH 8.6 and 70 °C, but after 72 h, no hydrolysis products could be detected [66].) As an alternative, Erxleben chose the organometallic compound molybdocene dichloride (Cp_2MoCl_2;

Cp = η^5-cyclopentadienyl). The metal center of Cp_2Mo is soft and forms relatively inert complexes with the thiolate side chain of cysteine and other soft ligands. Molybdocene dichloride was reacted under anaerobic conditions with several cysteine containing di- and tripeptides at pH values ranging from 2 to 9 [52]. (In an aqueous environment, the chloride ligands of Cp_2MoCl_2 are rapidly replaced by water, whereas the Cp-Mo bond is resistant to protolysis.) Analyses of ^{1}H NMR spectra revealed that the two dipeptides tested (Cys-Gly and Gly-Cys) formed stable complexes in which the Cp_2Mo^{2+} unit was bound to the thiolate group and to the free amino or free carboxylate group of Cys. Alternatively, the tripeptides glutathione (γ-Glu-Cys-Gly; GSH) and Gly-Cys-Gly reacted with Cp_2MoCl_2 at elevated temperatures to release free glycine. At a pD value of 7.4, hydrolysis of GSH was found to proceed in yields greater than 10% and 40% after ~ 40 h of treatment at 40 °C and 60 °C, respectively. The appearance of free glycine in the ^{1}H NMR spectra was accompanied by the formation of equivalent amounts of a stable dipeptide complex corresponding to the formula $[Cp_2Mo(\gamma\text{-Glu-Cys-}S,O)]^+$ (**11** in Fig. 1.12). Therefore, stoichiometric rather than catalytic amounts of Cp_2MoCl_2 are required to promote hydrolysis. Notwithstanding, observation of complex **11** supports a mechanism in which the anchoring thiolate of Cys facilities intramolecular attack of the Mo-OH nucleophile at an internal amide carbonyl (Fig. 1.12) [52]. In the case of Gly-Cys-Gly, analyses of ^{1}H NMR data indicated that free glycine release at 60 °C and pD values $\geq$ 7.0 was the result of an intramolecular aminolysis reaction. Erxleben proposed that chelation of the Cp_2Mo^{2+} unit to the deprotonated amide nitrogen and thiolate groups of Cys changed the configuration of the Gly-Cys peptide amide bond from *trans* to *cis*. This in turn would be expected to facilitate nucleophilic attack by the primary amino group of the tripeptide at the carbonyl group of the scissile Cys-Gly amide bond [52].

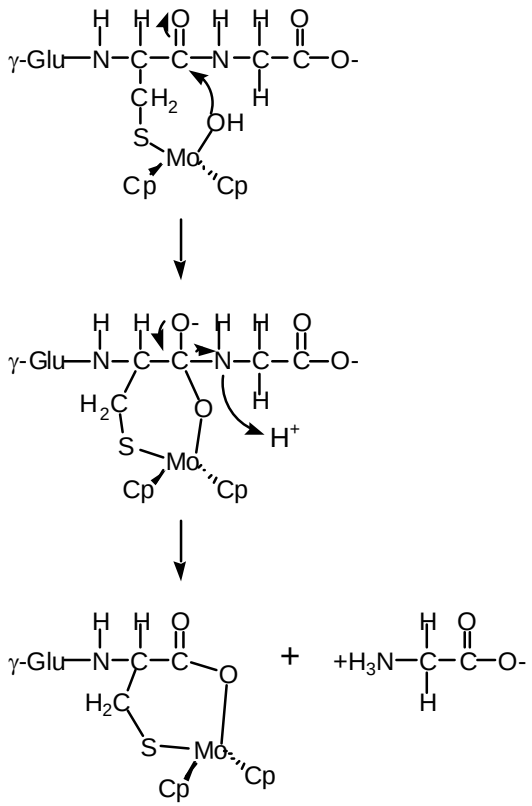


Fig. 1.12. Hydrolysis of GSH by Cp_2MoCl_2 releases free glycine and the dipeptide complex $[Cp_2Mo(\gamma\text{-Glu-Cys-}S,O)]^+$ (**11**) [52]).

Palladium(II) and Platinum(II)

In 1961, Bamann and coworkers reacted Gly-Leu with Pd^{II} and Pt^{II} at pH 8.6 and 70 °C, but after 72 h, no evidence of peptide hydrolysis was observed [66]. Notwithstanding, recent pioneering studies by the research groups of Kostić [41-43,70,71,73,76,77,79,81-83,85,86], Zhu [88], and Sheldrick [51] have proven palladium(II) and platinum(II) complexes to be extremely useful reagents for the sequence-specific hydrolysis of peptides. Although Pt^{II} is substitutionally inert, Pd^{II} is more labile and turnover is possible. In comparison to Ce^{IV}, Cu^{II}, Mo^{IV}, Ni^{II}, Zn^{II}, and Zr^{IV}, significant rate enhancements over background peptide hydrolysis have been reported. The sequence specificity of Pd^{II} and Pt^{II} complexes is derived from metal coordination to the soft

sulfur atom in the side chains of anchoring cysteine and methionine residues [43,70,71,73], and in the case of Pd^{II}, to the borderline imidazole nitrogen atom in the side chain of histidine [76,77]. Because Pt^{II} is a soft Lewis acid, Pt^{II} complexes preferentially hydrolyze peptides containing cysteine and methionine [93]. Alternatively, Pd^{II} is borderline and hydrolyzes peptides containing histidine, cysteine, and methionine [93]. In *N*-acetylated dipeptides of the type AcCysMe(His,Met)-Xaa, palladium(II) complexes are specific for hydrolysis of CysMe-Xaa [73], Met-Xaa [71], and His-Xaa bonds [76,77]. (The *N*-terminal amino groups of peptides containing fewer than four amino acids must be acetylated in order to avoid the formation of hydrolytically inactive chelate complexes in which the *N*-terminal nitrogen and one or more deprotonated amide nitrogens coordinate to palladium [82].) In larger peptides, cleavage by $[Pd(H_2O)_4]^{2+}$, *cis*-$[Pd(en)(H_2O)_2]^{2+}$, and other Pd^{II} complexes occurs at the second amide bond upstream from the anchoring residue (i.e., at the X-Y bonds in X-Y-His-Z and X-Y-Met-Z sequences, where X, Y, and Z are any noncoordinating amino acids) [82,83]. Alternatively, *cis*-$[Pt(en)(H_2O)_2]^{2+}$ hydrolyzes peptide amide bonds immediately downstream from the anchoring amino acid (i.e., the bond Met-Z in X-Y-Met-Z) [86]. With respect to *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and *cis*-$[Pt(en)(H_2O)_2]^{2+}$, the different regioselectivities can be attributed to the relatively labile nature of Pd^{II}. The "en" ligand in the Pd^{II} complex is rapidly replaced by water, whereas it remains coordinated to Pt^{II} throughout the cleavage reaction [86].

Hydrolysis of AcHis-Gly by *cis*-$[Pd(en)(H_2O)_2]^{2+}$ at pH 1.46 and 60 °C is modestly catalytic and proceeds with a half-life of ~ 7 h (k_{obsd} = 0.00166 min^{-1}) [76]. At pH 1.25 and 40 °C, the half-lives for hydrolysis of AcMet-Gly treated with *cis*-$[Pd(en)(H_2O)_2]^{2+}$ and $[Pd(H_2O)_3(OH)]^{+}$ are ~ 32 min (k_{obsd} = 0.022 min^{-1}), and ~ 19 min (k_{obsd} = 0.036 min^{-1}), respectively [73]. For *cis*-$[Pt(en)(H_2O)_2]^{2+}$, the half-live of AcMet-Gly hydrolysis at pH 0.94 and

40 °C is ~ 25 h (k_{obsd} = 0.00046 min^{-1}) [71]. The acidic solutions utilized in these reactions are needed to suppress the formation of oligomeric Pd^{II} complexes with hydroxo bridges [77] and/or the formation of hydrolytically inactive coordination modes involving deprotonated amide nitrogens in the peptide backbone [62,73]. (Palladium(II) lowers the pK_a value of the peptide amide nitrogen from ~ 15 to less than 2 [62].) As a result, hydrolysis yields decrease at higher pH values [71,73,76] and are negligible at near neutral pH. Notwithstanding, Kostić and coworkers have overcome this obstacle by targeting peptides containing X-Pro-Met and X-Pro-His sequences [41]. Proline participates in the formation of tertiary peptide amide bonds that are unable to form hydrolytically inactive complexes with Pd^{II} and other metals. At pH 7.0 and 40 °C, Kostić utilized $[Pd(H_2O)_4]^{2+}$ to hydrolyze a single Gly-Pro bond in the Gly-Pro-His sequence of an *N*-acetylated undecapeptide. The half-life of the reaction was only 4.2 h [41]. To extend the number of sequences selectively cleaved at neutral pH, Kostić synthesized a conjugate consisting of a Pd(II) aqua complex tethered to β-cyclodextrin (**12** in Fig. 1.13) [85]. (Cyclodextrin forms weak complexes with the aromatic side chains of Phe, Trp, and Tyr.) Treatment of the nonapeptide peptide bradykinin with **12** for 48 h at pH 7.0 and 60 °C resulted in selective hydrolysis of the Ser-Pro bond in the sequence Ser-Pro-Phe. Bradykinin (Arg-Pro-Pro-Gly-Phe-**Ser-Pro-Phe**-Arg) contains three X-Pro bonds, only one of which is adjacent to an aromatic amino acid. There was no evidence of hydrolysis at the remaining two proline residues in bradykinin [85].

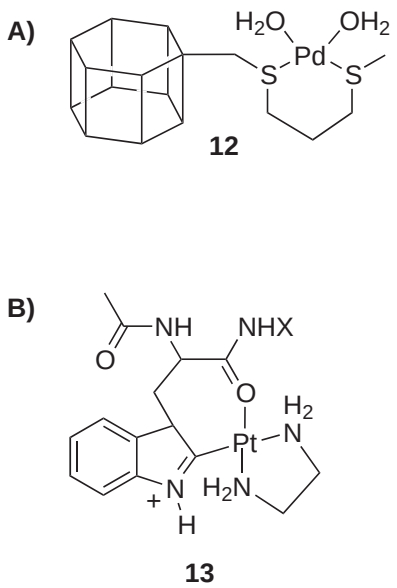


Fig. 1.13. Schematic diagrams of: **A)** a conjugate consisting of β-cyclodextrin and an PdII aqua complex [85]; **B)** a spiro complex formed between AcTrp-Xaa and cis-$[Pt(en)(sol)_2]^{2+}$ in acetone, where NHX represents the C-terminal amino acid [81].

Kostić and coworkers have designed a series of palladium(II) and platinum(II) complexes that are effective in promoting sequence-specific peptide hydrolysis in nonaqueous solutions [79,81]. Their goal was to develop useful tools for structural analyses of problematic, warter-insoluble hydrophobic proteins (e.g., membrane proteins) which are difficult to hydrolyze with endopeptidases. When *N*-acetylated dipeptides corresponding to the sequence AcTrp-Xaa were treated with *cis*-$[Pt(en)(sol)_2]^{2+}$ in acetone, hydrolysis of the Trp-Xaa peptide amide bond was readily achieved upon the addition of one equivalent of water. In the case of AcTrp-Gly, the observed rate constant for hydrolysis (k_{hyd}) at 40 °C was 0.30 min^{-1} [81]. Kostić demonstrated that initial interaction between Pt^{II} and the dipeptide substrate forms a spiro complex that involves bidentate coordination of tryptophan via the C(3) atom of its indole ring and the amide carbonyl oxygen (**13** in Fig. 1.13) [81]. Water inhibits tryptophan coordination, effectively

preventing peptide hydrolysis from occurring in neat, aqueous solutions.

A number of palladium(II) and platinum(II) complexes have been used to hydrolyze intact proteins [82,83,86,89,91-93,96]. For example, bovine serum albumin is cleaved by *trans*-$[Pd(py)_2(H_2O)_2]^{2+}$ at multiple sites including three Xaa-Ser bonds in **Xaa-Ser**-His sequences (in 60% yield at 50 °C, pH 4.5, 8 days [92]). Myoglobin is hydrolyzed by *cis*-$[Pd(dtco\text{-}OH)(H_2O)_2]^{2+}$ at multiple sites including the Xaa-Ser bonds in **Ala57-Ser58**-Glu59 and **Gln91-Ser92**-His93 sequences (in 39% yield at 60 °C, pH 6.2, 24 h [91]). Cytochrome *c* is hydrolyzed at (i) a single His-Thr bond in the presence of *cis*-$[Pd(en)(H_2O)_2]^{2+}$ (in 85% yield at 40 °C, pH 2.0, 2 days [89]), and at (ii) two Met-Xaa bonds in the presence of *cis*-$[Pt(en)(H_2O)_2]^{2+}$ (in 100% yield at 40 °C, pH 2.5, 24 h [93]). In a recent study, Kostić treated *cis*-$[Pt(en)(H_2O)_2]^{2+}$ with cytochrome *c* at pH 2.5. Microwave irradiation was then utilized to maintain the reaction temperature at 60 °C. (Microwave heating is a new method used to accelerate chemical reactions through a combination of thermal and nonthermal microwave effects.) As expected, cytochrome *c* was selectively hydrolyzed at two Met-Xaa bonds, but microwave-assisted heating at 60 °C approximately doubled the hydrolysis rate in comparison to thermal-assisted heating at the same temperature [86].

Concluding Remarks

Our major objective was to present a summary of significant advances that have contributed to the development of metal complexes that hydrolyze unactivated peptides and proteins under mild, near physiological conditions of temperature and pH. In our systematic review of Ce^{IV}, Co^{II}, Co^{III}, Cu^{II}, Mo^{IV}, Ni^{II}, Pd^{II}, Pt^{II}, Zn^{II}, and Zr^{IV}, we have described a number of complexes that produce outstanding rate enhancements over background hydrolysis. Several of these metals were shown to possess one or more of the following attributes: enhanced Lewis

acid strength, oxophilicity, the ability to form complexes with high coordination numbers and flexible ligand geometries, fast-ligand exchange kinetics, and the capacity to lower the pK_a value of coordinated water without producing a significant reduction in the pK_a of the amide nitrogen hydrogen atom. In the case of Cu^{II} and Ni^{II} ions, the formation of highly stable complexes with peptide fragments produced upon hydrolysis was shown to be important. Finally, sequence-specific hydrolysis of peptides and proteins is often achieved by coordination of the metal to anchoring amino acid side chains.

Although tremendous progress has been made in Co^{III}, Cu^{II}, Pd^{II} and in other metal systems, much work remains to be done. New regioselective reagents are needed that efficiently catalyze the hydrolysis of amino acid sequences in diverse sets of folded proteins. We envisage that future experiments will lead to the development of new synthetic metalloproteases. These will serve as versatile biochemical tools for use in a variety of applications ranging from protein structural studies to therapuetics.

ACKNOWLEDGEMENT

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Abbreviations

Ac	=	acetyl
Ala	=	alanine
Arg	=	arginine

Asn	=	asparagine
Asp	=	aspartate
BSA	=	bovine serum albumin
Cp	=	η^5-cyclopentadienyl
cyclen	=	1,4,7,10-tetraazacyclododecane
Cys	=	cysteine
DNA	=	deoxyribonucleic acids
Dtco-OH	=	dithiacyclooctan-3-ol
EDTA	=	ethylenediaminetetraacetate
en	=	ethylenediamine
ESI	=	electrospray ionization
Et	=	ethyl
Gln	=	glutamine
Glu	=	glutamate
Gly	=	glycine
GSH	=	glutathione
half-life	=	$t_{1/2}$
HEPES	=	*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid
His	=	histidine
Ile	=	isoleucine
Leu	=	leucine
Lys	=	lysine
MALDI-TOF	=	matrix-assisted laser desorption/ionization time-of-flight

M	=	metal
Me	=	methyl
Met	=	methionine
MS	=	mass spectrometry
N_4	=	four nitrogen donor atoms
PDF	=	peptide deformylase
Phe	=	phenylalanine
PNA	=	peptide nucleic acid
Pro	=	proline
PS	=	polystyrene
py	=	pyridine
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	serine
sol	=	coordinated solvent
TI	=	tetrahedral intermediate
tren	=	tris(2-aminoethyl)amine
trien	=	triethylenetetramine
Tris	=	tris(hydroxymethyl)aminomethane
Thr	=	threonine
Trp	=	tryptophan
Tyr	=	tyrosine
Val	=	valine
Xaa	=	any amino acid

[9]aneN_3 = 1,4,7-triazacyclononane

References

[1] Rana, T.M.; Meares, C.F. "Specific Cleavage of a Protein by an Attached Iron Chelate." *J. Am. Chem. Soc.*, **1990**, *112,* 2457.

[2] Rana, T.M.; Meares, C.F. "Iron Chelate Mediated Proteolysis-Protein-Structure Dependence." *J. Am. Chem. Soc.*, **1991**, *113,* 1859.

[3] Wu, J.; Perrin, D.M.; Sigman, D.S.; Kaback, H.R. "Helix Packing of Lactose Permease in *Escherichia coli* Studied by Site-Directed Chemical Cleavage." *Proc. Natl. Acad. Sci. USA*, **1995**, *92,* 9186.

[4] Greiner, D.P.; Hughes, K.A.; Gunasekera, A.H.; Meares, C.F. "Binding of the α^{70} Protein to the Core Subunits of *Escherichia coli* RNA Polymerase, Studied by Iron-EDTA Protein Footprinting." *Proc. Natl. Acad. Sci. USA*, **1996**, 93, 71.

[5] Heyduk, T.; Heyduk, E.; Severinov, K.; Tang, H.; Ebright, R.H. "Determinants of RNA Polymerase α Subunit for Interaction with β,βэ, and α Subunits: Hydroxyl-Radical Protein Footprinting." *Proc. Natl. Acad. Sci. USA*, **1996**, *93,* 10162.

[6] Goldshleger R.; Karlish, S.J.D. "Fe-Catalyzed Cleavage of the α Subunit of Na/K-ATPase: Evidence for Conformation-Sensitive Interactions between Cytoplasmic Domains." *Proc. Natl. Acad. Sci. USA*, **1997**, *94,* 9596.

[7] Bar Shimon, M.; Goldshleger, R.; Karlish, S.J.D. "Specific Cu^{2+} -Catalyzed Oxidative Cleavage of Na,K-ATPase at the Extracellular Surface." *J. Biol. Chem.*, **1998**, *273,* 34190.

[8] Colland, F.; Fujita, N.; Ishihama, A.; Kolb, A. "The Interaction between σs, the Stationary Phase σ Factor, and the Core Enzyme of *Escherichia coli* RNA Polymerase." *Genes Cells,*

2002, *7,* 233.

[9] Ermácora, M.R.; Delfino, J.M.; Cuenoud, B.; Schepartz, A.; Fox, R.O. "Conformation-Dependent Cleavage of Staphylococcal Nuclease with a Disulfide-Linked Iron Chelate." *Proc. Natl. Acad. Sci. USA*, **1992**, *89,* 6383.

[10] Mocz, G. "Vanadate-Mediated Photocleavage of Rabbit Skeletal Myosin." *Eur. J. Biochem.*, **1989**, *179,* 373.

[11] Mocz, G.; Gibbons, I.R. "Iron(III)-Mediated Photolysis of Outer Arm Dynein ATPase from Sea Urchin Sperm Flagella." *J. Biol. Chem.,* **1990,** *265,* 2917.

[12] Cremo, C.R.; Loo, J.A.; Edmonds, C.G.; Hatlelid, K.M. "Vanadate Catalyzes Photocleavage of Adenylate Kinase at Proline-17 in the Phosphate-Binding Loop." *Biochemistry*, **1992**, *31,* 491.

[13] Soundar, S.; Coleman, R.F. "Identification of Metal-Isocitrate Binding Site of Pig Heart NADP-Specific Isocitrate Dehydrogenase by Affinity Cleavage of the Enzyme by Fe^{2+}-Isocitrate." *J. Biol. Chem.*, **1993**, *268,* 5264.

[14] Wei, C.-H.; Chou, W.-Y.; Huang, S.-M.; Lin, C.-C.; Chang, G.-G. "Affinity Cleavage at the Putative Metal-Binding Site of Pigeon Liver Malic Enzyme by the Fe^{2+}-Ascorbate System." *Biochemistry*, **1994**, *33,* 7931.

[15] Grammer, J.C.; Loo, J.A.; Edmonds, C.G.; Cremo, C.R.; Yount, R.G. "Chemistry and Mechanism of Vanadate-Promoted Photoxidative Cleavage of Myosin." *Biochemistry*, **1996**, *35,* 15582

[16] Zaychikov, E.; Martin, E.; Denissova, L.; Kozlov, M.; Markovtsov, V.; Kashlev, M.; Heumann, H.; Nikiforov, V.; Goldfarb, A.; Mustaev, A. "Mapping of Catalytic Residues in the RNA Polymerase Active Center." *Science*, **1996**, *273,* 107.

[17] Gallagher, J.; Zelenko, O.; Walts, A.D.; Sigman, D.S. "Protease Activity of 1,10-Phenanthroline-Copper(I). Targeted Scission of the Catalytic Site of Carbonic Anhydrase." *Biochemistry*, **1998**, *37,* 2096.

[18] Grodsky, N. B.; Soundar, S.; Colman, R. F. "Evaluation by Site-Directed Mutagenesis of Aspartic Acid Residues in the Metal Site of Pig Heart NADP-Dependent Isocitrate Dehydrogenase." *Biochemistry,* **2000**, *39,* 2193.

[19] Hlavaty, J. J.; Benner, J. S.; Hornstra, L. J.; Schildkraut, I. "Identification of the Metal-Binding Sites of Restriction Endonucleases by Fe^{2+}-Mediated Oxidative Cleavage." *Biochemistry,* **2000**, *39,* 3097.

[20] Hua, S.; Inesi, G.; Toyoshima, C. "Distinct Topologies of Mono- and Decavanadate Binding and Photo-oxidative Cleavage in the Sarcoplasmic Reticulum ATPase." *J. Biol. Chem.,* **2000,** *275***,** 30546.

[21] Hoyer, D.; Cho, H.; Schultz, P.G. "A New Strategy for Selective Protein Cleavage." *J. Am. Chem. Soc.*, **1990**, *112,* 3249.

[22] Schepartz, A.; Cuenoud, B. "Site-Specific Cleavage of the Protein Calmodulin Using a Trifluoperazine-Based Affinity Probe." *J. Am. Chem. Soc.*, **1990**, *112,* 3247.

[23] Cuenoud, B.; Tarasow, T.M.; Schepartz, A. "A New Strategy for Directed Protein Cleavage." *Tetrahedron Lett.*, **1992**, *33,* 895.

[24] Ettner, N. E.; Metzger, J.W.; Lederer, T.; Hulmes, J.D.; Kisker, C.; Hinrichs, W.; Ellestad, G.A.; Hillen, W. "Proximity Mapping of the Tet Repressor-Tetracycline-Fe^{2+} Complex by Hydrogen Peroxide Mediated Protein Cleavage." *Biochemistry*, **1995**, *34,* 22.

[25] Frank, O.; Schwanbeck, R.; Wisniewski, J.R. "Protein Footprinting Reveals Specific Binding Modes of a High Mobility Group Protein I to DNAs of Different Conformation."

J. Biol. Chem., **1998**, *273,* 20015.

[26] Cheng, X.; Shaltiel, S.; Taylor, S.A. "Mapping Substrate-Induced Conformational Changes in cAMP-Dependent Protein Kinase by Protein Footprinting." *Biochemistry*, **1998**, *37,* 14005.

[27] Baichoo, N.; Heyduk, T. "Mapping Cyclic Nucleotide-Induced Conformational Changes in Cyclic AMP Receptor Protein by a Protein Footprinting Technique Using Different Chemical Proteases." *Protein Sci.*, **1999**, *8,* 518.

[28] Montigny, C.; Jaxel, C.; Shainskaya, A.; Vinh, J.; Labas, V.; Moller, J. V.; Karlish, S. J. D.; le Maire, M. "Fe^{2+}-catalyzed Oxidative Cleavages of Ca^{2+}-ATPase Reveal Novel Features of Its Pumping Mechanism." *J. Biol. Chem.,* **2004**, *279,* 43971.

[29] Loizos, N. "Mapping Protein-Ligand Interactions by Hydroxyl-Radical Protein Footprinting." *Method Mol. Biol.*, **2004**, *261,* 199.

[30] Thomas, J.J.; Bakhtiar, R.; Siuzdak, G. "Mass Spectrometry in Viral Proteomics." *Acc. Chem. Res.*, **2000**, *33,* 179.

[31] Wallace, C.J.A. *Protein Engineering by Semisynthesis*, CRC: Florida, **2000**.

[32] Humphreys, D.P.; Smith, B.J.; King, L.M.; West, S.M.; Reeks, D.G.; Stephens, P.E. "Efficient Site-Specific Removal of a C-terminal FLAG Fusion from a Fab' using Copper(II) Ion Catalysed Protein Cleavage." *Protein Eng.*, **1999**, *12,* 179.

[33] Humphreys, D.P.; King, L.M.; West, S.M.; Chapman, A.P.; Sehdev, M.; Redden, M.W.; Glover D.J.; Smith B. J.; Stephens P.E. "Improved Efficiency of Site-Specific Copper(II) Ion-Catalysed Protein Cleavage Effected by Mutagenesis of Cleavage Site." *Protein Eng.*, **2000**, *13,* 201.

[34] Jeon, J.W.; Son, S.J.; Yoo, C.E.; Hong, I.S.; Suh, J. "Toward Protein-Cleaving Catalytic

Drugs: Artificial Protease Selective for Myoglobin." *Bioorg. Med. Chem.*, **2003**, *11,* 2901.

[35] Kassai, M.; Ravi, R.G.; Shealy, S.J.; Grant, K.B. "Unprecedented Acceleration of Zirconium(IV)-Assisted Peptide Hydrolysis at Neutral pH." *Inorg. Chem.*, **2004**, *43,* 6130.

[36] Chae, P. S.; Kim, M.-S.; Jeung, C.-S.; Lee, S. D.; Park, H.; Lee, S.; Suh, J. "Peptide-Cleaving Catalyst Selective for Peptide Deformylase." *J. Am. Chem. Soc.,* **2005**, *127*, 2396.

[37] Bryant, R.A.; Hansen, D.E. "Direct Measurement of the Uncatalyzed Rate of Hydrolysis of a Peptide Bond." *J. Am. Chem. Soc.*, **1996**, *118,* 5498.

[38] Radzicka, A.; Wolfenden, R. "Rates of Uncatalyzed Peptide-Bond Hydrolysis in Neutral Solution and the Transition-State Affinities of Proteases." *J. Am. Chem. Soc.*, **1996**, *118,* 6105.

[39] Smith, R.; Hansen, D.E. "The pH-Rate Profile for the Hydrolysis of a Peptide Bond." *J. Am. Chem. Soc.*, **1998**, *120*, 8910.

[40] Walker, J.M. *The Protein Protocols Handbook*, Humana Press: New Jersey, **2002**.

[41] Milovic, N.M.; Kostić, N.M. "Palladium(II) Complex as a Sequence-Specific Peptidase: Hydrolytic Cleavage under Mild Conditions of X-Pro Peptide Bonds in X-Pro-Met and X-Pro-His Segments." *J. Am. Chem. Soc.*, **2003**, *125,* 781.

[42] Chen, X.; Zhu, L.; Yan, H.; You, X.; Kostić, N.M. "Kinetic Study of Stereochemical and Other Factors Governing Hydrolytic Cleavage of a Peptide Ligand in Binuclear Palladium(II) Complexes." *J. Chem. Soc. Dalton Trans.*, **1996**, 2653.

[43] Korneeva, E.N.; Ovchinnikov, M.V.; Kostić, N.M. "Peptide Hydrolysis Promoted by Polynuclear and Organometallic Complexes of Palladium(II) and Platinum(II)." *Inorg. Chim. Acta*, **1996**, *243,* 9.

[44] Buranaprapuk, A.; Leach, S.P.; Kumar, C.V.; Bocarsly, J.R. "Protein Cleavage by Transition Metal Complexes Bearing Amino Acid Substituents." *Biochim. Biophys. Acta*, **1998**, *1387,* 309.

[45] Shrivastava, H. Y.; Nair, B. U. "Chromium(III)-Mediated Structural Modification of Glycoprotein: Impact of the Ligand and the Oxidants." *Biochem. Biophys. Res. Commun.,* **2001**, *285,* 915.

[46] Shrivastava, H. Y.; Unni, N. B. "Cleavage of Human Orosomucoid by a Chromium(V) Species: Relevance in Biotoxicity of Chromium." *Biochem. Biophys. Res. Commun.*, **2000**, *279,* 980.

[47] Bentley, K.W.; Creaser, E.H. "Qualitative Determination of *N*-Terminal Amino Acids of Peptides and Proteins with Cobalt(III) Chelates." *Biochem. J.*, **1973**, *135,* 507.

[48] Bal, W.; Lukszo, J.; Bialkowski, K.; Kasprzak, K.S. "Interactions of Nickel(II) with Histones: Interaction of Nickel(II) with CH_3CO-Thr-Glu-Ser-His-His-Lys-NH_2, a Peptide Modeling the Potential Metal Binding Site in the 'C-Tail' Region of Histone H2A." *Chem. Res. Toxicol.*, **1998**, *11,* 1014.

[49] Bal, W.; Liang, R.; Lukszo, J.; Lee, S.-H.; Dizdaroglu, M.; Kasprzak, K.S. "Ni(II) Specifically Cleaves the C-terminal Tail of the Major Variant of Histone H2A and Forms an Oxidative Damage-Mediating Complex with the Cleaved-off Octapeptide." *Chem. Res. Toxicol.*, **2000**, *13,* 616.

[50] Kumar, C.V.; Buranaprapuk, A.; Cho, A.; Chaudhari, A. "Artificial Metallopeptidases: Regioselective Cleavage of Lysozyme." *J. Chem. Soc. Chem. Commun.*, **2000**, 597.

[51] Manka, S.; Becker, F.; Hohage, O.; Sheldrick, W.S. "Cisplatin-Mediated Selective Hydrolytic Cleavage of Methionine-Containing Peptides with Neighboring Serine or

Histidine Residues." *J. Inorg. Biochem.*, **2004**, *98,* 1947.

[52] Erxleben, A. "Interaction of Molybdocene Dichloride with Cysteine-Containing Peptides: Coordination, Regioselective Hydrolysis, and Intramolecular Aminolysis." *Inorg. Chem.*, **2005**, *44,* 1082.

[53] Halliwell, B.; Gutteridge, J.M.C. "The Role of Iron in Radical Reactions." *Methods Enzymol.*, **1984,** *105,* 47.

[54] Johnson, G.R.A.; Nazhat, N.B. "Kinetics and Mechanism of the Reaction of the Bis(1,10-phenanthroline)copper(I) Ion with Hydrogen Peroxide in Aqueous Solution." *J. Am. Chem. Soc.*, **1987**, *109,* 1990.

[55] Masarwa, M.; Cohen, H.; Meyerstein, D.; Hickman, D.L.; Bakac, A.; Espenson, J.H. "Reactions of Low-Valent Transition-Metal Complexes with Hydrogen Peroxide. Are They "Fenton-like" or Not? 1. The Case of Cu^{+} aq and Cr^{2+} aq." *J. Am. Chem. Soc.*, **1988**, *110,* 4293.

[56] Shi, X.; Dalal, N.S. "Vanadate-Mediated Hydroxyl Radical Generation from Superoxide Radical in the Presence of NADH: Haber-Weiss vs Fenton Mechanism." *Arch. Biochem. Biophys*., **1993**, *307,* 336.

[57] Bateman, R.C.; Youngblood, W.W.; Busby, W.H.; Kizer, J.S. "Nonenzymatic Peptide α-Amidation." *J. Biol. Chem.*, **1985**, *260,* 9088.

[58] Platis, I.E.; Ermácora, M.R.; Fox, R.O. "Oxidative Polypeptide Cleavage Mediated by EDTA-Fe Covalently Linked to Cysteine Residues." *Biochemistry*, **1993**, *32,* 12761.

[59] Sayre, L. M. "Metal Ion Catalysis of Amide Hydrolysis." *J. Am. Chem. Soc.,* **1986,** *108,* 1632.

[60] Chin, J. "Developing Artificial Hydrolytic Metalloenzymes by a Unified Mechanistic

Approach." *Acc. Chem. Res.*, **1991**, *24,* 145.

[61] Polzin, G. M.; Burstyn, J. N. "Synthetic Cu(II) and Ni(II) Peptidases." *Metal Ions Biol. Syst.*, **2001,** *38,* 103.

[62] Martin, R. B. "Peptide Bond Characteristics." *Metal Ions Biol. Syst.,* **2001,** *38,* 1.

[63] Meriwether, L.; Westheimer, F.H. "Metal Ion Promoted Hydrolysis of Glycine Amide and of Phenylalanylglycine Amide." *J. Am. Chem. Soc.,* **1956,** *78,* 5119.

[64] Bamann, E.; Rother, A.; Trapmann, H. "Metal-Catalyzed Cleavage of the Peptide Bond." *Naturwiss.,* **1956,** *43,* 326.

[65] Bamann, E.; Trapmann, H.; Rother, A. "The Hydrolysis of Dipeptides in the Presence of Lanthanum, Cerium(III), and Cerium(IV) Ions." *Chem. Ber.*, **1958**, *91,* 1744.

[66] Bamann, E.; Hass, J.G.; Trapmann, H. "Cation-Catalyzed Decomposition of the CONH Group in Peptides and *N*-acetyl Amino Acids." *Arch. Pharm.*, **1961**, *294,* 569.

[67] Grant, I.J.; Hay, R.W. "Copper(II) Catalysis of the Hydrolysis of Glycylglycine." *Aust. J. Chem.*, **1965**, *18,* 1189.

[68] Buckingham, D.A.; Collman, J.P. "The Reaction of the Hydroxoaquobis(ethylenediamine)-cobalt(III) Ion with Amino Acids and Dipeptides and their Esters and Amides." *Inorg. Chem.*, **1967**, *6,* 1803.

[69] Buckingham, D.A.; Collman, J.P.; Happer, D. A. R.; Marzilli, L.G. "Hydrolysis of *N*-Terminal Peptide Bonds and Amino Acid Derivatives by the β-Hydroxoaquotriethylenetetraminecobalt(III) ion." *J. Am. Chem. Soc.,* **1967,** *89,* 1082.

[70] Burgeson, I.E.; Kostić, N.M. "Selective Hydrolysis of Unactivated Peptide Bonds, Promoted by Platinum(II) Complexes Anchored to Amino Acid Side Chains." *Inorg. Chem.*, **1991**, *30,* 4299.

[71] Zhu, L.; Kostić, N.M. "Toward Artificial Metallopeptidases: Mechanisms by which Platinum(II) and Palladium(II) Complexes Promote Selective, Fast Hydrolysis of Unactivated Amide Bonds in Peptides." *Inorg. Chem.*, **1992**, *31,* 3994.

[72] Yashiro, M.; Takarada, T.; Miyama, S.; Komiyama, M. "Cerium(IV)-Cyclodextrin Complex for Peptide Hydrolysis in Neutral Homogeneous Solutions." *J. Chem. Soc. Chem. Commun.*, **1994**, 1757.

[73] Zhu, L.; Kostić, N.M. "Hydrolytic Cleavage of Peptides by Palladium(II) Complexes is Enhanced as Coordination of Peptide Nitrogen to Palladium(II) is Suppressed." *Inorg. Chim. Acta,* **1994**, *217,* 21.

[74] Hegg, E.L.; Burstyn, J.N. "Hydrolysis of Unactivated Peptide Bonds by a Macrocyclic Copper(II) Complex: Cu([9]aneN$_3$)Cl$_2$ Hydrolyzes both Dipeptides and Proteins." *J. Am. Chem. Soc.*, **1995**, *117,* 7015.

[75] Allen, G.; Campbell, R.O. "Specific Cleavage of Histidine-Containing Peptides by Copper(II)." *Int. J. Peptide Protein Res.*, **1996**, *48,* 265.

[76] Parac, T.N.; Kostić, N.M. "New Selectivity and Turnover in Peptide Hydrolysis by Metal Complexes. A Palladium(II) Aqua Complex Catalyzes Cleavage of Peptides Next to the Histidine Residue." *J. Am. Chem. Soc.*, **1996**, *118,* 51.

[77] Parac, T.N.; Kostić, N.M. "Effects of Linkage Isomerism and of Acid-Base Equilibria on Reactivity and Catalytic Turnover in Hydrolytic Cleavage of Histidyl Peptides Coordinated to Palladium(II). Identification of the Active Complex Between Palladium(II) and the Histidyl Residue." *J. Am. Chem. Soc.*, **1996**, *118,* 5946.

[78] Bordignon Luiz, M.T.; Szpoganicz, B.; Rizzoto, M.; Basallote, M.G.; Martell, A.E. “Molecular Recognition of Dipeptides. Catalysis of Deuteration and Hydrolysis of

Glycylglycine by Dinuclear OBISDIEN Zn(II) Complexes." *Inorg. Chim. Acta*, **1999**, *287,* 134.

[79] Kaminskaia, N.V.; Johnson, T.W.; Kostić, N.M. "Regioselective Hydrolysis of Tryptophan-Containing Peptides Promoted by Palladium(II) Complexes." *J. Am. Chem. Soc.*, **1999**, *121,* 8663.

[80] Takarada, T.; Yashiro, M.; Komiyama, M. "Catalytic Hydrolysis of Peptides by Cerium(IV)." *Chem. Eur. J.*, **2000**, *6,* 3906.

[81] Kaminskaia, N.V.; Kostić, N.M. "New Selectivity in Peptide Hydrolysis by Metal Complexes. Platinum(II) Complexes Promote Cleavage of Peptides Next to the Tryptophan Residue." *Inorg. Chem.*, **2001**, *40,* 2368.

[82] Milovic, N.M.; Kostić, N.M. "Interplay of Terminal Amino Group and Coordinating Side Chains in Directing Regioselective Cleavage of Natural Peptides and Proteins with Palladium(II) Complexes." *Inorg. Chem.*, **2002**, *41,* 7053.

[83] Milovic, N.M.; Kostić, N.M. "Palladium(II) Complexes, as Synthetic Peptidases, Regioselectively Cleave the Second Peptide Bond "Upstream" from Methionine and Histidine Side Chains." *J. Am. Chem. Soc.*, **2002**, *124,* 4759.

[84] Yashiro, M.; Sonobe, Y.; Yamamura, A.; Takarada, T.; Komiyama, M.; Fuji, Y. "Metal-Ion-Assisted Hydrolysis of Dipeptides Involving a Serine Residue in a Neutral Aqueous Solution." *Org. Biomol. Chem.*, **2003**, *1,* 629.

[85] Milovic, N.M.; Badjic, J.D.; Kostić, N.M. "Conjugate of Palladium(II) Complex and β-Cyclodextrin Acts as a Biomimetic Peptidase." *J. Am. Chem. Soc.*, **2004**, *126,* 696.

[86] Dutca, L.-M.; Ko, K.-S.; Pohl, N.L.; Kostić, N.M. "Platinum(II) Complex as an Artificial Peptidase: Selective Cleavage of Peptides and a Protein by *cis*-$[Pt(en)(H_2O)_2]^{2+}$ Ion under

Ultraviolet and Microwave Irradiation." *Inorg. Chem.*, **2005**, *44,* 5141.

[87] Mylonas, M.; Krezel, A.; Plakatouras, J.C.; Hadjiliadis, N.; Bal, W. "Interactions of Transition Metal Ions with His-Containing Peptide Models of Histone H2A." *J. Mol. Liq.,* **2005**, *118,* 119.

[88] Yang, G.; Miao, R.; Li, Y.; Hong, J.; Zhao, C.; Guo, Z.; Zhu, L. "Synergic Effect of Two Metal Centers in Catalytic Hydrolysis of Methionine-Containing Peptides Promoted by Dinuclear Palladium(II) Hexaazacyclooctadecane Complex." *J. Chem. Soc. Dalton Trans.*, **2005**, 1613.

[89] Zhu, L.; Qin, L.; Parac, T.N.; Kostić, N.M. "Site-Specific Hydrolytic Cleavage of Cytochrome *c* and of Its Heme Undecapeptide, Promoted by Coordination Complexes of Palladium(II)." *J. Am. Chem. Soc.*, **1994**, *116,* 5218.

[90] Smith, M.A.; Easton, M.; Everett, P.; Lewis, G.; Payne, M.; Riveros-Moreno, V.; Allen, G. "Specific Cleavage of Immunoglobulin G by Copper Ions." *Int. J. Peptide Protein Res.*, **1996**, *48,* 48.

[91] Zhu, L.; Bakhtiar, R.; Kostić, N.M. "Transition Metal Complexes as Alternatives to Proteolytic Enzymes. Regioselective Cleavage of Myoglobin by Palladium(II) Aqua Complexes." *J. Biol. Inorg. Chem.*, **1998**, *3,* 383.

[92] Zhu, L.; Kostić, N.M. "Sequence-Dependent Cleavage of Albumins with Palladium(II) Complexes: Role of Serine Residue in Controlling the High Regioselectivity of Protein Cleavage." *Inorg. Chim. Acta*, **2002**, *339,* 104.

[93] Milovic, N.M.; Dutca, L.-M.; Kostić, N.M. "Transition-Metal Complexes as Enzyme-Like Reagents for Protein Cleavage: Complex *cis*-$[Pt(en)(H_2O)_2]^{2+}$ as a New Methionine-Specific Protease." *Chem. Eur. J.*, **2003**, *9,* 5097.

[94] Yoo, C.E.; Chae, P.S.; Kim, J.E.; Jeong, E.J.; Suh, J. "Degradation of Myoglobin by Polymeric Artificial Metalloproteases Containing Catalytic Modules with Various Catalytic Group Densities: Site Selectivity in Peptide Bond Cleavage." *J. Am. Chem. Soc.*, **2003**, *125,* 14580.

[95] Zhang, L.; Mei, Y.; Zhang, Y.; Li, S.; Sun, X.; Zhu, L. "Regioselective Cleavage of Myoglobin with Copper(II) Compounds at Neutral pH." *Inorg. Chem.*, **2003**, *42,* 492.

[96] Sun, X.; Jin, C.; Mei, Y.; Yang, G.; Guo, Z.; Zhu, L. "Interaction of Palladium(II) and Platinum(II) Complexes with Microperoxidase-11 Studied by Electrospray Mass Spectrometry and MS/MS Analysis." *Inorg. Chem.*, **2004**, *43,* 290.

[97] de Oliveira, M.C.B.; Scarpellini, M.; Neves, A.; Terenzi, H.; Bortoluzzi, A.J.; Szpoganics, B.; Greatti, A.; Mangrich, A.S.; de Souza, E.M.; Fernandez, P.M.; Soares, M.R. "Hydrolytic Protein Cleavage Mediated by Unusual Mononuclear Copper(II) Complexes: X-ray Structures and Solution Studies." *Inorg. Chem.*, **2005**, *44,* 921.

[98] Yoo, S. H.; Lee, B. J.; Kim, H.; Suh, J. "Artificial Metalloprotease with Active Site Comprising Aldehyde Group and Cu(II)Cyclen Complex." *J. Am. Chem. Soc.*, **2005**, *127,* 9593.

[99] Bamann, E.; Trapmann, H. "Metal Ion (Particularly Rare Earth Metal Ion)-Catalyzed Processes. Enzyme and Enzyme Model Catalysis." *Adv. Enzymol.*, **1959**, *21,* 169.

[100] Nakahara, A.; Hamada, K.; Nakao, Y.; Higashiyama, T. "Reactions of Metal Chelates. II. Hydrolytic Cleavage of Peptides by Bis(salicylaldehydato)-Copper(II), -Nickel(II), and -Cobalt(II). *Coord. Chem. Rev.*, **1968**, *3,* 207.

[101] Hay, R.W.; Morris, P.J. "Metal Ion-Promoted Hydrolysis of Amino Acid Esters and Peptides." *Met. Ions Biol. Syst.*, **1976**, *5,* 173.

[102] Sutton, P.A.; Buckingham, D.A. "Cobalt(III)-Promoted Hydrolysis of Amino Acid Esters and Peptides and the Synthesis of Small Peptides." *Acc. Chem. Res.*, **1987**, *20,* 357.

[103] Hegg, E.L.; Burstyn, J.N. "Toward the Development of Metal-Based Synthetic Nucleases and Peptidases: a Rationale and Progress Report in Applying the Principles of Coordination Chemistry." *Coord. Chem. Rev.*, **1998**, *173,* 133.

[104] Bal, W.; Kozlowski, H.; Kasprzak, K.S. "Molecular Models in Nickel Carcinogenesis." *J. Inorg. Biochem.*, **2000**, *79,* 213.

[105] Allen G. "Specific Protein Degradation by Copper(II) Ions." *Met. Ions Biol. Syst.*, **2001**, *38,* 197.

[106] Buckingham, D.A.; Clark, C.R. "Co(III)-Promoted Hydrolysis of Amides and Small Peptides." *Met. Ions Biol. Syst.*, **2001**, *38,* 43.

[107] Komiyama, M. "Lanthanide Ion-Mediated Peptide Hydrolysis." *Met. Ions Biol. Syst.*, **2001**, *38,* 25.

[108] Milovic, N.M.; Kostić, N.M. "Palladium(II) and Platinum(II) Complexes as Synthetic Peptidases." *Met. Ions Biol. Syst.*, **2001**, *38,* 145.

[109] Kasprzak, K.S.; Bal, W.; Karaczyn, A.A. "The Role of Chromatin Damage in Nickel-Induced Carcinogenesis. A Review of Recent Developments." *J. Environ. Monitor.*, **2003**, *5,* 183.

[110] Komiyama, M.; Takarada, T. "Lanthanide-Promoted Peptide Bond Hydrolysis." *Met. Ions in Biol. Syst.*, **2003**, *40,* 355.

[111] Suh, J. "Synthetic Artificial Peptidases and Nucleases Using Macromolecular Catalytic Systems." *Acc. Chem. Res.*, **2003**, *36,* 562.

[112] Komiyama, M.; Kodama, T.; Takeda, N.; Sumaoka, J.; Shiiba, T.; Matsumoto, Y.; Yashiro,

M. "Catalytically Active Species for $CeCl_3$ - Induced DNA Hydrolysis." *J. Biochem.,* **1994,** *115,* 809.

[113] Komiyama, M.; Takeda, N.; Takahashi, Y.; Uchida, H., Shiiba, T.; Kodama, T.; Yashiro, M. "Efficient and Oxygen-Independent Hydrolysis of Single-Stranded DNA by Ce(IV) Ion." *J. Chem. Soc. Perkin Trans.,* **1995,** *2,* 269.

[114] Kajimura, A.; Sumaoka, J.; Komiyama, M. "DNA Hydrolysis by Cerium(IV)-Saccharide Complexes." *Carbohydrate Res.,* **1998,** *309,* 345.

[115] Branum, M. E.; Tipton, A. K.; Zhu, S.; Que, L., Jr. "Double-Strand Hydrolysis of Plasmid DNA by Dicerium Complexes at 37 °C." *J. Am. Chem. Soc.*, **2001,** *123,* 1898.

[116] Berg, J.M.; Shi, Y. "The Galvanization of Biology: a Growing Appreciation for the Roles of Zinc." *Science,* **1996**, 271, 1081.

[117] Buckingham, D.A.; Marzilli, L.G.; Sargeson, A.M. "Peptide Bond Formation and Subsequent Hydrolysis at a Cobalt (3) Center." *J. Am. Chem. Soc.,* **1967,** *89,* 2772.

[118] Moss, R.A.; Zhang, J.; Ragunathan, K.G. "Zirconium and Hafnium Cations Rapidly Cleave Model Phosphodiesters in Acidic Aqueous Solutions." *Tetrahedron Lett.*, **1998**, *39,* 1529.

[119] Ott, R.; Krämer, R. "Rapid Phosphodiester Hydrolysis by Zirconium(IV)." *Angew. Chem. Int. Ed.*, **1998**, *37,* 1957.

[120] Zelder, F.H.; Mokhir, A.A.; Krämer, R. "Sequence Selective Hydrolysis of Linear DNA Using Conjugates of Zr(IV) Complexes and Peptide Nucleic Acids." *Inorg. Chem.*, **2003**, *42,* 8616.

[121] Fay, R.C. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Gillard, R.D., McCleverty, J.A., Eds.; Pergamon: Oxford, **1987**; Vol. *3*, pp. 364, 384-387.

[122] Burgess, J. In *Metal Ions in Solution*, Halsted Press (Wiley): New York, **1978**; pp. 259*f*.

[123] Singhal, A.; Toth, L.M.; Lin, J.S.; Affholter, K. “Zirconium(IV) Tetramer/Octamer Hydrolysis Equilibrium in Aqueous Hydrochloric Acid Solution.” *J. Am. Chem. Soc.*, **1996**, *118,* 11529.

[124] Sigel, H.; Martin, R.B. "Coordinating Properties of the Amide Bond. Stability and Structure of Metal Ion Complexes of Peptides and Related Ligands." *Chem. Rev.*, **1982**, *82,* 385.

[125] Clearfield, A. “The Mechanism of Hydrolytic Polymerization of Zirconyl Solutions.” *J. Mater. Res.*, **1990**, *5,* 161.

[126] Arnaud-Neu, F.; Loufouilou, E.L.; Schwing-Weill, M.J. “Thermodynamic Parameters of the Complexation of Trivalent Lanthanides by Monocyclic and Bicyclic Cryptands and the Tripodal Ligand Tris(3,6-dioxaheptyl)amine in Propylene Carbonate.” *J. Chem. Soc. Dalton Trans.,* **1986**, 2629.

[127] Schneider, H.-J.; Rammo, J.; Hettich, R. “Supramolecular Chemistry. 38. Catalysis of Phosphate Diester Hydrolysis by Lanthanide Ions and the Effect of Ligands.” *Angew. Chem., Int. Ed. Engl.*, **1993**, *32,* 1716.

[128] Lee, L.; Berg, D.J.; Bushnell, G.W. “Cationic Zirconium Dialkyl and Alkyl Complexes Supported by DAC (Deprotonated 4,13-Diaza-18-crown-6) Ligation.” *Organometallics*, **1997**, *16,* 2556.

[129] Mylonas, M.; Plakatouras, J.C.; Hadjiliadis, N. “Interactions of Ni(II) and Cu(II) Ions with the Hydrolysis Products of the C-Terminal -ESHH- Motif of Histone H2A Model Peptides. Association of the Stability of the Complexes Formed with the Cleavage of the -E-S- Bond.” *J. Chem. Soc. Dalton Trans.,* **2004**, 4152.

[130] Karaczyn, A.A.; Bal, W.; North, S.L.; Bare, R.M.; Hoang, V.M.; Fisher, R.J.; Kasprzak, K.S. "The Octapeptidic End of the C-Terminal Tail of Histone H2A is Cleaved Off in Cells Exposed to Carcinogenic Nickel(II)." *Chem. Res. Toxicol.*, **2003**, *16,* 1555.

Epilogue

After publication of the preceding *Current Organic Chemistry* manuscript, several new metal complexes were used to hydrolyze peptide and protein amide bonds [1-5]. The pertinent literature describing these complexes is summarized below.

Palladium(II)

Djuran et al. used the new palladium complexes *cis*-[Pd(dpa)Cl_2] and *cis*-[Pd(dpa)$(H_2O)_2]^{2+}$ (dpa = the bidentate ligand 2,2'-dipyridylamine) to hydrolyze two dipeptides and their *N*-acetylated peptide analogs [1]. The hydrolysis reactions were investigated by ^{1}H NMR spectroscopy. When *cis*-[Pd(dpa)Cl_2] and *cis*-[Pd(dpa)$(H_2O)_2]^{2+}$ were allowed to react with Met-Gly and His-Gly in the pH range 2.0-2.5 at 25 ^{o}C and at 60 ^{o}C, no peptide hydrolysis was observed. Hydrolytically inactive Pd(II)-intermediates were formed in which the Pd(II) ion was bound to the nitrogen atom of the *N*-terminal dipeptide amino group and to the sulfur atom of methionine (in Met-Gly) or to the N3 imidazole nitrogen atom of histidine (in His-Gly). After the formation of each of these Pd(II)-peptide intermediates, the dpa ligand was replaced by two water molecules. The released dpa ligand was then free to react with unreacted *cis*-[Pd(dpa)Cl_2] to form a [Pd(dpa)$_2$]Cl_2·4H_2O complex. The crystal structure of [Pd(dpa)$_2$]Cl_2·4H_2O was confirmed to be square-planar. Because no peptide hydrolysis occurred using Met-Gly and His-Gly, the *N*-acetylated dipeptides AcMet-Gly and AcHis-Gly were reacted with *cis*-[Pd(dpa)$(H_2O)_2]^{+2}$ at 25 and 60 ^{o}C and pH 2.0 - 2.5. It was found that 25% of AcMet-Gly was

hydrolyzed at 60 ^{o}C after 2 h and that a 90% cleavage yield was obtained after 72 h. At 25 ^{o}C, cleavage was 40% after 10 days. However, AcHis-Gly showed no hydrolysis, presumably due to steric hindrance arising from interactions between the two bulky dpa pyridine rings and the imidazole ring in the side chain of histidine.

Iron(III)

Liu et al. successfully used the diiron(III) complex Fe_2(DTPB)-(μ_2-O)(μ_2-OAc)Cl(BF_4)$_2$ [DTPB = 1,1,4,7,7-pentakis(2'-benzimidazol-2-yl-methyl)triazaheptane, OAc = acetate] to promote protein degradation [2]. The five intact, folded proteins hemoglobin (Hb), bovine serum albumin (BSA), lysozyme (Lyso), RNase, and Cu,Zn superoxide dismutase (SOD) were reacted with Fe_2(DTPB)-(μ_2-O)(μ_2-OAc)Cl(BF_4)$_2$ for 48 h at 50 ^{o}C in 20 mM HAc-NaAc buffer pH 5.6 under both aerobic and anaerobic conditions. In both cases, sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis showed progressive degradation of intact Hb, BSA, and SOD as the concentration of Fe_2(DTPB)-(μ_2-O)(μ_2-OAc)Cl(BF_4)$_2$ was raised. There was no relationship between degradation yields and secondary structure content. It was suggested that under the anaerobic conditions, the degradation reactions occurred by a hydrolytic pathway. Then, the five proteins were partially denatured in 1.9 M guanidinium chloride and 0.54% 2-mercaptoethanol and were reacted with Fe_2(DTPB)-(μ_2-O)(μ_2-OAc)Cl(BF_4)$_2$ to see the effects of protein unfolding on degradation patterns. The partly denatured proteins were more easily degraded than the folded proteins. The highest hydrolytic yields occurred in the order Hb > BSA > Lyso > RNase > SOD as the content of α-helices decreased, or as the content of β-sheets or coiled structures increased. Thus, the hydrolysis of these partially denatured proteins was affected by secondary structure content.

Platinum(II) and Copper(II)

Zhu et al. used the following Pt(II) and Cu(II) complexes to hydrolyze peptide amide bonds in oxidized insulin B chain [3]. Oxidized insulin B was allowed to react with *cis*-$[Pt(en)(H_2O)_2]^{2+}$ and with $[CuL(H_2O)]^{2+}$ (en = ethylenediamine, L = 2-[bis(2-aminoethyl)amino]ethanol) at pH 2.5 and 37 ^oC. The reactions were analyzed by LC-electrospray ionization mass spectrometry (LC-ESI-MS) and tandem mass spectrometry (MS/MS). The mass spectral data showed that the two complexes were bound to the *N*-terminal amino group of the peptide as well as to the N3 imidazole atoms of His5 and His10. After 14 days of treatment at pH 2.5 and 40 ^oC in the presence of 5 mol equiv of *cis*-$[Pt(en)(H_2O)_2]^{2+}$, oxidized insulin B chain was hydrolyzed at only one position: His10-Leu11. Platinum(II) complexes are usually specific for cleavage of the Met-X peptide amide bond. Thus, this is the first report in which *cis*-$[Pt(en)(H_2O)_2]^{2+}$ was successfully used to hydrolyze the sequence His-X. Alternatively, when 5 mol equiv of $[CuL(H_2O)]^{2+}$ was reacted with insulin B chain at 40 ^oC and pH 2.5 for 14 days, a total of three amide bonds was hydrolyzed: Gly8-Ser9, Asn3-Gln4, and Phe1-Val2.

Cobalt(III)

Masuda et al. described peptide hydrolysis by ternary Co(III) complexes of $[Co(bcmpa)(dp)]^-$ (bcmpa = the tripodal tetradentate ligand bis-*N*-*N*-carboxymethyl-L-phenylalanine, dp = dipeptide) [4]. Inter-ligand interactions occurred between the *N*-terminal amino hydrogen of the dipeptide and carboxyl oxygen atom of bcmpa and between the α-hydrogen of bcmpa and the amide carbonyl group of the dipeptide. In the case of dipeptides with a *C*-terminal aromatic side chain, a CH-π inter-ligand interaction occurred between the aromatic ring and the α-hydrogen of bcmpa. The Co(III) bcmpa complex was allowed to react with dipeptides containing glycine at the *N*-terminal position. After 3 h of treatment at 40 ^oC and pH 9.0, the yields of the hydrolysis product $[Co(bcmpa)](gly)]^-$ were determined by HPLC. The

dipeptides Gly-Gly and Gly-Ala were hydrolyzed more readily than Gly-Leu and Gly-Val. Thus, dipeptides with a bulky *C*-terminus gave only poor yields and when an aromatic side chain was present (e.g., in Gly-Phe) there was no hydrolysis. Hydrophobic interactions between the benzene ring of bcmpa and the *C*-terminal aromatic side chain of the dipeptide were thought to inhibit the approach of OH^- and prevent peptide cleavage.

Nickel(II)

In experiments conducted by Bal et al., the sequence AcThr-Glu-Thr-His-His-Lys-NH_2 was allowed to react with $Ni(NO_3)_2$ at pH 9.5 and 25 ^oC [5]. The rate of hydrolysis of this peptide was higher in comparison to hydrolysis of the human histone H2A hexapeptide sequence AcThr-Glu-Ser-His-His-Lys-NH_2: the half-life of AcThr-Glu-Thr-His-His-Lys-NH_2 was 29 h and that of its serine analog was 96 h. The pK_a of formation of a four-nitrogen square-planar Ni(II) complex with the threonine peptide was lower by 0.38 log units than that of the serine analog. This was thought to arise from additional shielding of amide nitrogen from water provided by Thr side chain methyl group.

References

1. Živković, M.D.; Rajković, S.; Rychlewska, U.; Warżajtis, B.; Djuran, M.I. "A Study of the Reactions of Methionine- and Histidine-Containing Peptides with Palladium(II) Complexes: The Key Role of Steric Crowding on Palladium(II) in the Selective Cleavage of the Peptide Bond." *Polyhedron*, **2007**, *26*, 1541.
2. Pan, Q.; Jiang, W.; Liao, Z.; Zhang, T.; Liu, C. "Recognition of Secondary Structures in Proteins by a Diiron(III) Complex via a Hydrolytic Pathway." *Inorg. Chem. Commun.*, **2006**, *45*, 490.
3. Hong, J.; Miao, R.; Zhao, C.; Jiang, J.; Tang, H.; Guo, Z.; Zhu, L. "Mass Spectrometry

Assisted Assignments of Binding and Cleavage Sites of Copper(II) and Platinum(II) Complexes Towards Oxidized Insulin B Chain." *J. Mass. Spectrom.*, **2006**, *41*, 1061.

4. Jitukawa, K.; Mabuchi, T.; Einaga, H.; Masuda, H. "Site-Specific Recognition of Dipeptides Through Non-Covalent Inter-Ligand Interactions for the Hydrolysis of Dipeptide to Amino Acid Ligands Mediated by Ternary Cobalt(III) Complexes." *Eur. J. Inorg. Chem.*, **2006**, 4254.

5. Krezel, A.; Mylonas, M.; Kopera, E.; Bal, W. "Sequence-Specific Ni(II)-Dependent Peptide Bond Hydrolysis in a Peptide Containing Threonine and Histidine Residues." *Act. Biochim. Polonica*, **2006**, *53*, 721.

CHAPTER II

Zirconium(IV)-Assisted Hydrolysis of the Dipeptide Glycylglycine and its Blocked Dipeptide Analogs

(This chapter was written by Miki Kassai with assistance from Prof. Kathryn B. Grant. The fluorescamine-based microplate assay was developed by Sowmya Patthabi. Positional scanning experiments were conducted by Dr. Tjaša Bantan-Polak. The blocked dipeptide analogs AcGG, AcGGOMe, and GGOMe were synthesized by Dr. R. Gnana Ravi. All other experiments were performed by Miki Kassai.)

Abstract

Zirconium(IV)-assisted hydrolysis of AcGly-Gly was analyzed using a microplate reader to record the fluorescence produced by the reaction of fluorescamine with amino acids released upon cleavage of the peptide amide bond. Our experiments showed that the highest relative fluorescent intensity values were observed between pH 4.4 and 4.7 and increased as a function of increasing temperature (37 °C to 60 °C) and increasing Zr^{IV} concentration (0.2 mM to 20 mM). At pH values above 5.0, decreased hydrolysis yields were accompanied by increasing levels of Zr^{IV} precipitation. HPLC and electrospray ionization mass spectrometry were then used to identify hydrolysis products. It was apparent that Zr^{IV} cleaved all of the amide bonds and/or ester linkages in Gly-Gly and in the *N*- and/or *C*- blocked peptide analogs AcGly-Gly, AcGly-GlyOMe, and Gly-GlyOMe. We then attempted to enhance the reactivity of Zr^{IV} at physiological pH by addition of the solubilizing reagents α, β, γ-cyclodextrin and Brij 35. However, we were unable to prevent Zr^{IV} precipitation and were unable to increase hydrolysis yields.

Introduction

Early research in our laboratory was focused on creating a positional scanning-based combinatorial method to enable rapid identification and characterization of new metal ions and complexes capable of hydrolyzing the peptide amide bond under non-denaturing conditions of temperature and pH. The first step was to develop and optimize a microplate assay to detect the fluorescence produced upon reaction of fluorescamine with the primary amino groups of amino acids liberated upon metal-assisted hydrolysis of the peptide amide bond [1,2]. Fluorescamine is a well-established fluorophore used for the quantitative determination of amino acids, peptides, and proteins [3-5]. It is intrinsically nonfluorescent, but at room temperature reacts in seconds with primary aliphatic amines to produce highly fluorescence derivatives (λ_{ex} = 381 nm, λ_{em} = 470 nm; Fig. 2.1). Thus, to develop a model system to produce cleaved peptide fragments that could be visualized with fluorescamine, Sowmya Patthabi reacted the acetylated dipeptides AcGly-Gly and AcMet-Gly with $K_2[PdCl_4]$ under conditions that promote sequence-specific peptide hydrolysis adjacent to methionine residues (4 mM peptide, 10 mM Pd^{II}, 100 mM TFA pH 2.1) [1,6]. The peptide solutions were kept at 45 °C for 20 h, after which the palladium was precipitated out by the addition of ethanedithiol. In the control reactions, metal solutions were replaced by water. Aliquots taken at t = 0 h and t = 20 h were then transferred to the wells of a microplate, treated with fluorescamine, and the 8 samples were quantitated in a FLUOstar microplate reader in under 60 s. Our results showed that hydrolysis was specific for AcMet-Gly and was easily detected by the fluorescamine microplate assay with an excellent signal to noise ratio. There was a sharp increase in fluorescence as a function of time in the AcMet-Gly reaction relative to AcGly-Gly and relative to the control reactions conducted in the absence of $K_2[PdCl_4]$.

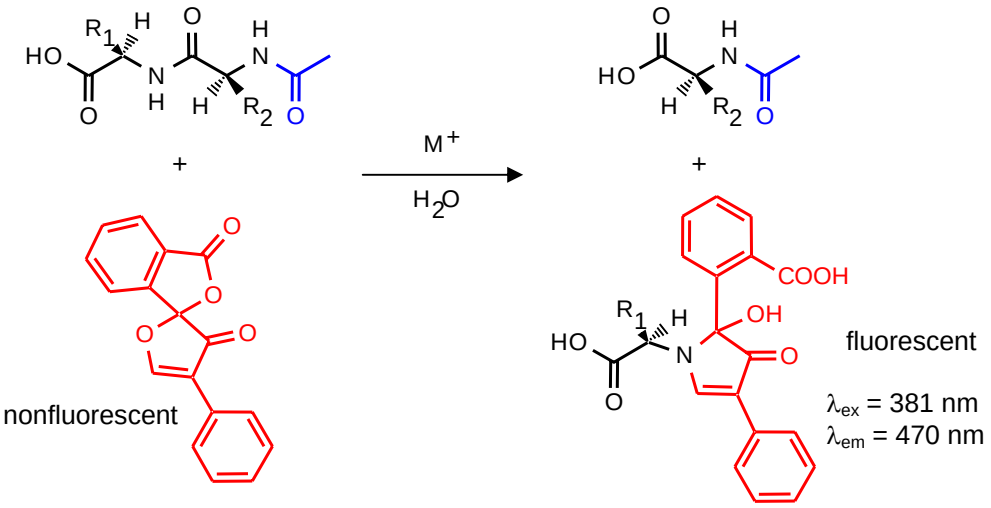


Fig. 2.1. A primary amino group released upon metal-assisted hydrolysis of an acetylated dipeptide reacts with fluorescamine. (R1 and R2 represent amino acid side chains.)

After the microplate assay had been optimized, Dr. Tjaša Bantan-Polak synthesized a spatially addressed positional scanning peptide library containing 121 acetylated dipeptides in a total of 22 library samples [7]. The 22 samples were then reacted with a number of metals including Pd^{II} and Zr^{IV} [7], after which the hydrolyzed peptides were identified by treatment with of the samples with fluorescamine and subsequent quantitation in the microplate reader. Because the samples in the library were spatially addressed, the screening automatically identified the original amino acid sequences of the hydrolyzed peptides within the library [7]. Thus, the fluorescamine microplate assay permitted the rapid detection of peptide cleavage within all positional scanning library samples and rapid screening of numerous metal salts. In the case of Zr^{IV}, preliminary positional scanning data gave encouraging results, indicating that the commercially available salt $ZrCl_4$ was capable of hydrolyzing acetylated dipeptides in the library, with a strong preference for glycine at the first amino acid position (Fig. 2.2). This led to the following study of glycylglycine (GG), the acetylated dipeptide AcGG, and of blocked dipeptide analogs GGOMe and AcGGOMe. The goal of this work was to optimize pH, temperature, and

other reaction conditions with the aim of enhancing yields of zirconium(IV)-assisted peptide hydrolysis under physiologically relevant conditions of temperature and pH.

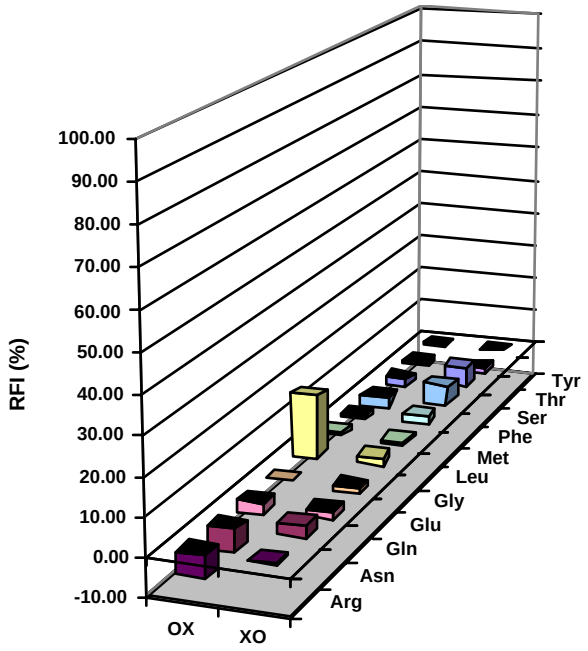


Fig. 2.2. Fluorescamine-based microplate assay: a total of 2 mM of each of 22 individual positional scanning library "samples" was reacted in the presence of 10 mM $ZrCl_4$ for 40 h at pH 7.0 and 60° C. The hydrolysis reactions were transferred to a microplate and treated with fluorescamine. The relative fluorescence intensities shown above were then recorded in a fluorescence microplate reader. The sub-libraries OX and XO were comprised of 11 "samples" that each contain 11 acetylated dipeptides in which position O is fixed and X represents equimolar quantities of the 11 amino acids Arg, Asn, Gly, Glu, Gln, Leu, Met, Phe, Ser, Thr, and Tyr. The total number of acetylated dipeptides in the positional scanning library was 121. To reduce background fluorescence, relative fluorescence intensities at t = 0 h were subtracted from relative fluorescence intensities at t = 40 h.

Material and Methods

All reagents were of the highest available purity. Distilled, deionized water was utilized in the preparation of all buffers. Metal solutions were freshly prepared in all cases. Dimethylaminoazobenzenesulfonyl chloride (dabsyl chloride, DABS-Cl) and fluorescamine were obtained from Molecular Probes. Sodium bicarbonate, sodium phosphate monobasic and dibasic salts, *N*,*N*-dimethylformamide (DMF), glycine (Gly), glycylglycine (Gly-Gly), and α–, β–, and γ-cyclodextrins were purchased from Sigma. HPLC standard glycine methyl ester hydrochloride (H-Gly-OMe•HCl) was obtained from Novabiochem. HPLC grade acetone was

from EMD and Optima grade acetonitrile was from Caledon. Trifluoroacetic acid (TFA) was obtained from Halocarbon, and molecular biology grade ethylenediaminetetraacetic acid disodium salt (EDTA) was from the Eastman Kodak Company. Other chemicals, including zirconium chloride ($ZrCl_4$), Brij 35 and 1,2-ethanedithiol (EDT), were purchased from the Aldrich Chemical Company. Known compounds *N*-acetylglycylglycine (AcGly-Gly), *N*-acetylglycylglycine methyl ester (AcGly-GlyOMe), and glycine methyl ester (Gly-GlyOMe) were synthesized by Dr. R. Gnana Ravi.

Peptide Hydrolysis Reactions

In a typical reaction, a total of 4 μL of acetylated AcGly-Gly (100 mM stock concentration) in deionized water was allowed to react at 60 °C in the absence and presence of $ZrCl_4$. (The reaction pH was pre-adjusted by adding NaOH.) At specific time intervals, 70 μL aliquots of the reaction were quenched at room temperature by a 60 min treatment with EDTA pH 8.0 (50 mM final concentration). In control reactions, $ZrCl_4$ was substituted by equivalent volumes of pH-adjusted water.

Fluorometric Microplate Detection of Peptide Hydrolysis

A total of 4 μL of hydrolysis reaction, 90 μL of 0.1 % (wt/v) fluorescamine dissolved in acetonitrile, 15 μL of 100 mM sodium borate buffer pH 8.0, and 191 μL of water were transferred to the wells of a flat-bottomed microplate (COSTAR 96). The solutions were allowed to react at room temperature for 5 min after which the microplate was read for fluorescence in a FLUOstar microplate reader equipped with a high energy xenon flash lamp (BMG Lab Technologies; 380 nm excitation filter, 520 nm emission filter). To correct for background fluorescence, blanks consisting of water (to substitute for amino acid), buffer, and fluorescamine dissolved in acetonitrile were loaded into microplate wells. In general, relative fluorescence

intensity (RFI) values corresponding to each amino acid were expressed as a percent relative to the amino acid producing the highest fluorescence intensity.

HPLC Analysis of Peptide Hydrolysis Reaction Products

Amino acid products of the Zr^{IV}-assisted peptide hydrolysis reactions were identified by HPLC analysis of their dabsylated derivatives. In a total volume of 120 μL, a 45 μL aliquot of each hydrolysis reaction was treated with 60 μL of 25 mM dabsyl chloride in acetone in the presence of 15 μL of 1 M sodium bicarbonate buffer pH 10. Solutions were allowed to reacte at 70 °C for 15 min, chilled on ice, and diluted with 120 μL of ethanol. Twenty μL of each sample were analyzed on a Beckman System Gold High Performance Liquid Chromatograph (HPLC) system equipped with a Varian MICROSORB-MV™ C_{18} 5 μm, 100 Å, 4.6 x 250 mm reversed-phase column. Using gradient elution (Table 2.1), separations were conducted at 50 °C with a flow rate of 1 mL/min of mobile phase A (2% DMF in 20 mM sodium phosphate buffer pH 6.5), and of mobile phase B (6% DMF in acetonitrile). Dabsylated hydrolysis products were detected by UV absorption at 466 nm and their identities were confirmed by comparison of retention times to corresponding dabsylated amino acid standards. Appropriate dabsylated standards were then included in the reaction mixtures and were shown to enhance HPLC peaks corresponding to hydrolysis products.

Table 2.1: Reversed-phase HPLC gradient elution scheme

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
25	55	45
35	20	80
40	80	20

Results and Discussion

The effects of temperature and pH were studied first. Hydrolysis of 1 mM AcGly-Gly in 5 mM $ZrCl_4$ was carried out at 45 °C and 60 °C in unbuffered solutions adjusted to pH 3.3, 3.8, 5.5, and 7.1 at 25 °C. In control reactions, $ZrCl_4$ was substituted by an equivalent volume of pH adjusted water. Aliquots taken from each sample at t = 0 h, t = 20 h, and t = 40 h were transferred to 48 wells of a 96 well microplate. A final concentration of 50 mM EDTA pH 8.0 was added in order to minimize the influence of zirconium on subsequent derivatization reactions. Following the addition of fluorescamine in acetonitrile, the microplate was quantitated in a fluorescence microplate reader. As can be seen in Fig. 2.3, AcGly-Gly produced significant levels of fluorescence which increased with time and temperature only when Zr^{IV} was present. We hypothesized that this phenomenon might arise from the hydrolytic release of a primary amine group, most likely free glycine. Maximal reactivity occurred at pH values of 3.8 and 5.5, with notable levels of fluorescence under nondenaturing conditions (pH 7.1, 45 °C). To estimate percent conversion, a comparison to a glycine standard indicated that 1 mM AcGly-Gly, 5 mM $ZrCl_4$ at pH 3.8, 60 °C released one mole equivalent of glycine after 40 h (data not shown). In the optimal pH range (3.8 - 5.5), solutions of $ZrCl_4$ are predominated by a soluble cationic polynuclear polyhydroxo species [8]. It is therefore conceivable for hydrolysis to proceed by attack of a zirconium-coordinated hydroxide on the peptide amide carbonyl. Zirconium hydroxide is known to precipitate in aqueous solutions greater than pH 5.0, an observation that is in agreement with our data: we observed a substantial increase in the degree of zirconium(IV) precipitation and a substantial decrease in hydrolysis yields as pH values were raised above the optimal pH range (3.8 - 5.5).

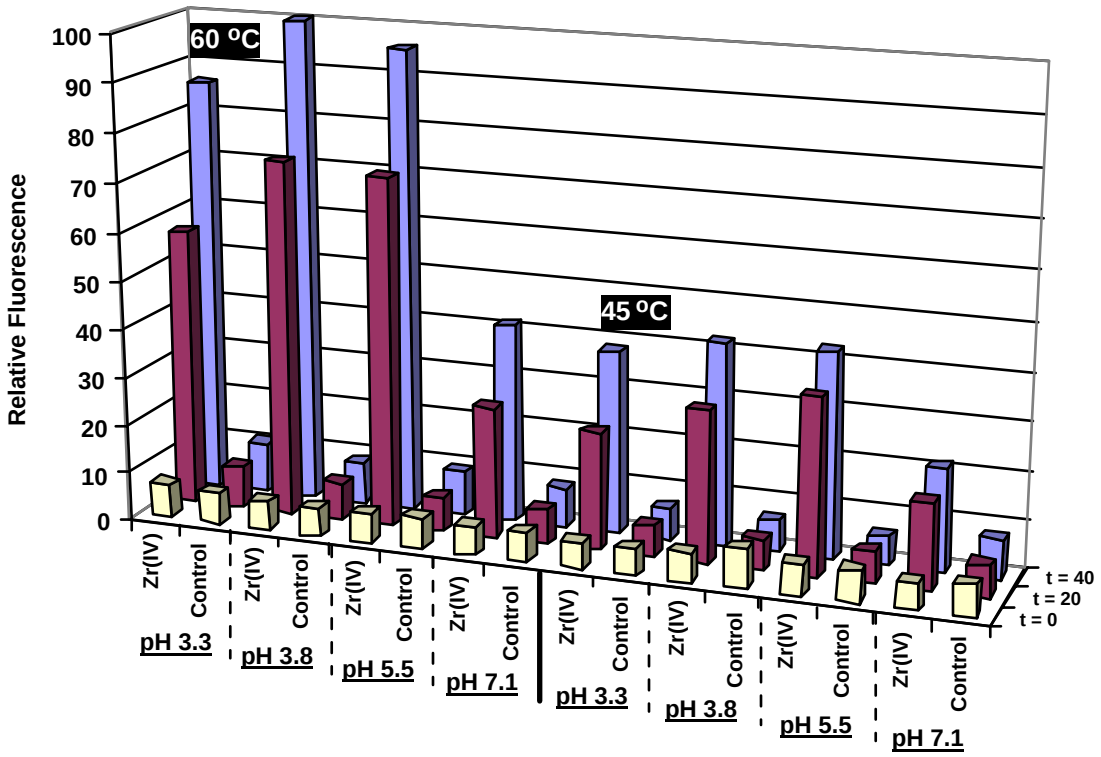


Fig. 2.3. Fluorescamine-based microplate assay: relative fluorescence intensities produced by reaction aliquots taken at t = 0 h, t = 20 h, and t = 40 h in which 1 mM of the acetylated dipeptide AcGly-Gly was treated at 45 °C and at 60 °C in the presence and absence of 5 mM $ZrCl_4$. Parallel reactions in which $ZrCl_4$ was substituted by equivalent volumes of water appear to the immediate right of each $ZrCl_4$ reaction.

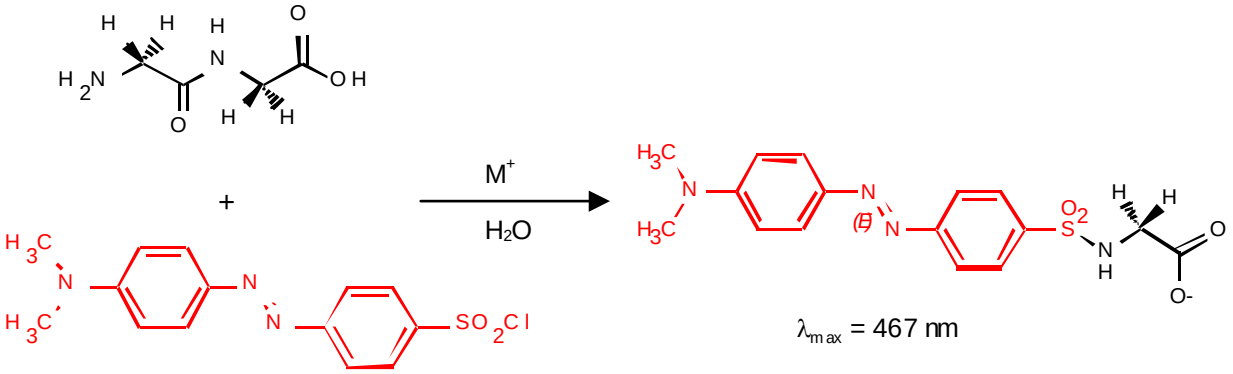


Fig. 2.4. A primary amino group released upon metal-assisted hydrolysis of dipeptide Gly-Gly reacts with dabsyl chloride. The resulting dabsylated hydrolysis product is detected by UV absorption at 466 nm.

The presence of free glycine was subsequently confirmed by reversed-phase HPLC analysis in which peptide hydrolysis products were derivatized with dabsyl chloride (Fig. 2.4).

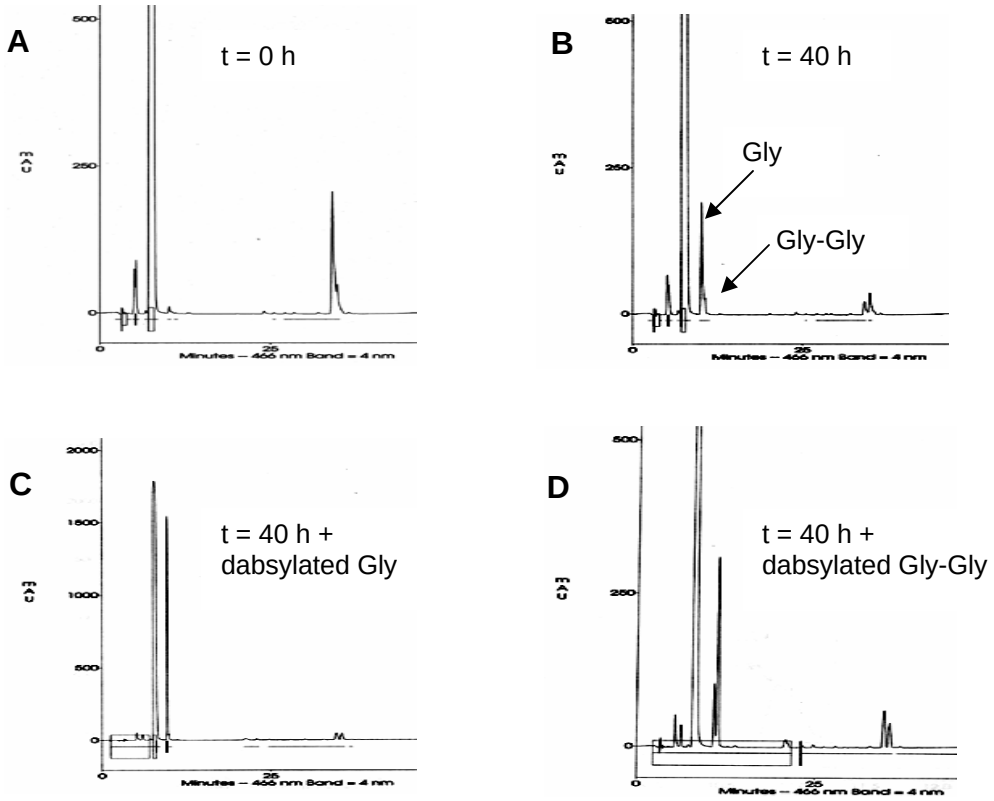


Fig. 2.5. Representative HPLC chromatograms of an acetylated peptide hydrolysis reaction (1 mM AcGly-Gly, 5 mM $ZrCl_4$, 60 °C, pH 5) dabsylated at two time points: **A)** t = 0 h, **B)** t = 40 h. The peaks corresponding to dabsylated hydrolysis product Gly and dabsylated Gly-Gly are identified; **C)** aliquot at t = 40 h is spiked with a dabsylated Gly standard; **D)** aliquot at t = 40 h is spiked with a dabsylated Gly-Gly standard.

For example, in the AcGly-Gly reaction treated with $ZrCl_4$ for 40 h at 60 °C and pH 5, a major, dabsylated peptide hydrolysis product was observed for which the HPLC retention time of a dabsyl derivatized glycine standard was is exact agreement (Fig. 2.5, chromatogram B). In the same reaction, a trace dabsylated hydrolysis product was observed for which the HPLC retention time of a dabsyl derivatized glycylglycine standard was is exact agreement (Fig. 2.5, chromatogram B). To confirm the peak assignments, the dabsylated hydrolysis reaction at t = 40 h (chromatogram B) was spiked with dabsylated glycine to produce chromatogram C and spiked with dabsylated glycylglycine to produce chromatogram D (Fig. 2.5). (There was no peak for

glycine at t = 0 h as seen in Fig. 2.5, chromatogram A.) Thus, in chromatogram C, the major, putative glycine peak has been enhanced upon addition of the dabsylated glycine standard, whereas in chromatogram D, the trace, putative glycylglycine peak has been enhanced by the glycylglycine standard. Therefore, we can conclude that Zr^{IV} has hydrolyzed AcGly-Gly to produce glycine as a major product and glycylglycine as a trace hydrolysis product. To our knowledge, this HPLC data represented the first direct evidence of successful, zirconium-assisted hydrolysis of an acetylated dipeptide.

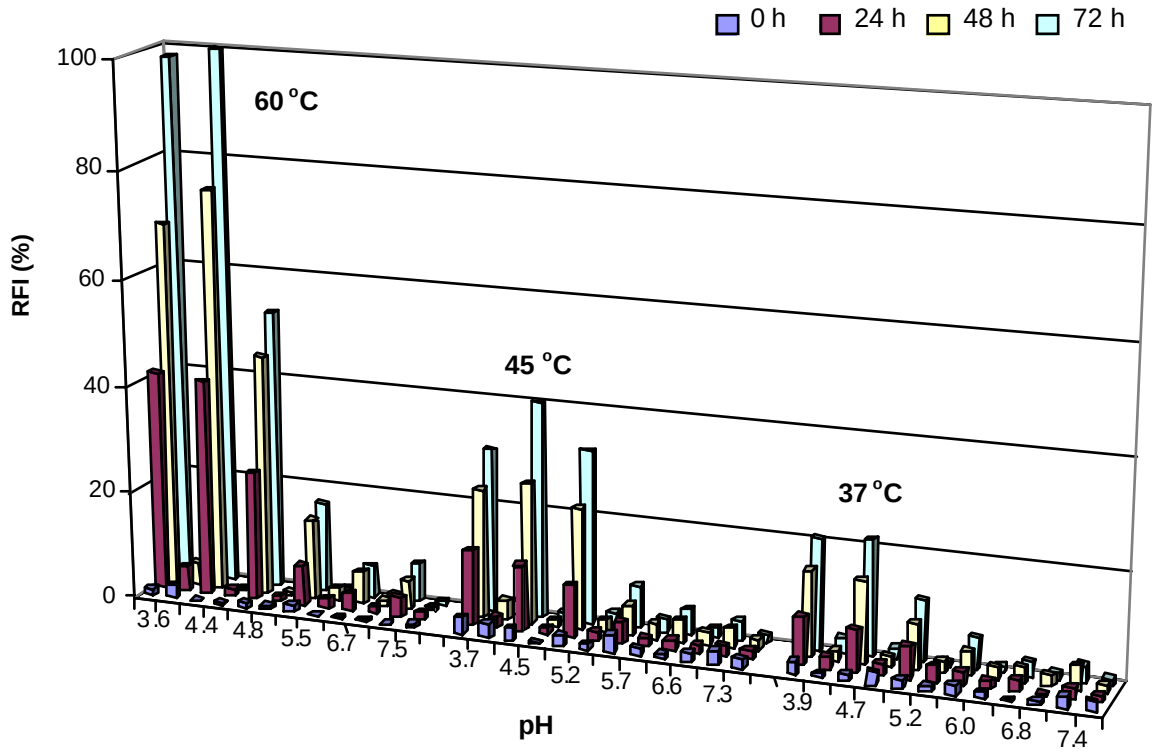


Fig. 2.6. Fluorescamine-based microplate assay: relative fluorescence intensities produced by reaction aliquots taken at t = 0 h, t = 24 h, t = 48 h, and t = 72 h in which 1 mM of the acetylated dipeptide AcGly-Gly was reacted at 37 °C, 45 °C and at 60 °C in the presence and absence of 5 mM $ZrCl_4$. Parallel reactions in which $ZrCl_4$ was substituted for by an equivalent volume of water appear to the immediate right of each $ZrCl_4$ reaction.

The preliminary pH profile conducted above indicated that zirconium(IV) effected maximum levels of amide bond hydrolysis at pH values between 3.8 and 5.5. In order to better

access the versatility of zirconium(IV), we extended our profile to include several additional measurements. Hydrolysis of 1 mM of the acetylated dipeptide AcGly-Gly in 5 mM $ZrCl_4$ was carried out at 37 °C, 45 °C, and 60 °C in unbuffered solutions in which pH values were increased in smaller increments ranging from 3.6 to pH 7.5. (The reported pH values were an average of pre- and post-reaction measurements.) In control reactions, $ZrCl_4$ was substituted by an equivalent volume of pH adjusted water. Aliquots were taken from each sample at 0 h, 24 h, 48 h, and 72 h time points and were transferred to the wells of a microplate. A final concentration of 50 mM EDTA pH 8.0 was then added in order to minimize the influence of zirconium on subsequent derivatization. Following the addition of fluorescamine, a microplate containing the reactions was quantitated using the fluorescence microplate reader. Again, AcGly-Gly produced significant levels of fluorescence which increased with time and temperature only when Zr^{IV} was present (Fig. 2.6). The highest fluorescence intensities were observed at pH values between 4.4 and 4.7 with maximal reactivity occurring at pH 4.4 and 60 ^{o}C. The fluorescence intensity at all temperatures was diminished as the pH was increased above pH 4.4, presumably due to the formation of insoluble Zr^{IV} precipitates: as in our previous pH profile, all of our zirconium(IV) hydrolysis reactions became more and more turbid as reaction pH was raised. The presence of significant amounts free Gly in the zirconium(IV)–assisted peptide hydrolysis reactions was subsequently confirmed by HPLC analysis of dabsylated hydrolysis products. It is important to note that measurable, but low levels of fluorescence were produced at physiological temperature (37 °C) at pH values ranging from 3.9 to 6.0.

In our next experiment, the acetylated dipeptide AcGly-Gly in deionized water reacted with 5, 10, 20 mM $ZrCl_4$ for 0 h, 24 h, and 48 h at 60 °C. The final pH was about 3.5. As can be seen in Fig. 2.7, AcGly-Gly showed an increase in fluorescence as a function of time only when

Zr^{IV} was present. As expected, the relative fluorescence increased as the concentration of $ZrCl_4$ was increased. After 48 h, the RFI produced by 5 mM of Zr^{IV} was about 75%, whereas the RFI was 100% when 20 mM $ZrCl_4$ was reacted with 1 mM AcGly-Gly for 48 h. There was minimum fluorescence observed in the absence of Zr^{IV}.

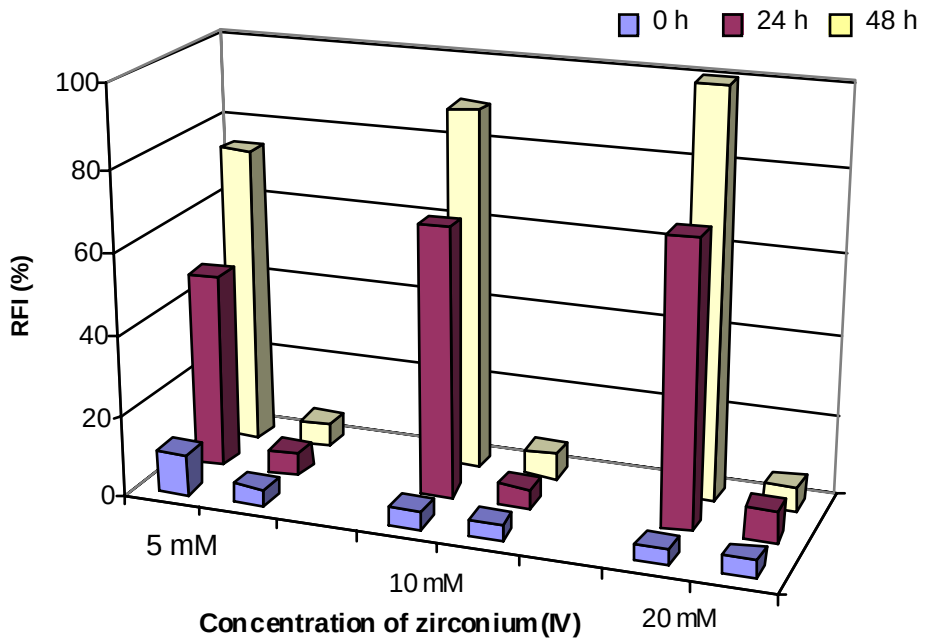


Fig. 2.7. Fluorescamine-based microplate assay: relative fluorescence intensities produced by reaction aliquots taken at t = 0 h, t = 24 h, and t = 48 h, in which 1 mM of the acetylated dipeptide AcGly-Gly was treated at 60 °C in the presence and absence of 5 mM, 10 mM, and 20 mM $ZrCl_4$. Parallel reactions in which $ZrCl_4$ was substituted for by an equivalent volume of water appear to the immediate right of each $ZrCl_4$ reaction.

We further changed peptide to metal ratio by reacting AcGly-Gly (1 mM) with 5, 2, 1, 0.5, and 0.2 mM $ZrCl_4$ at 60 ^{o}C. The final pH was 4.5. According to Fig. 2.8, the results showed that 5 mM Zr^{IV} produced the highest fluorescent intensity. While a peptide to metal ratio of 5:1 (0.2 mM $ZrCl_4$) showed almost no hydrolysis even after 48 h, a time-dependant increase in fluorescence could be detected by using concentrations of Zr^{IV} as low as 0.5 M. (At the peptide to metal ratio of 5:1, it is conceivable that hydrolysis may have been detected if the reaction time had been extended beyond 48 h.)

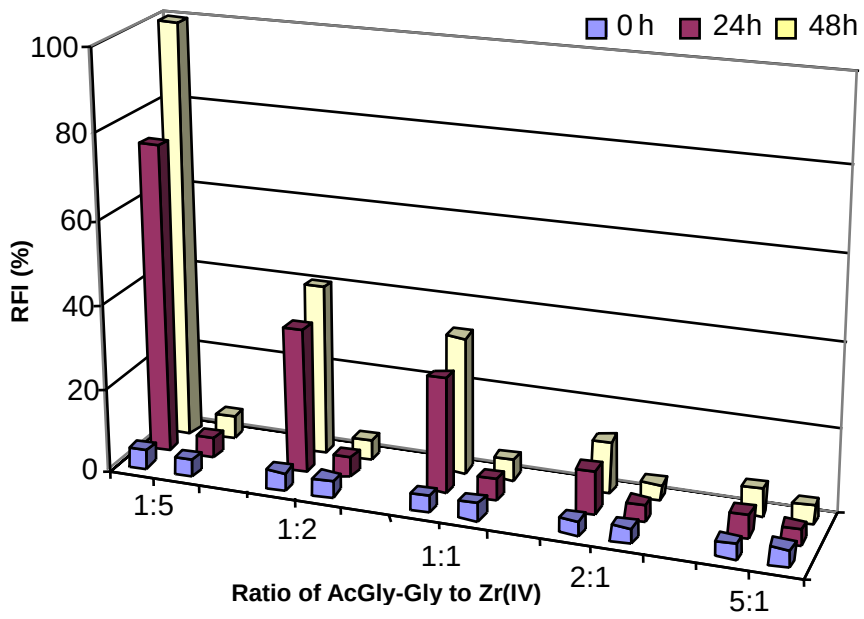


Fig. 2.8. Fluorescamine-based microplate assay: relative fluorescence intensities produced by reaction aliquots taken at t = 0 h, t = 24 h, and t = 48 h, in which 1 mM of the acetylated dipeptide AcGly-Gly was treated at 60 °C in the presence and absence of 5 mM, 2 mM, 1 mM, 0.5 mM and 0.2 mM $ZrCl_4$, respectively. Parallel reactions in which $ZrCl_4$ was substituted for by an equivalent volume of water appear to the immediate right of each $ZrCl_4$ reaction.

Table 2.2: Zirconium(IV)-assisted hydrolysis of dipeptides, and blocked peptide analogs: HPLC analysis of product yields

Peptides (1 mM):		**mM Product:**		
	pH	Gly	X	XX
GG	4.7	1.2	*na*	0.6 **(GG)**
AcGG	4.8	0.4	*na*	0.1 **(GG)**
GGOMe	4.7	*na*	0.7 **(G, GOMe)**	0.4 **(GG, GGOMe)**
AcGGOMe	4.9	*na*	0.6 **(G, GOMe)**	0.2 **(GG, GGOMe)**

Note. All peptides were reacted at a final concentration of 1 mM in the presence of 5 mM $ZrCl_4$ (40 h, 60 °C). Hydrolysis products were derivatized with dabsyl chloride, resolved by HPLC, and identified by spiking with dabsylated amino acid standards. Reaction yields were then estimated by comparing HPLC peak heights to appropriate standard curves. Not determined is indicated by *nd*. Not applicable is indicated by *na*. Identities of products X and XX are indicated in parentheses. Because G and GOMe possess the same HPLC retention time, their yields are reported together. Because GG and GGOMe possess the same HPLC retention time, their yields are reported together.

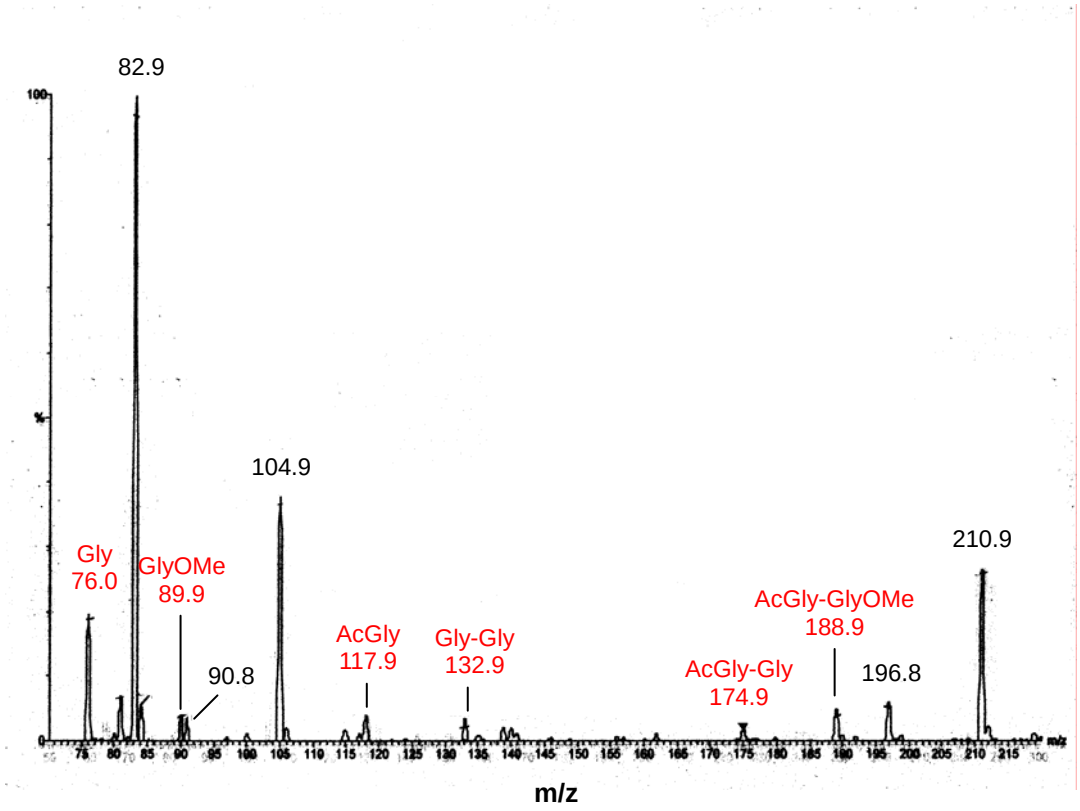


Fig. 2.9. Representative ESI mass spectrum of a zirconium(IV)-assisted peptide hydrolysis reaction (1 mM AcGly-GlyOMe, 5 mM $ZrCl_4$, t = 40 h, pH 4.7, 60 °C).

Our previous experiments were limited to the study of the acetylated dipeptide AcGly-Gly. We therefore conducted additional hydrolysis reactions in which we treated 1 mM of the dipeptide Gly-Gly and 1 mM of the blocked peptide analogs AcGly-Gly, AcGly-GlyOMe, Gly-GlyOMe with 5 mM $ZrCl_4$ (60 °C, 40 h, pH 4.7 to 4.9). Subsequent analyses by HPLC and/or by electrospray ionization (ESI) mass spectrometry demonstrated that all of the amide and ester linkages in Gly-Gly and in the blocked peptide analogs were hydrolyzed by Zr^{IV}. Representative hydrolysis yields based on the total mM of free amino acid and peptide released are shown in Table 2.2. (Essentially no hydrolysis was observed for negative controls in which Zr^{IV} was

substituted by equivalent volumes of water; data not shown.) A representative ESI mass spectrum is in Fig. 2.9.

It is important to note that zirconium(IV) hydrolyzes GG and the peptide analogs AcGG, GGOMe, and AcGGOMe irrespective of the presence of free and blocked *N*-terminal and *C*-terminal groups. This is significant in light of the fact that certain Co^{III} [9] and Ce^{IV} [10,11] complexes require a free *N*-terminal amino group to effect peptide hydrolysis. Furthermore, many Pd^{II} and Pt^{II} complexes have limitations which involve an absolute requirement for a blocked *N*-terminus [12].

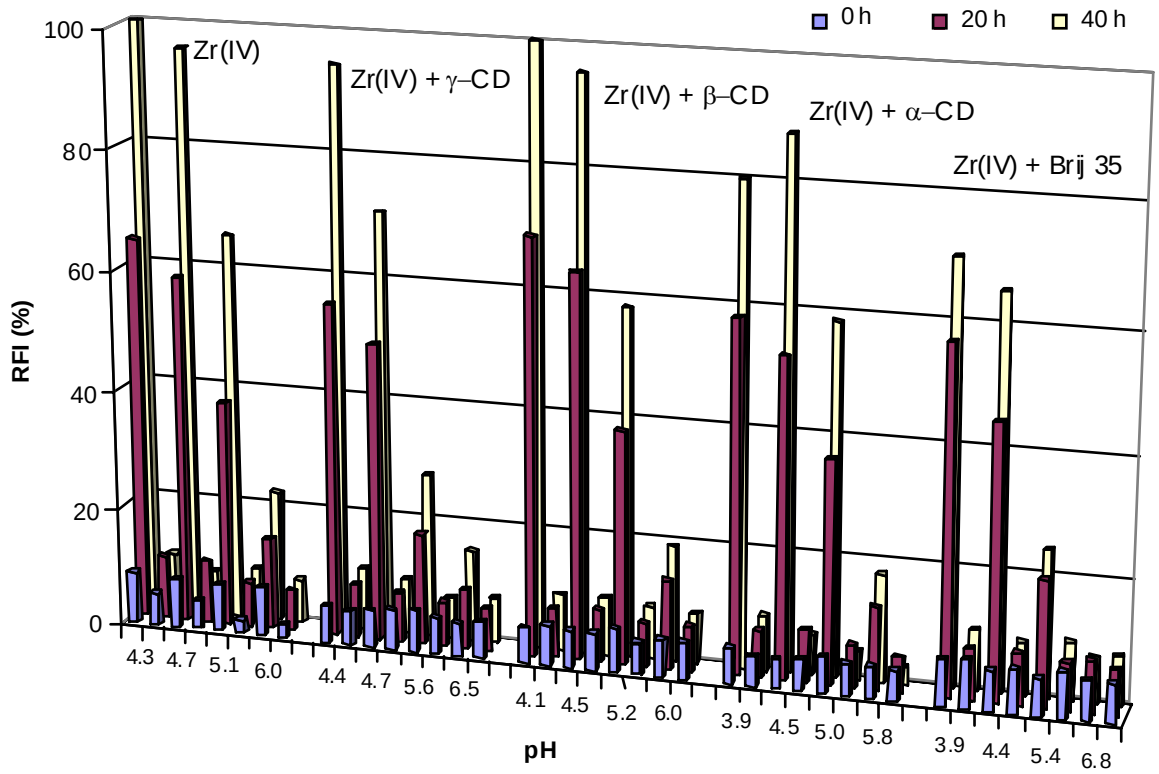


Fig. 2.10. Fluorescamine-based microplate assay: relative fluorescence intensities produced by reaction aliquots taken at t = 0 h, t = 20 h, and t = 40 h in which 1 mM of the acetylated dipeptide AcGly-Gly was treated at 60 °C in the presence and absence of 5 mM $ZrCl_4$ and 0.5 mM of α–, β–, γ-cyclodextrin or of Brij 35. Parallel reactions in which $ZrCl_4$ was substituted for by an equivalent volume of water appear to the immediate right of each $ZrCl_4$ reaction.

In the pH profiles described in this chapter, we observed a significant increase in the degree of zirconium(IV) precipitation as reaction pH was increased. This was accompanied by a significant decrease in peptide hydrolysis yields. Therefore, the goal of our next experiment was to attempt to enhance the reactivity of Zr^{IV} by utilizing Brij micelles as well as α-, β-, and γ-cyclodextrin (CD) as solubilizing reagents. Komiyama had successfully employed γ-cyclodextrin to solubilize Ce^{IV} and was able to achieve homogeneous hydrolysis of the dipeptides Gly-Phe in 39% yield after 24 h at pH 8.0 and 60 ^{o}C [11]. Moss et al. used Brij micelles in thorium(IV) phosphodiester hydrolysis at pH 7.0 and 37 ^{o}C and reported 2.8 billion-fold acceleration in the hydrolysis of bis(*p*-nitrophenyl)phosphate compared to the unanalyzed reaction in the absence of thorium(IV) [13]. Therefore, we reasoned that the use of solubilizing reagents such as α–, β–, and γ-cyclodextrin and Brij 35 might help to dissolve Zr^{IV} precipitates and increase hydrolysis yields at mildly acidic to neutral pH values. We accordingly added 5 mM of α–, β–, and γ-cyclodextrins and of Brij 35 to 50 mM stock solutions of $ZrCl_4$. The dipeptide AcGly-Gly was then reacted in the absence and presence of 5 mM $ZrCl_4$ and 0.5 mM of each solubilizing agent at pH values ranging from 3.9 to 6.8. We expected to see a reduction in precipitation and an improvement in hydrolysis yields at high pH values, especially in the case of γ-cyclodextrin, which should have prevented the precipitation of $Zr(OH)_4$ due to its relatively large radius. However, our results showed zirconium precipitation was unaffected and that no significant improvements in hydrolysis were produced by the solubilizing agents tested (Fig. 2.10).

Conclusion

In summary, we have shown that fluorescamine detects amino acids liberated upon Zr^{IV}-assisted hydrolysis of AcGly-Gly and that Zr^{IV} is capable of hydrolyzing peptides under

relatively mild conditions. A preliminary pH profile conducted at 45 ^{o}C and 60 ^{o}C showed that the highest relative fluorescent intensity values were observed between pH 3.8 and 5.5. When the pH approached 7, the hydrolysis yields were decreased and amounts of zirconium precipitation were increased proportionately. The hydrolysis products were then dabsylated with DABS-Cl and identified by HPLC. This analysis confirmed that AcGly-Gly was hydrolyzed by zirconium(IV) to release glycine and Gly-Gly as major and minor hydrolysis products, respectively. In a second pH profile, we ran zirconium-assisted peptide hydrolysis reactions at 37 ^{o}C, 45 ^{o}C and 60 ^{o}C. While the highest levels of fluorescence were observed at pH values ranging from 4.4 to 4.7, it is important to note that zirconium(IV) was indeed capable of hydrolyzing acetylated peptides at physiological temperature (37 ^{o}C), even though the yields were relatively low. (Our optimal reactions conditions in this experiment were 1 mM acetylated peptide hydrolyzed in the presence of 5 mM $ZrCl_4$ at 60 ^{o}C and pH 4.4 for 48 h.) We then demonstrated that hydrolysis yields could be improved by increasing the concentration of Zr^{IV} from 0.2 mM up to 20 mM. HPLC and electrospray ionization mass spectrometry were utilized to show that all of the peptide amide bonds and/or ester linkages in Gly-Gly and in the *N*- and/or *C*- blocked peptide analogs AcGly-Gly, AcGly-GlyOMe, and Gly-GlyOMe could be hydrolyzed by Zr^{IV}. Thus, it was apparent that Zr^{IV}-assisted peptide hydrolysis was not significantly affected by the presence of free and/or blocked *N*-terminal and *C*-terminal groups. We were also interested in enhancing the reactivity of Zr^{IV} by reducing its precipitation at physiological pH. The solubilizing reagents α, β, γ-cyclodextrin and Brij 35 were used for this purpose, but at near neutral pH, they were unable to prevent the formation of zirconium precipitates and were unable to increase peptide hydrolysis yields.

Abbreviations

AcMet-Gly	=	*N*-acetylmethylglycine
AcGly-Gly	=	*N*-acetylglycylglycine
AcGly-GlyOMe	=	*N*-acetylglycylglycine methyl ester
CD	=	cyclodextrin
DABS-Cl	=	dabsyl chloride, dimethylaminoazobenzenesulfonyl chloride
DMF	=	*N,N*-dimethylformamide
EDT	=	1,2-ethanedithiol
EDTA	=	ethylenediaminetetraacetic acid disodium salt
Gly	=	glycine
Gly-Gly	=	glycylglycine
GlyOMe	=	glycine methyl ester
HPLC	=	high performance liquid chromatography
TFA	=	trifluoroacetic acid
RFI	=	relative fluorescence intensity

References

1. K.B. Grant, S. Pattabhi, Anal. Biochem. 289 (2001) 196-201.
2. T. Bantan-Polak, M. Kassai, K.B. Grant, Anal. Biochem. 297 (2001) 128-136.
3. S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, Science 133 (1972) 871-872.
4. S. Stein, P. Bohlen, J. Stone, W. Dairman, S. Udenfriend, Arch. Biochem. Biophys. 155 (1973) 202-212.
5. A. Lorenzen, S.W. Kennedy, Anal. Biochem. 214 (1993) 346-348.
6. L. Zhu, N.M. Kostić, J. Am. Chem. Soc. 115 (1993) 4566.

7. R.G Ravi, T Bantan-Polak, K.B. Grant, Contributed Lecture, “American Chemical Society 55th Southeast Regional Meeting," Atlanta, November 18, 2003, Abstract 450.

8 R. Ott, R. Krämer, Angew. Chem. Int. Ed. 37 (1998) 1957-1960.

9. D.A. Buckingham, J.P. Collman, Inorg. Chem. 6 (1967) 1803-1807.

10. T. Takarada, M. Yashiro, M. Komiyama, Chem. Eur. J. 6 (2000) 3906-3913.

11. M. Yashiro, T. Takarada, S. Miyama, M. Komiyama, J. Chem. Soc. Chem. Commun. (1994) 1757-1758.

12. L. Zhu, N.M. Kostić, Inorg. Chem. 31 (1992) 3994-4001.

13. R.A. Moss, J. Zhang, K. Bracken, J. Chem. Soc. Chem. Commun. (1997) 1639-1640.

CHAPTER III

Unprcedented Acceleration of Zirconium(IV)-Assisted Peptide Hydrolysis at Neutral pH

(This chapter is verbatim as it appears in Kassai, M.; Ravi, R. G.; Shealy, S. J.; Grant, K. B. *Inorganic Chemistry* **2004**, *43*, 6130-6132. The syntheses of acetylated dipeptides were conducted by Dr. R. Gnana Ravi. Mrs. Sarah S. Capeda conducted the characterization of the Zr(IV)-complex by mass spectroscopy (data not shown). All other experiments were performed by Miki Kassai and the manuscript was written by Prof. Kathryn B. Grant with assistant from Miki Kassai.)

Abstract

4,13-Diaza-18-crown-6 substantially increases the rate of zirconium(IV) hydrolysis of unactivated peptide amide bonds under near physiological conditions of temperature and pH. In the presence of this azacrown ether, $ZrCl_4$ efficiently hydrolyses both neutral and negatively charged peptides (pH 7.0 - 7.3, 37 - 60 ^{o}C).

Introduction

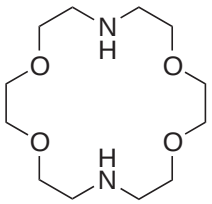
The design and synthesis of metal complexes that hydrolyze peptide amide bonds under nondenaturing conditions of temperature and pH has become an area of intensive study. These reagents show great promise for use in protein bioengineering and protein structural studies, and might one day lead to the development of new and powerful therapeutic agents. Interest has focused on metal ions and/or complexes of Ce^{IV}, Co^{III}, Cu^{II}, Ni^{II}, Pd^{II}, Pt^{II}, and Zn^{II}, which have been used to effect hydrolytic cleavage of unactivated amide bonds in small peptides.[1] In the case of Cu^{II}, Co^{III}, Ni^{II}, and Pd^{II}, intact proteins have also been cleaved.[1f,g,h, 2] Although efficient hydrolysis is sometimes accomplished under physiologically relevant conditions (~ pH 7.0, 37 °C),[1c,h,i, 2b,d] low pH and/or elevated temperatures are often required.[1a,b,d-g,j, 2a,c] There is now a growing need to discover optimal metal ions and complexes that target diverse amino acid

sequences.

The early transition metal zirconium(IV) has enhanced Lewis acid strength imparted by its stable +4 oxidation state, enabling Zr^{IV} ions to efficiently hydrolyze DNA and activated phosphodiesters.[3] It should also be possible for Zr^{IV} to effect efficient hydrolysis of unactivated peptide amide bonds. Because Zr^{IV} is oxophilic and preferentially forms complexes with high coordination numbers,[4] we envisage that this metal center should be capable of coordinating an amide carbonyl oxygen in the peptide backbone (activating the carbon toward nucleophilic attack), while simultaneously delivering a hydroxide nucleophile to the scissile amide bond. (The pKa values of Zr^{IV} bound water molecules are $\leq$ 0.6, and as a result, Zr-OH readily exists in both acidic and neutral media.[5]) The preference of Zr^{IV} for oxygen should avoid hydrolytically inactive peptide amide nitrogen coordination at neutral pH[1a,d] Furthermore, the fast-ligand exchange kinetics characteristic of Zr^{IV} [6] should facilitate catalytic turnover by promoting release of the hydroxide nucleophile at the scissile amide bond and release of coordinated peptide hydrolysis products. Despite the numerous advantages of Zr^{IV}, evidence of efficient peptide hydrolysis by this metal center is lacking.[7]

In aqueous solutions with H^+ concentrations of $\leq$ 0.5 M, Zr^{IV} ions form an octanuclear $[Zr_8(OH)_{20}(H_2O)_{24}]^{12+}$ species,[6] whereas at pH values above 5.0, the production of insoluble gels and precipitates[3b,8] is thought to substantially reduce the efficiency of phosphodiester hydrolysis.[3b] In preliminary experiments, we had demonstrated that Zr^{IV} could hydrolyze the acetylated dipeptide AcGly-Gly between pH 4.4-4.7 (60 °C, 1 mM AcGly-Gly, 5 mM $ZrCl_4$; data not shown). However, as with Zr^{IV}-assisted phosphodiester hydrolysis, we found that peptide hydrolysis yields were significantly diminished at pH values approaching 7.0. To circumvent this difficulty, we employed 4,13-diaza-18-crown-6 (**1**) and are now pleased to report

this reagent dramatically accelerates Zr^{IV}-assisted peptide hydrolysis at pH 7.0 - 7.3 (37 - 60 °C). To the best of our knowledge, we are the first research group to present evidence of efficient Zr^{IV} hydrolysis of unactivated peptide amide bonds.



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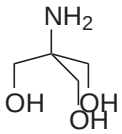

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Results and Discussion

A series of 16 dipeptides was studied first (Table 3.1, entries 1-16). In a total volume of 400 μl, 2 mM of each dipeptide was reacted in either 10 mM $ZrCl_4$, 19 – 22 mM 4,13-diaza-18-crown-6 or 10 mM $ZrCl_4$ in 19 – 22 mM 4,13-diaza-18-crown-6. The pH was adjusted at 25 °C to 7.0 – 7.3 by direct addition of the crown ether,[9] whereas in the absence of the ether, pH was adjusted to 6.9 – 7.4 with NaOH. (Because the pKa_1 of 4,13-diaza-18-crown-6 is 7.94 at 25 ^{o}C,[10] we utilized the azacrown ether to buffer the reaction pH. An expected, pre- and postreaction measurements revealed minimal pH drift; Table 3.1.) After 20 h at 60 °C, each reaction was equilibrated with 1/5 volume of 0.5 M EDTA pH 8 (1 h, at 25 °C). Amino acids released upon peptide amide bond hydrolysis were then derivatized with dimethylaminoazobenzenesulfonyl chloride (dabsyl chloride) and identified and quantitated by reversed-phase HPLC analysis (Supporting Information).

Table 3.1. Extent of $ZrCl_4$–Assisted Peptide Hydrolysis in the Absence and Presence of 4,13-Diaza-18-crown-6 (t = 20 h, 60 °C)[a]

		Zr^{IV}		azacrown ether		Zr^{IV} +azacrown ether		
	peptide	**pH**	**yield (%)**	**pH**	**yield (%)**	**pH**	**yield (%)**	**increase (%)**
1	KG	7.4	12	7.2	0	7.1	17	42
2	GK	7.1	16	7.0	0	7.1	17	6
3	GL	7.0	5	7.2	0	7.2	35	600
4	LG	7.0	3	7.2	1	7.2	54	1700
5	GH	7.3	15	7.2	0	7.1	56	273
6	PG	7.2	7	7.2	1	7.1	63	800
7	HG	7.0	10	7.2	0	7.0	66	560
8	GQ	7.2	6	7.2	0	7.2	68	1033
9	GM	7.2	4	7.3	0	7.3	75	1775
10	MG	7.2	5	7.3	0	7.3	77	1440
11	DG	6.9	19	7.1	0	7.1	85	347
12	GD	7.2	30	7.1	0	7.1	87	190
13	GS	7.2	28	7.2	0	7.0	88	214
14	GG	7.1	26	7.2	1	7.0	90	246
15	SG	7.1	7	7.3	0	7.1	91	1200
16	GE	6.9	10	7.2	0	7.2	97	870
17	GG[b]	7.1	26	6.9	0	7.0	22	-15
18	GG	4.2	42	4.8	0	4.2	65	55
19	AcGGOMe[c]	7.2	1	7.1	0	7.0	26	2500
20	GE[d]	6.7	6	*nd*	*nd*	7.3	39	550
21	GE[e]	7.0	16	7.0	0	7.2	77	381

[a]$[peptide]_0$ = 2 mM, $[ZrCl_4]_0$ = 10 mM, $[4,13\text{-diaza-18-crown-6}]_0$ = 19 - 22 mM. Yield (%) = percent of Gly released. Ether increase (%) = [(yield of Zr^{IV} with 4,13-diaza-18-crown-6 – yield of Zr^{IV})/ yield of Zr^{IV}] x 100. Reported pH values are an average of pre- and postreaction measurements. Average pH drifts were 1.3 $\pm$ 0.4, 0.1 $\pm$ 0.1, and 0.1 $\pm$ 0.2 for the reactions conducted in the presence of $ZrCl_4$, 4,13-diaza-18-crown-6, and $ZrCl_4$ with 4,13-diaza-18-crown-6, respectively (n = 17, pH 6.9 - 7.4, 60 °C). Not determined = *nd*. [b]40 mM tris(hydroxymethyl)aminomethane used to substitute for the ether. [c]Yield (%) = percent of Gly + Gly-OMe released. [d]Reactions are at 37 °C. [e]10 mM $ZrOCl_2{\cdot}8H_2O$ in 7 mM 4,13-diaza-18-crown-6 used to substitute for 10 mM $ZrCl_4$ in 19 - 22 mM 4,13-diaza-18-crown-6.

Wheareas zirconium(IV)-assisted peptide cleavage of the 16 dipeptides was minimal in the absence of 4,13-diaza-18-crown-6, zirconium hydrolysis of all neutral and negatively charged dipeptides was increased by 190 - 17775% upon addition of the azacrown ether (Table 3.1, entries 1 - 16). Amounts of glycine released ranged from 35% for Gly-Leu to 97% for Gly-Glu (entries 3 – 16). Interestingly, both positively charged dipeptides displayed low levels of hydrolysis in the absence and presence of the azacrown ether, perhaps due to unfavorable electrostatic interactions with positively charged Zr^{IV} and/or Zr^{IV}/azacrown ether complex (entries 1 and 2). Overall, the data in Table 3.1 show that Zr^{IV}/4,13-diaza-18-crown-6 shows a

marked preference for efficient hydrolysis of neutral and negatively charged peptides containing glycine and amino acids with oxygen-rich side chains. Essentially no hydrolysis was observed for azacrown ether controls in which $ZrCl_4$ was substituted by equivalent volumes of water (Table 3.1). It is also important to note that insoluble Zr^{IV} precipitates were formed in all $ZrCl_4$ reactions. To our surprise, 4,13-diaza-18-crown-6 did not appear to influence the extent of Zr^{IV} precipitation.

Tris(hydroxymethyl)aminomethane (**2**) reduces the formation of zirconium precipitates at pH > 5 and has been shown to significantly increase $ZrCl_4$ phosphodiester hydrolysis in acidic and neutral solutions[3b,c]. In an attempt to further enhance peptide cleavage yields and decrease precipitation, a reaction was conducted in which 40 mM **2** was used to substitute for the crown ether (60 °C, 2mM Gly-Gly, 10 mM $ZrCl_4$, 20 h). Because the pKa of Tris ligand is 8.1 at 25 ^{o}C, the reaction pH was adjusted to 7.0 by direct addition of **2**.[9] Interestingly, **2** helped to reduce Zr^{IV} precipitation, peptide hydrolysis was slightly decreased under the experimental conditions we employed (Table 3.1, entry 17). This observation led us to speculate that hydrolysis of peptides by Zr^{IV}/4,13-diaza-18crown-6 might have a heterogeneous component similar to peptide hydrolysis by lanthanide hydroxide gels.[1e] (Upon addition of 40 mM EDTA to a typical Zr^{IV}/4,13-diaza-18-crown-6 reaction, we found that Zr^{IV} precipitation was almost completely cleared while hydrolysis was reduced 94%.)

The dipeptide Gly-Gly was then treated with Zr^{IV} at pH 4.2 (60 °C, 2 mM peptide, 10 mM $ZrCl_4$, 20 h). As expected, hydrolysis by Zr^{IV} alone was more efficient at pH 4.2 than pH 7.1 (Table 3.1, entries 14 and 18). However, in the presence of 10 mM Zr^{IV} and 20 mM 4,13-diaza-18-crown-6, hydrolysis was higher at pH 7.1, indicating that the azacrown ether is more effective at near-neutral pH.

Hydrolysis of the blocked peptide analogue AcGly-GlyOMe[11] was studied next (pH 7.0 - 7.2, 60 °C, 2 mM peptide, 10 mM $ZrCl_4$, 20 h). Although levels of cleavage were reduced in comparison to unblocked Gly-Gly, Zr^{IV}-hydrolysis of AcGly-GlyOMe was increased by 2500% in the presence of 19 mM azacrown ether (Table 3.1, entries 14 and 19). The ability of Zr^{IV}/4,13-diaza-18-crown-6 to hydrolyze Gly-Gly irrespective of the presence of free and/or blocked *N*- and *C*-terminal peptide groups is significant in light of the fact that many applications in biochemistry require internal cleavage of peptide amide bonds. In a reaction containing 4mM of the tetrapeptide Ala-Gly-Asp-Val, 20 mM $ZrCl_4$, and 40 mM 4,13-diaza-18-crown-6, 70% free Ala, 71% free Gly, 7% free Asp, and 41% free Val were released, reflecting preferential hydrolysis of both the Ala-Gly and Gly-Asp peptide amide bonds (pH 7.2, 60 °C, 20 h).

To evaluate Zr^{IV} activity under physiologically relevant conditions, the dipeptide Gly-Glu was reacted at pH 7.3, 37 °C (2 mM peptide, 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, 20 h). We are pleased to report that 39% of the dipeptide was hydrolyzed and that the azacrown ether increased levels of Zr^{IV} hydrolysis by 550% (Table 3.1, entry 20). Time course experiments were then conducted at 37 and 60 ^{o}C to monitor reaction kinetics. Products obtained at individual time points were derivatized with dabsyl chloride, hydrolysis yields were determined by subsequent reverse-phase HPLC analysis (Figure 3.1 and Supporting Information). At 37 °C and pH 7.3, the half-life ($t_{1/2}$) for Zr^{IV}/4,13-diaza-18-crown-6 hydrolysis of Gly-Glu was estimated 36.6$\pm$2.7 h. This represents a significant rate enhancement in comparison to the average half-life of ~200 years estimated for spontaneous hydrolysis of unactivated peptide amide bonds under nearly identical conditions (pH 6.8 – 7.0, 37 ^{o}C).[12] At pH 7.1 and 60 ^{o}C, $t_{1/2}$ was 69.3$\pm$5.5 h and 5.3$\pm$0.1 h for Zr^{IV} hydrolysis of Gly-Glu without and with 4,13-diaza-18-crown-6, respectively. (As shown in Figure 3.1, levels of background hydrolysis produced in the absence of $ZrCl_4$ were very

low at both temperatures). To test for catalytic turnover, 10 mM Gly-Glu, 5 mM $ZrCl_4$, and 15 mM of 4,13-diaza-18-crown-6, were reacted at 60 °C, pH 7. Yields of free glycine were 56%, 75%, and 83% after 45 h, 94 h, and 138 h respectively. Because there was 0% glycine at 138 h when Zr^{IV} was omitted, the greater than stoichiometric levels of hydrolysis indicate modest levels of catalytic activity.

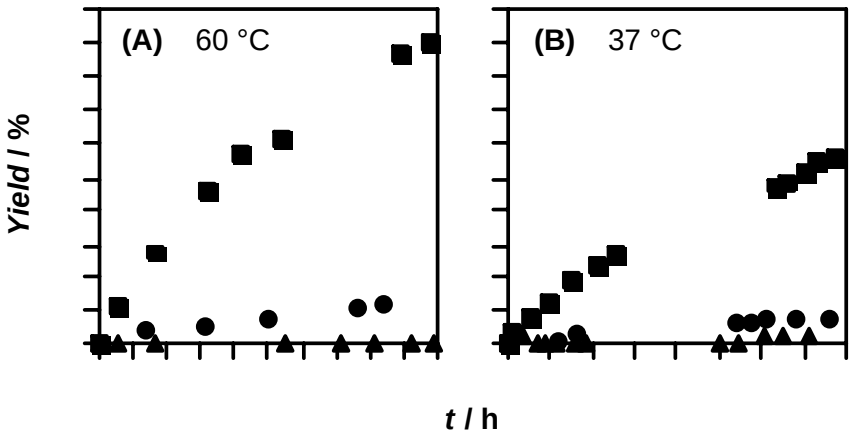

Figure 3.1. Time course plots for hydrolysis of 2 mM Gly-Glu. Percent yield = (mM free Gly released/2 mM) x 100. **a)** 60 °C: (■) 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, pH 7.1; (●) 10 mM $ZrCl_4$, pH 7.1; (▲) 20 mM 4,13-diaza-18-crown-6, pH 7.0. **b)** 37 °C: (■) 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, pH 7.3; (●) 10 mM $ZrCl_4$, pH 7.0; (▲) 20 mM 4,13-diaza-18-crown-6, pH 7.0.

Although 4,13-diaza-18-crown-6 forms stable zirconium(IV) complexes in organic solvents,[13] interactions between Zr^{IV} and the azacrown ether are likely to be exceedingly complicated in aqueous solutions. This is due to the strong propensity of Zr^{IV} to form polynuclear polyhydroxo species, insoluble gels, and precipitates.[6,8] Nevertheless, we employed ^{1}H NMR spectroscopy to obtain preliminary evidence of Zr^{IV}/4,13-diaza-18-crown-6 complex formation in D_2O. Spectra of the azacrown ether were recorded without and with 1 equiv of the $ZrCl_4$ (pH 7, 23.5 ^{o}C). In the presence of Zr^{IV}, all of the ^{1}H NMR azacrown ether resonances were shifted with respect to those of the free ligand, a feature that can be indicative of metal binding (Supporting Information).

The formation of polynuclear polyhydroxo species by $ZrCl_4$ involves the production of excess HCl.[14] To circumvent this complication, we used 10 mM $ZrCl_2 \bullet H_2O$ to substitute for 10 mM $ZrCl_4$ and reacted the oxide chloride with 2 mMGly-Glu in the absence and presence of 4,13-diaza-18-crown-6 (pH 7.0 – 7.2, 60 ^oC, 20 h). Hydrolysis yields were 16% and 77%, respectively (Table 3.1, entry 21). In addition, only 7mM 4,13-diaza-18-crown-6 was required to achieve a final pH of 7.2, indicating that $ZrCl_2 \bullet H_2O$ likely avoids excess HCl production.

In summary, 4,13-diaza-18-crown-6 dramatically enhances the rate of zirconium-assisted peptide hydrolysis in neutral solutions (pH 7.0 - 7.3; 37 - 60 °C). We found that Zr^{IV}/4,13-diaza-18-crown-6 displays a preference for cleavage of neutral and negatively charged peptides containing glycine and amino acids with oxygen-rich side chains. The reaction is catalytic and does not require the presence of free and/or blocked *N*- and *C*-terminal groups. To our knowledge, we are the first research group to present evidence of efficient Zr^{IV} hydrolysis of unactivated peptide amide bonds. Our future work will focus on additional mechanistic studies and on the design of crown ether derivatives that will impart additional Lewis acidity to the Zr^{IV} metal center. We envisage that Zr^{IV} complexes might one day represent promising reagents for use in a variety of biochemical applications.

Acknowledgement

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Supporting Information Available: additional experimental details and data concerning the identification and quantitation of peptide hydrolysis products, HPLC analysis of reaction kinetics,

and NMR analysis of complex formation. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Grant, I. J.; Hay, R. W. *Aust. J. Chem.* **1965**, *18*, 1189. (b) Buckingham, D. A.; Collman, J. P. *Inorg. Chem.* **1967**, *6*, 1803. (c) Bentley, K. W.; Creaser, E. H. *Biochem. J.* **1973**, *135*, 507. (d) Zhu, L.; Kostić, N. M. *Inorg. Chem.* **1992**, *31*, 3994. (e) Yashiro, M.; Takarada, T.; Miyama, S.; Komiyama, M. *Chem. Commun.* **1994**, 1757. (f) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015. (g) Allen, G.; Campbell, R. O. *Int. J. Peptide Protein Res.* **1996**, *48*, 265. (h) Bal, W.; Liang, R.; Lukszo, J.; Lee, S.-H.; Dizdaroglu, M.; Kasprzak, K. S. *Chem. Res. Toxicol.* **2000**, *13*, 616. (i) Milovic, N. M.; Kostić, N. M. *J. Am. Chem. Soc.* **2003**, *125*, 781. (j) Yashiro, M.; Sonobe, Y.; Yamamura, A.; Takarada, T.; Komiyama, M.; Fuji, Y. *Org. Biomol. Chem.* **2003**, *1*, 629. (k) Milovic, N. M.; Badjic, J. D.; Kostić, N. M. *J. Am. Chem. Soc.* **2004**, *126*, 696.

(2) (a) Smith, M. A.; Easton, M.; Everett, P.; Lewis, G.; Payne, M.; Riveros-Moreno, V.; Allen, G. *Int. J. Peptide Protein Res.* **1996**, *48*, 48. (b) Kumar, C. V.; Buranaprapuk, A.; Cho, A.; Chaudhari, A. *Chem. Commun.* **2000**, 597. (c) Zhu, L.; Kostić, N. M. *Inorg. Chim. Acta* **2002**, *339*, 104. (d) Jeon, J. W.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Suh, J. *Bioorg. Med. Chem.* **2003**, *11*, 2901.

(3) (a) Moss, R. A.; Zhang, J.; Ragunathan, K. G. *Tetrahedron Lett.* **1998**, *39*, 1529. (b) Ott, R.; Krämer, R. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1957. (c) Zelder, F. H.; Mokhir, A. A.; Krämer, R. *Inorg. Chem.* **2003**, *42*, 8616.

(4) Fay, R. C. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Gillard, R. D., McCleverty, J. A., Eds.; Pergamon Press: Oxford, U.K., 1987; Vol. 3, p 364.

(5) Burgess, J. *Metal Ions in Solution;* Halsted Press (Wiley): New York, 1978; p 259.

(6) Singhal, A.; Toth, L. M.; Lin, J. S.; Affholter, K. *J. Am. Chem. Soc.* **1996**, *118*, 11529.

(7) In a paper focusing on Ce^{IV}, Takarada et al. reported that Zr^{IV} effects low levels of peptide hydrolysis (~25% conversion of Gly-Phe after 24 h at 80 °C, pH 7.0: Takarada, T.; Yashiro, M.; Komiyama, M. *Chem. Eur. J.* **2000**, *6*, 3906).

(8) Clearfield, A. *J. Mater. Res.* **1990**, *5*, 161.

(9) To minimize the formation of zirconium precipitates, reaction pH was adjusted in the absence of NaOH.

(10) Dantz, D. A.; Buschmann, H.-J.; Schollmeyer, E. *Thermochim. Acta* **1997**, *294*, 133.

(11) Grassmann, W.; Hormann, H.; Endres, H. *Chem. Ber.* **1953**, *86*, 1477.

(12) (a) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105. (b) Smith, R.; Hansen, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 8910.

(13) Lee, L.; Berg, D. J.; Bushnell, G. W. *Organometallics* **1997**, *16*, 2556.

(14) Cotton, F. A.; Wilkinson, G.; Murillo, C. A.; Bochmann, M. *Advanced Inorganic Chemistry*, 6th ed.; John Wiley and Sons: New York, 1999; p 880.

Supporting Information

Identification and quantitation of peptide hydrolysis products:

Upon the addition of EDTA to peptide reactions, Zr^{IV} was chelated and the hydrolysis products free arginine, aspartate, glutamine, glutamate, glycine, histidine, leucine, lysine, proline, methionine, and serine could then be derivatized with a chromophore suitable for HPLC analysis. In a typical experiment, a total of 45 μL of each peptide hydrolysis reaction in 15 μL of 1 M

sodium bicarbonate buffer pH 9.9 was reacted with 60 μL of 24 mM dabsyl chloride in acetone (70 °C for 15 min).[1] The solution was cooled on ice, after which 120 μL of ethanol were added. Twenty μL of the sample were then analyzed on a Beckman System Gold HPLC system equipped with a Varian MICROSORB-MV™ C_{18} 5 μm, 100 Å, 4.6 x 250 mm reversed-phase column using the gradient elution scheme shown in Table 3S1. Dabsylated amino acid hydrolysis products were then detected by UV absorption at 466 nm (Figure 3.S1). In each case, the reversed phase HPLC retention times of the derivatized hydrolysis products were in exact agreement with corresponding dabsyl derivatized amino acid standards. In addition, the dabsylated standards were added to the Zr^{IV} reaction mixtures and were shown to enhance HPLC peaks corresponding to the free amino acids and peptides (Figure 3.S1). Hydrolysis yields were then estimated based on the percent of free glycine released in each reaction. (The free glycine was quantitated by comparing the HPLC peak height of the dabsylated glycine hydrolysis product to a titration curve of a dabsylated glycine standard.)

Table 3.S1. Reversed-phase HPLC gradient elution scheme.[a]

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	80	20
25	55	45
35	20	80
40	80	20

[a]Separations were conducted at 50 °C with a flow rate of 1 mL/min of Mobile Phase A (2% DMF in 20 mM sodium phosphate buffer pH 6.5), and Mobile Phase B (6% DMF in CH_3CN).

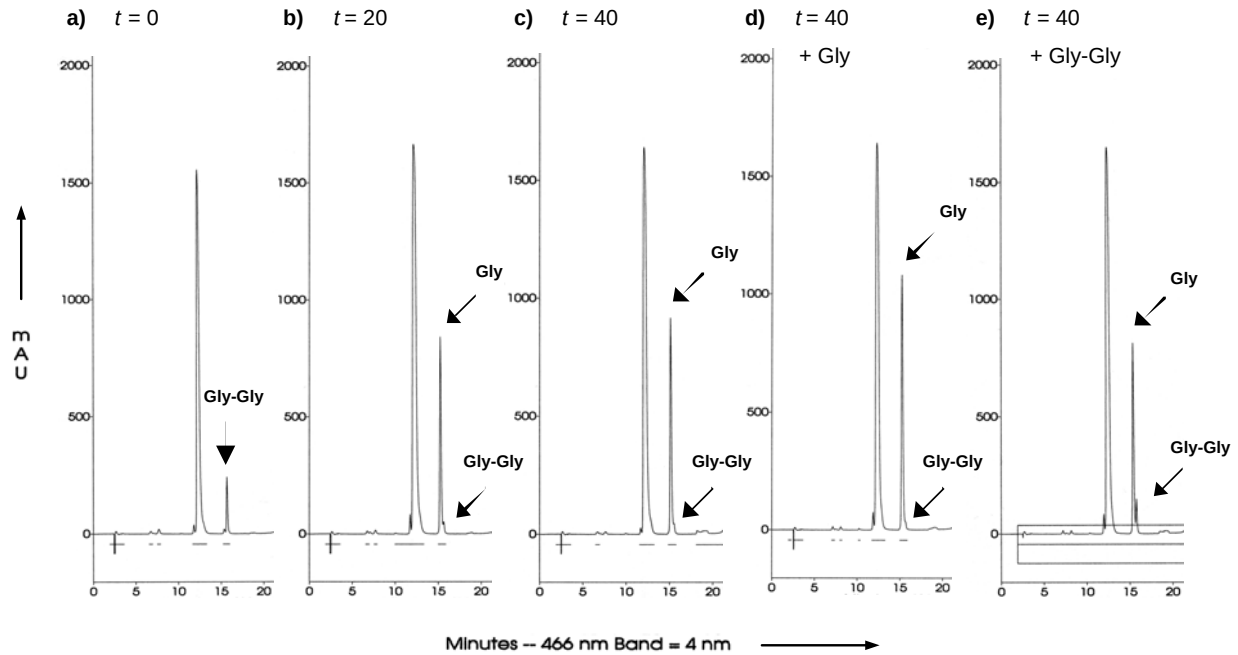

Figure 3.S1. Representative HPLC chromatograms of a peptide hydrolysis reaction (pH 7.3, 60 °C, 2 mM Gly-Gly, 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6) dabsylated at various time points: **A)** *t* = 0 h, **B)** *t* = 20 h, **C)** *t* = 40 h. The peaks corresponding to dabsylated hydrolysis product Gly and dabsylated starting material Gly-Gly are identified: **D)** aliquot at *t* = 40 h is spiked with a dabsylated Gly standard, **E)** aliquot at *t* = 40 h is spiked with a dabsylated Gly-Gly standard.

HPLC analysis of reaction kinetics:[2]

In a total volume of 1200 μL, 2 mM of the dipeptide Gly-Glu was reacted with 10 mM $ZrCl_4$ in the presence and absence of 20 mM 4,13-diaza-18-crown-6 at 37 °C and/or at 60 ° C. An Eppendorf Thermomixer R was used to control temperature to ± 0.1 °C. Seventy μL reaction aliquots were removed at suitable time points, quenched with 14 μL of 0.5 M EDTA pH 8 (1 h, at 25 °C), and stored at -20 °C. At the conclusion of the time course experiment, the aliquots were derivatized with dabsyl chloride and the release of free glycine (P_t) was monitored on a Beckman System Gold HPLC system as described above. Values of P_∞ were estimated from HPLC chromatograms of dabsylating standard solutions of 2 mM glycine in the presence of 20 mM 4,13-diaza-18-crown-6 and/or 10 mM $ZrCl_4$. For each set of reaction conditions, half-lives were estimated from duplicate time course experiments in which plots of $\ln(P_\infty - P_t)$ as a

function of time were linear with R ≥ 0.996 ($t_{1/2}$ = 0.693/k, k = -slope; Figure 3.S2).

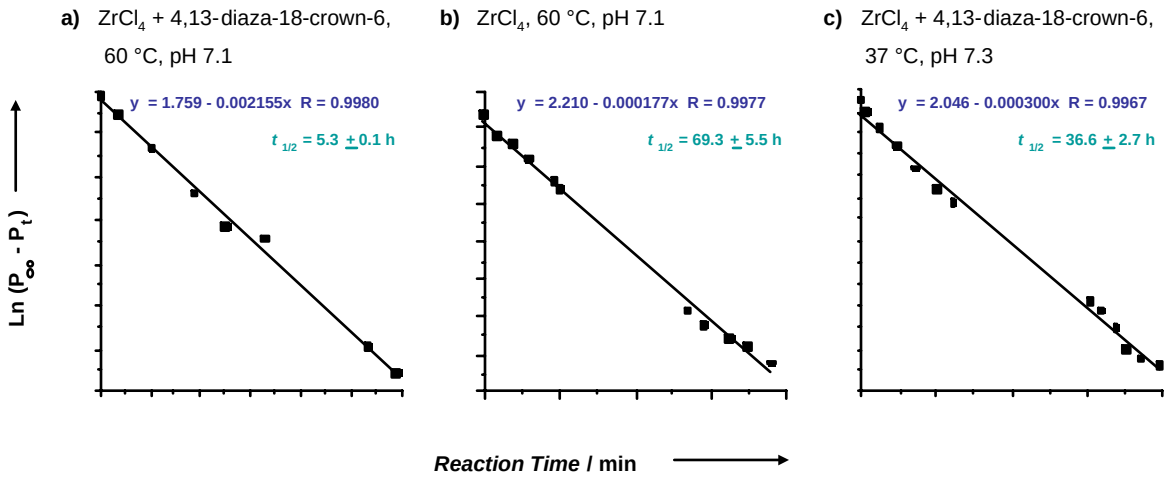

Figure 3.S2. Representative kinetic plots derived from release of free glycine by Zr^{IV}-hydrolysis of the dipeptide Gly-Glu. Ln(P_∞ - P_t) is plotted as a function of time for: **A)** 2 mM Gly-Glu, 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, 60 °C, pH 7.1; **B)** 2 mM Gly-Glu, 10 mM $ZrCl_4$, 60 °C, pH 7.1; and **C)** 2 mM Gly-Glu, 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6 , 37 °C, pH 7.3.

NMR spectrometry:

In order to obtain evidence of complex formation, ^{1}H NMR spectra of 10 mM 4,13-diaza-18-crown-6 and 10 mM $ZrCl_4$ at L:M ratios of 1:0 and 1:1 were recorded in D_2O using a 300 MHz Varian Unity+ Spectrometer at 23.5 °C. Solutions were adjusted to a pH of 7.0 with 0.1 M CF_3COOH in D_2O and 0.1 M NaOH in D_2O. In the presence of Zr^{IV}, small downfield shifts were observed for all ^{1}H NMR azacrown ether resonances with respect to free ligand (Table 3.S2, Figures 3.S3A and B). These changes are similar in magnitude to those produced by potassium(I) in the ^{1}H NMR spectrum of a potassium 4,13-diaza-18-crown-6 complex[3] and could be therefore be indicative of zirconium(IV) complex formation. Nevertheless, because Zr^{IV} is relatively acidic, the downfield shifts we observed might also have resulted from an increase in ligand pK_a due to the change in ionic strength arising from the addition of 10 mM $ZrCl_4$ to 10 mM 4,13-diaza-18-crown-6. To test these two alternative hypotheses, we utilized the metal chelating agent EDTA,

which forms a stable 1:1 complex with zirconium(IV) at pH 7.0. In the presence of 20 mM EDTA, downfield shifts created by the addition of 10 mM $ZrCl_4$ to 10 mM 4,13-diaza-18-crown-6 were significantly smaller in magnitude. This was the case for two out of three ^{1}H NMR azacrown ether resonances (Table 3.S3, Figures 3.S4A and B). Most notably, the 0.023 ppm shift associated with 4,13-diaza-18-crown-6 $NHCH_2$ protons was reduced to 0.0002 ppm. This result can be considered to be indicative of Zr^{IV}/4,13-diaza-18-crown-6 complex formation in the absence of EDTA.

Table 3.S2. ^{1}H NMR chemical shift data for 4,13-diaza-18-crown-6. Spectra were recorded in the absence and presence of $ZrCl_4$ (300 MHz Varian Unity+ Spectrometer, D_2O, pH 7, 23.5 °C). [a]

	4,13-diaza-18-crown-6		$ZrCl_4$ + 4,13-diaza-18-crown-6	
	δ (ppm)	^3J H-H (Hz)	δ (ppm)	^3J H-H (Hz)
OCH_2	3.650: t, 8 H	4.80	3.658 (0.008): t, 8 H	4.95
OCH_2CH_2O	3.583: s, 8 H	-	3.585 (0.002): s, 8 H	-
$NHCH_2$	3.148: t, 8 H	4.80	3.171 (0.023): t, 8 H	4.80

[a] [$ZrCl_4$] = 10 mM, [4,13-diaza-18-crown-6] = 10 mM. Numeric values in parenthesis indicate downfield shifts relative to free ligand. Solutions were adjusted to pH 7 with 0.1 M CF_3COOH in D_2O and 0.1 M NaOH in D_2O.

Table 3.S3. ^{1}H NMR chemical shift data for 4,13-diaza-18-crown-6. Spectra were recorded with EDTA, in the absence and presence of $ZrCl_4$ (300 MHz Varian Unity+ Spectrometer, D_2O, pH 7, 23.5 °C). [a]

	4,13-diaza-18-crown-6 + EDTA		$ZrCl_4$ + 4,13-diaza-18-crown-6 + EDTA	
	δ (ppm)	^3J H-H (Hz)	δ (ppm)	^3J H-H (Hz)
OCH_2	3.660: t, 8 H	4.95	3.663 (0.003): t, 8 H	4.80
OCH_2CH_2O	3.586: s, 8 H	-	3.589 (0.003): s, 8 H	-
$NHCH_2$	3.175: t, 8 H	4.95	3.177 (0.002): t, 8 H	4.80

[a] [$ZrCl_4$] = 10 mM, [4,13-diaza-18-crown-6] = 10 mM, [EDTA] = 20 mM. Numeric values in parenthesis indicate downfield shifts relative to free ligand. Solutions were adjusted to pH 7 with 0.1 M CF_3COOH in D_2O and 0.1 M NaOH in D_2O.

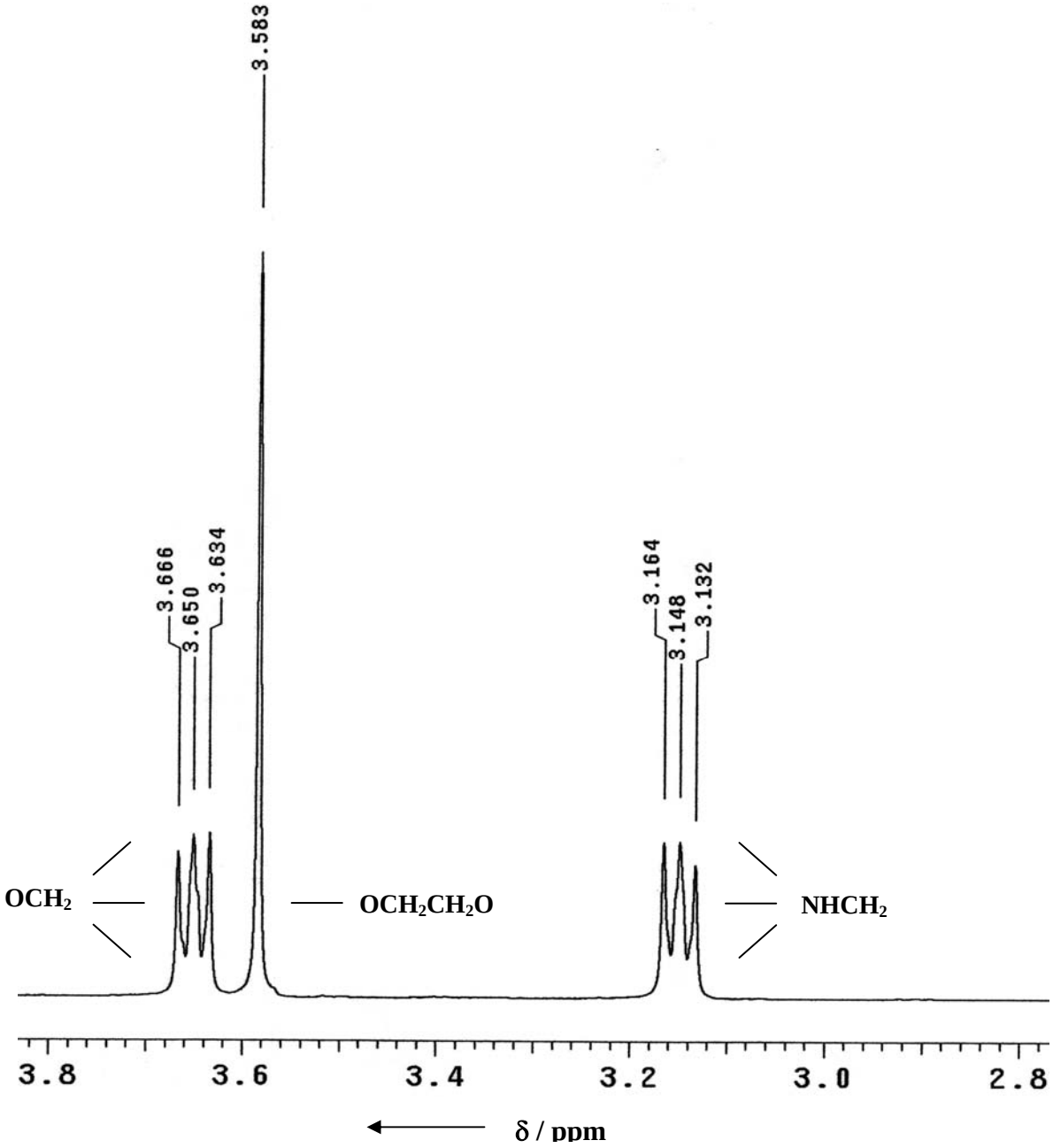

Figure 3.S3. A) L:M 1:0. ^{1}H NMR spectrum of 10 mM 4,13-diaza-18-crown-6 in D_2O at pH 7, 23.5 °C.

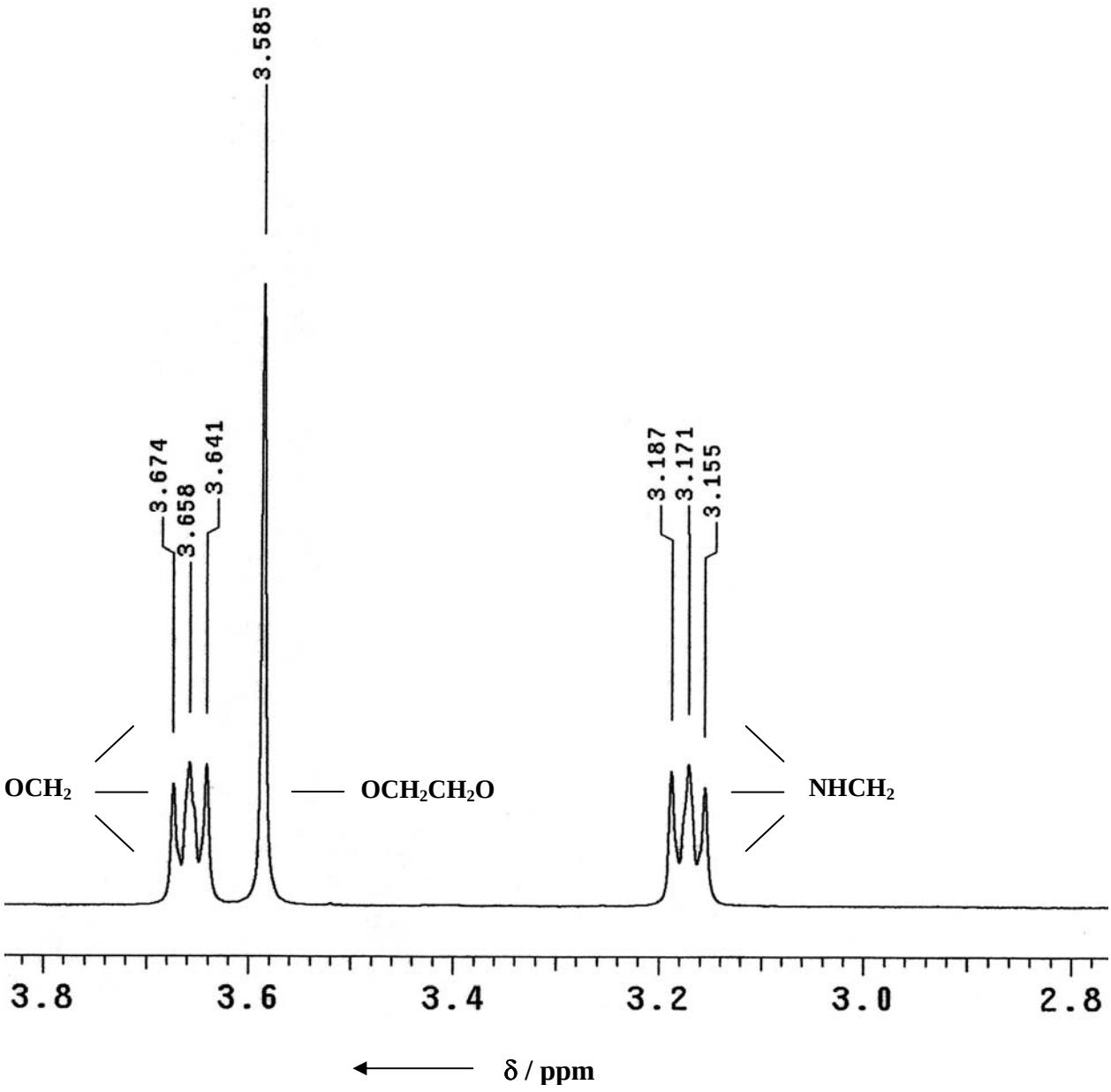

Figure 3.S3. B) L:M 1:1. ^{1}H NMR spectrum of 10 mM $ZrCl_4$ and 10 mM 4,13-diaza-18-crown-6 in D_2O at pH 7, 23.5 °C.

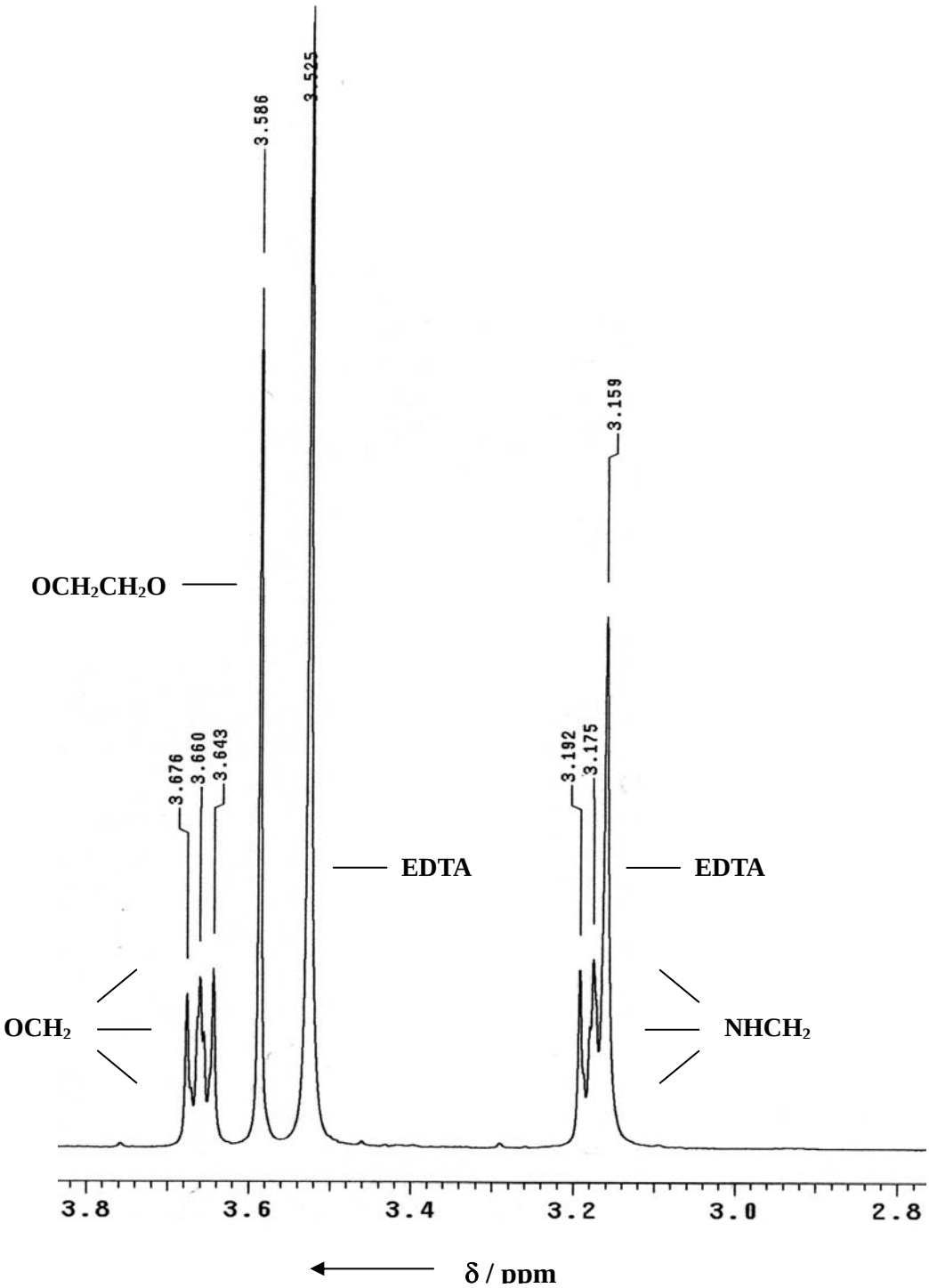

Figure 3.S4. A) ^{1}H NMR spectrum of 10 mM 4,13-diaza-18-crown-6 and 20 mM EDTA in D_2O at pH 7, 23.5 °C. (EDTA resonances were identified by comparison to an NMR spectrum of 20 mM EDTA in D_2O, pH 7.)

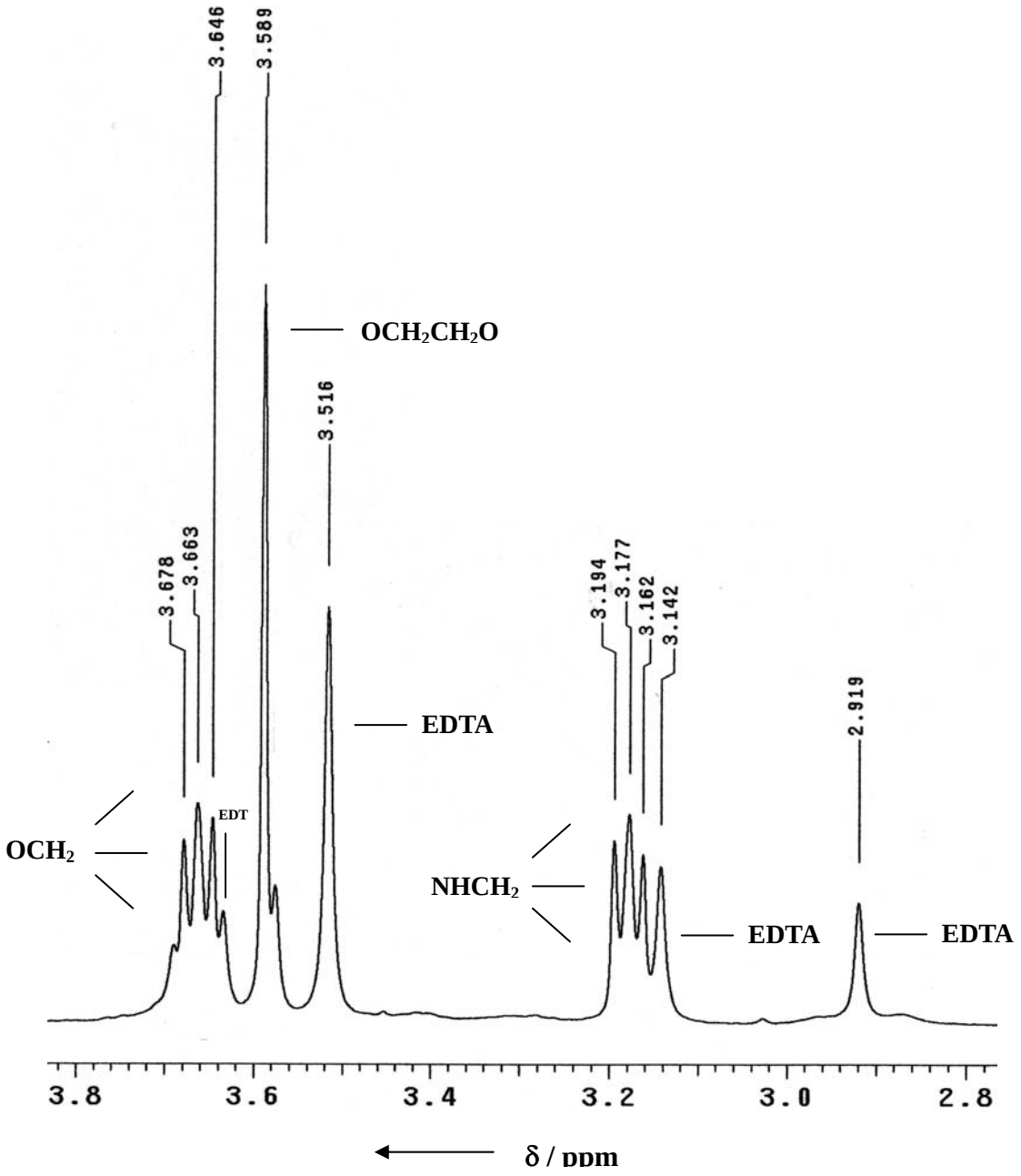

Figure 3.S4. B) ^{1}H NMR spectrum of 10 mM $ZrCl_4$, 10 mM 4,13-diaza-18-crown-6, and 20 mM EDTA in D_2O at pH 7, 23.5 °C. (EDTA resonances were identified by comparison to an NMR spectrum of 10 mM $ZrCl_4$, 20 mM EDTA in D_2O, pH 7.)

References:

(1) Chang, J.-Y.; Knecht, R.; Braun, D.G. *Methods Enzymol.* **1983**, *91*, 41.

(2) Grant, K.B.; Pattabhi, S. *Anal. Biochem.* **2001**, *289*, 196.

(3) Knöchel, A.; Oehler, J.; Rudolph, G.; Sinnwell, V. *Tetrahedron* **1977**, *33*, 119.

CHAPTER IV

Tuning Zr(IV)-Assisted Peptide Hydrolysis at Neutral pH

(This chapter was written by Miki Kassai with assistance from Prof. Kathryn B. Grant. All experiments were performed by Miki Kassai. The chapter will be submitted to *Inorganic Chemistry Communications* and as been written in the format of this journal.)

Abstract

The present study has compared a total of 17 ligands to observe their relative effects on Zr(IV)-assisted hydrolysis of the dipeptide Gly-Gly (60 °C, pH 6.8 - 7.4, t = 4 h and t = 10 h). While it was not necessary to have a ring structure to enhance Zr(IV) reactivity, the macrocyclic azacrown ether ligands 4,13-diaza-18-crown-6 and 4,10-trioxa-7,13-diazacyclopentadecane produced the overall highest hydrolysis yields. The potential ability of open-chain ligands to form multiple chelate rings appeared to coincide with substantially reduced levels of peptide hydrolysis and Zr(IV) precipitation.

Introduction

Metal complexes of Ce(IV), Co(II), Co(III), Cu(II), Fe(III), Mo(IV), Ni(II), Pd(II), Pt(II), Zn(II), and Zr(IV) can be used as non-enzymatic reagents to efficiently hydrolyze peptides and/or proteins [1]. The design and synthesis of these artificial metallopeptidases has been focused on effective cleavage of unactivated peptide amide bonds. A number of metal-based model systems that incorporate bidentate, tridentate, multidentate and/or macrocyclic ligands have been reported [1b-e, k,l,n,o,q-u]. For example, the Kostić group has shown that *cis*-

$[Pd(en)(H_2O)_2]^{2+}$ and other Pd(II) complexes cleave the X-Y bond in the sequences X-Y-Met-Z and X-Y-His-Z, in which X, Y, and Z can be any amino acid in a weakly acidic solution [1k]. A new bidentatethioether complex of Pd(II), *cis*-$[Pd(CH_3SCH_2CH_2CH_2SCH_3)(H_2O)_2]^{2+}$, displays the same selectivity as $[Pd(en)(H_2O)_2]^{2+}$. However, the complex reacts more slowly due to the steric bulk of the thioether ligand [1q]. Djuran et al. reported that the Pd(II) complex *cis*-$[Pd(dpa)(H_2O)_2]^{2+}$ is able to hydrolyze the *N*-acetylated dipeptide AcMet-Gly under acidic condition [1t]. It was found that 25% of the dipeptide was cleaved at 60 ^{o}C after 2 h and that a 90% cleavage yield was obtained after 72 h. However, AcHis-Gly showed no hydrolysis because of steric hindrance arising from interactions between the two bulky dpa pyridine rings and the imidazole ring in the side chain of histidine. Thus, the reactivity of a metal complex towards specific peptide sequences can vary greatly. When designing an artificial metallopeptidase, it is therefore important to evaluate and compare the effects of a variety of different metal chelating ligands.

We have found that the metal ion zirconium(IV) efficiently hydrolyzes amide bonds in peptides at neutral pH [1o,r,s]. Other groups have shown that Zr(IV) hydrolyzes phosphodiester bonds in *p*-nitrophenol activated phosphodiesters, in nucleic acids, and in nucleotides [2]. Our research has focused on Zr(IV) for a number of major reasons. Because Zr(IV) is oxophilic and forms complexes with high coordination numbers [3], it should be able to interact with peptide amide carbonyl oxygens (activating the carbonyl carbon towards nucleophilic attack), while delivering a hydroxide nucleophile to the scissile amide bond. (The pK_a values of Zr(IV) bound water molecules are lowered from $\approx$ 15.7 to $\leq$ 0.6 [4].) In addition, Zr(IV) possesses low cellular toxicity [5], enhanced Lewis acid strength, and rapid-ligand exchange kinetics [6], a requirement for efficient catalytic turnover.

Results and Discussion

We have shown that the macrocyclic ligand 4,10,13-tetraoxa-7,16-diazacyclooctadecane (4,13-diaza-18-crown-6) markedly enhances the reactivity of Zr(IV): $t_{1/2}$ values at 60 °C and pH 7.1 are 5.3 ± 0.1 h and 69.3 ± 5.5 h for Zr(IV)-assisted hydrolysis of the dipeptide Gly-Glu in the presence and absence of 4,13-diaza-18-crown-6, respectively [1o]. We then compared the relative effects of 4,13-diaza-18-crown-6 to *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)aminomethane (Tris) [1s]. When 78 mM of HEPES was added to a typical hydrolysis reaction (60 ^{o}C and pH 6.6 – 7.1, 2 mM Gly-Glu, 10 mM $ZrCl_4$, 20 h), Gly-Glu was hydrolyzed in high yield (80%), similar to the cleavage produced using 20 mM of 4,13-diaza-18-crown-6 (90%). However, in the presence of 40 mM of Tris, hydrolysis was only 22% complete, similar to Zr(IV)-assisted hydrolysis of Gly-Glu when 4,13-diaza-18-crown-6, HEPES, and Tris were omitted (26%). Thus, 4,13-diaza-18-crown-6 and HEPES were show to facilitate Zr(IV)-assisted peptide hydrolysis [1o,s], while Tris produced an inhibitory effect [1o]. Peptide cleavage was not observed in negative controls in which Zr(IV) was replaced by an equivalent volume of water.

With the goal of developing synthetic Zr(IV)-based metallopeptidases with superior reactivities at neutral pH, the present study has systematically compared a total of 17 ligands (Fig. 4.1; Figs. 4.S3 and 4.S4 in Supporting Information) to observe their effects on dipeptide hydrolysis. We have examined 4,13-diaza-18-crown-6 and similar macrocyclic ligands as well as ligands that have been used to facilitate metal-assisted hydrolysis of phosphodiester bonds in nucleotides and/or in nucleic acids. We have also studied compounds obtained by breaking 4,13-diaza-18-crown-6, HEPES, and Tris down into their component parts. This was done to identify

key structural features that might be responsible for either promoting or inhibiting peptide hydrolysis.

Individual hydrolysis solutions contained 2 mM of the dipeptide Gly-Gly, 10 mM of $ZrCl_4$, and 20 mM of ligand (Supporting Information). Prior to each reaction, pH was adjusted at 25 °C to ~ 7.0 by direct addition of NaOH and/or HCl. The solutions were treated at 60 °C, after which aliquots were removed at 0 h, 4 h, and 10 h time points. Amino acids released upon peptide amide bond hydrolysis where then derivatized with dimethylaminoazobenzenesulfonyl chloride (dabsyl chloride) and identified by reversed-phase HPLC (Supporting Information). Relative product yields were estimated by calculating the ratio of the HPLC peak heights of the hydrolysis product glycine to the peak heights of unreacted Gly-Gly. Peptide cleavage was minimal at all t = 0 h time points.

In our first experiment, we compared Tris (**1**) to its component parts 2-amino-1,3-propanediol (**1a**) and ethanolamine (**1b**) (Fig. 4.1a, Fig. 4.S3a). Ligand **1** substantially reduced the formation of zirconium precipitates. However, it produced the lowest levels of hydrolysis at t = 4 h and t = 10 h. (This is in contrast to Zr(IV)-assisted phosphodiester hydrolysis where ligand **1** has been shown to significantly increase hydrolysis yields in acidic and neutral solutions [2b].) At pH values above 5.0-5.2, Zr(IV) forms insoluble gels and precipitates [7]. While these precipitates are thought to substantially reduce the efficiency of phosphodiester hydrolysis [2b,c], they appear to coincide with enhanced peptide hydrolysis in our Zr(IV) experiments. Ligand **1a** produced intermediate levels of hydrolysis and precipitation, followed by **1b**, which produced the most precipitation and the highest peptide hydrolysis yields. Ligands **1**, **1a**, and **1b** form complexes with a wide variety of metals including Eu(III) (**1**), Cu(II) (**1a**), and Zr(IV) (**1b**) [8]. (In the case of **1b**, Zr(IV) forms a bis complex in which each ethanolamine ligand is

bidentate and coordinates through its N and O donor atoms.) When comparing the three ligands, it is apparent that levels of Zr(IV) precipitation and levels of peptide hydrolysis are reduced according to the potential ability of each ligand to form multidentate chelate rings (**1** > **1a** > **1b**).

HEPES (**2**) and component parts 2-amino-ethanesulfonic acid (**2b**), 1-piperazineethanol (**2c**) and derivative 1,4-piperazinediethanol (**2a**) were studied next (Fig. 4.1b, Fig. 4.S3b). It has long been assumed that the biological buffer HEPES does not possess binding affinity for metal ions. However, there is now evidence that HEPES interacts weakly with Cu(II) and other metals [9]. Furthermore, HEPES analog **2a** undergoes weak complexation with Mg(II), Ca(II), Sr(II), Ba(II), Pr(III), Ni(II), Cu(II), and Zn(II) [10]. While ligand **2d** has not been studied, **2c** forms a ternary complex with [Cu(II)(Gly-Gly)] in which **2c** acts as a monodenate N donor ligand [11]. In the presence of Zr(IV), HEPES and its analogs hydrolyzed Gly-Gly in the following order: **2** > **2a** ≥ **2b** ~ **2c.** At t = 10 h, it is apparent that any change made to the HEPES framework reduces the effectiveness of this ligand. Significant Zr(IV) precipitation was observed in all of the above hydrolysis reactions.

We then made a comparison of macrocyclic azacrown ether 4,13-diaza-18-crown-6 (**3**) to its open-chain component parts 2-(2-aminoethoxy)-ethanol (**3a**) and 2,2'-iminobis-ethanol (**3b**) and to derivative 2,2',2"-nitrilotris-ethanol (**3c**) (Fig. 4.1c, Fig. 4.S3c). In a previous paper, we employed ^{1}H NMR to obtain preliminary evidence of weak complex formation between Zr(IV) and azacrown ether **3** [1o]. With respect to **3b** [8a], Zr(IV) forms a mono complex in which the ligand produces a single, eight membered chelate ring by coordinating through its two oxygen donor atoms. Interestingly, composite formation constants show that the complex formed between vanadium(V) and multidentate ligand **3c** is 4-fold more stable than the complex formed between V(V) and **3b** [12]. In the presence of Zr(IV), ligands **3** and **3a** produced approximately

the same levels of peptide hydrolysis within experimental error at the t = 4 h time point, but at t = 10 h, **3** produced significantly more cleavage than **3a**. Ligand **3b** showed much lower amounts of hydrolysis at 4 h and 10 h. Taken together, the above data suggest that the structural feature "$ROCH_2CH_2OCH_2CH_2NR$" may contribute to the efficiency of Zr(IV)-assisted peptide hydrolysis. It is also important to note that the multidentate, chelating ligand **3c** dramatically reduced Zr(IV) precipitation and peptide cleavage yields.

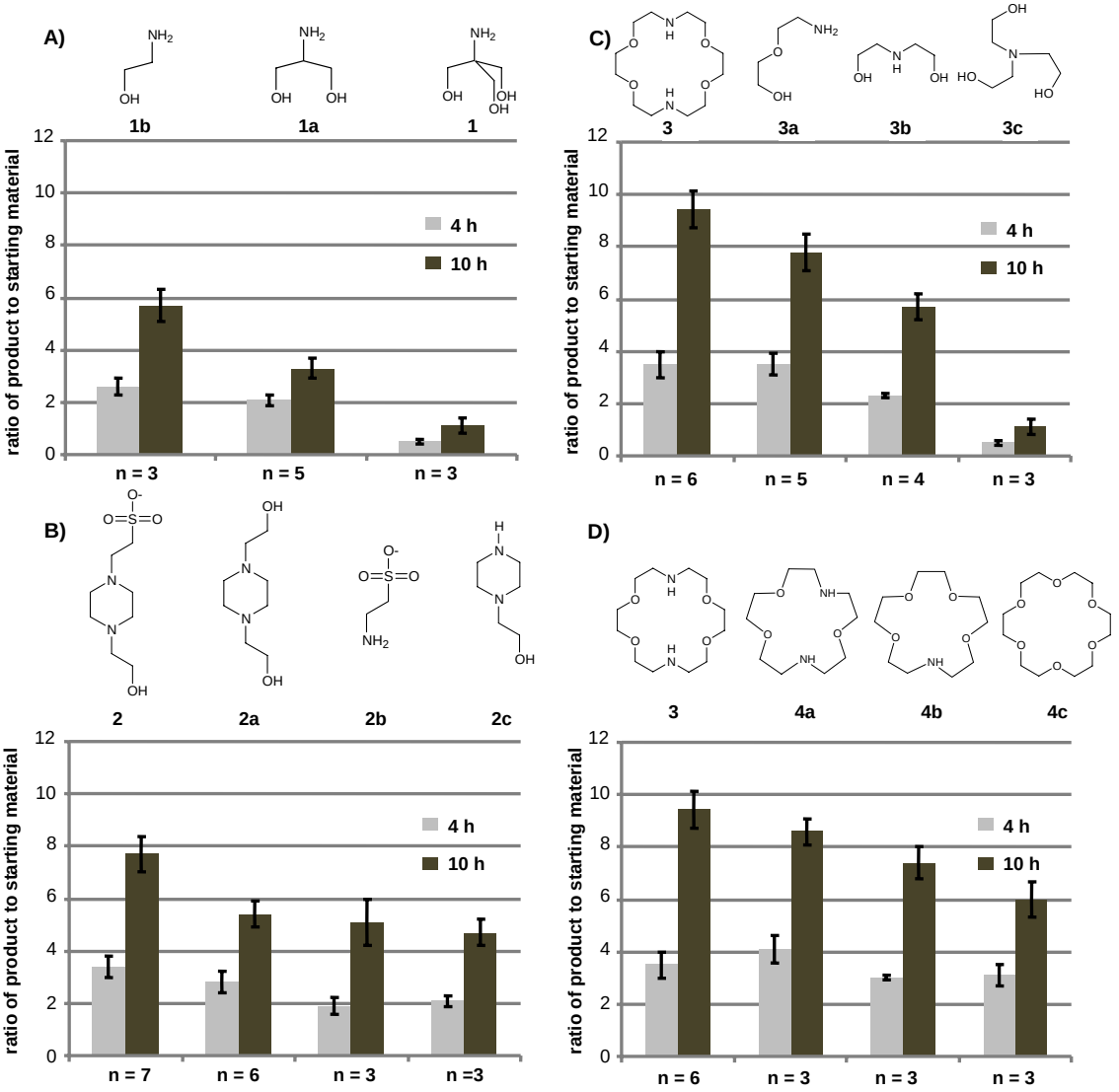

Fig. 4.1. The ratio of the peak height of the hydrolysis product glycine to the peak height of unreacted Gly-Gly plotted as a function of time for hydrolysis reactions containing 2 mM of Gly-Gly, 10 mM of $ZrCl_4$, and 20 of ligand (60 °C and pH 6.8-7.4). The letter **n** indicates the number of trials. Error bars indicate standard deviation.

Our next comparison was of four macrocyclic 18-menbered and 15-menbered crown ether ring systems containing the hard donor atoms N and/or O: 4,13-diaza-18-crown-6 (**3**), 4,10-trioxa-7,13-diazacyclopentadecane (**4a**), 1,4,7,10-tetraoxa-13-azacyclopentadecane (**4b**), and 18-crown-6 (**4c**) (Fig. 4.1d, Fig. 4.S3d). These macrocycles coordinate with a wide variety of transition metal ions, including La(II), Eu(III) and other lathanides [13]. (As mentioned above, we previously obtained ^{1}H NMR evidence of weak complex formation between Zr(IV) and azacrown ether **3** [1o]). Our current data has revealed that relative Zr(IV)-assisted peptide hydrolysis yields decrease in the following order: **3** ~ **4a** > **4b** ≥ **4c** . Ligands **3** and **4a** possess two nitrogen donor ligands, while **4b** and **4c** have 1 and 0, respectively. Thus, the presence of nitrogen atoms appears to have had a positive impact on Zr(IV)-assisted peptide hydrolysis yields. Significant Zr(IV) precipitation was observed in all reactions.

We ranked the above 14 ligands at 4 h and 10 h time points in order of decreasing hydrolysis yields (Figs. 4.S5 and 4.S6). The macrocyclic ligands **3** and **4**a produced the most hydrolysis, followed by open-chain compounds **3a** and then **2** (**3** ~ **4a** ≥ **3a** ≥ **2)**. The lowest levels of cleavage were produced by open-chain, multidentate ligands **1** and **3c** (**1** ~ **3c**).

In our final experiment, we investigated three open-chain ligands which facilitate either Ce(IV)- or Zr(IV)-assisted phosphodiester bond hydrolysis [2b,c; 14]: D-glucamine (**5**), 2,6-pyridinedimethanol (**6**), and *N,N'*-[(2-hydroxy-1,3-phenylene)bis(methylene)]bis[*N*-(carboxymethyl)-glycine] (**7**) (Fig. 4.S4). Similar to Zr(IV), Ce(IV) has a strong tendency to form catalytically active metal hydroxide gels and precipitates. To address this problem, Komiyama and coworkers utilized glucamine (**5**). They showed that this ligand could be used to reduce Ce(IV) precipitation and increase levels of Ce(IV)-assisted DNA hydrolysis 10^{9}-fold over background [14]. Moreover, Jagoda and Krämer employed the multidentate, chelating

ligands **6** and **7** to respectively reduce Zr(IV) precipitation at neutral pH and increase the rate of Zr(IV)–assisted ATP hydrolysis ~ two and three times more than Tris (**1**) [2c]. Therefore, we were interested in testing the effects of ligands **5**, **6**, and **7**, but Zr(IV)-assisted peptide hydrolysis was not observed. However, as seen with the multidentate, chelating ligands Tris (**1**) and 2,2',2''-nitrilotris-ethanol (**3c**), which produced extremely low levels of peptide hydrolysis, zirconium precipitation was dramatically reduced. Taken together, these data suggest that, at pH 7.0, Zr(IV)-assisted peptide hydrolysis might be heterogeneous in nature, similar to peptide hydrolysis by lanthanide hydroxide gels. It is conceivable that strong, multidentate chelating ligands might fully occupy all of the coordination sites on Zr(IV), hindering the interaction of this metal with peptide substrates, hydroxide nucleophiles, and other competing ligands.

In conclusion, a comparison of 17 ligands in hydrolysis reactions containing 2 mM of the dipeptide Gly-Gly, 10 mM of $ZrCl_4$, and 20 mM of ligand (60 °C at t = 4 h and t = 10 h, pH 6.8-7.4) showed that the following four compounds produced the most glycine hydrolysis product at both of the time points: 4,13-diaza-18-crown-6 (**3**) ~ 4,10-trioxa-7,13-diazacyclopentadecane (**4a**) ≥ 2-(2-aminoethoxy)-ethanol (**3a**) ≥ HEPES (**2**) (Figs. S5 and S6). Our results demonstrated that while it was not necessary to have a ring structure to enhance Zr(IV) reactivity, the macrocyclic azacrown ether ligands (**3**) and (**4a**) produced the overall highest hydrolysis yields. The apparent, subtle advantage conferred by the macrocyclic framework of ligands **3** and **4a** may be due to the presence of free axial coordination sites on Zr(IV) that are available to associate with hydroxide nucleophiles as well as with oxygen and nitrogen donor atoms in Gly-Gly. With respect to open-chain ligands, the potential ability to form multiple chelate rings appears to coincide with reduced levels of Zr(IV) precipitation as well as reduced levels of hydrolysis. Our future work will focus on mechanistic and structural studies aimed at testing our hypotheses.

Acknowledgment

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References

[1] (a) L. Meriwether, F.H. Westheimer, J. Am. Chem. Soc. 78 (1956) 5119;

(b) D.A. Buckingham, J.P. Collman, D.A.R. Happer, L.G. Marzilli, J. Am. Chem. Soc. 89 (1967) 1082;

(c) K.W. Bentley, E.H. Creaser, Biochem. J. 135 (1973) 507;

(d) L. Zhu, M. Kostić, Inorg. Chem. 31 (1992) 3994;

(e) E.L. Hegg, J.N. Burstyn, J. Am. Chem. Soc. 117 (1995) 7015;

(f) G. Allen, R.O. Campbell, Int. J. Peptide Protein Res. 48 (1996) 265;

(h) W. Bal, R. Liang, J. Lukszo, S.-H. Lee, M. Dizdaroglu, K.S. Kasprzak, Chem. Res. Toxicol. 13 (2000) 616;

(i) C.V. Kumar, A. Buranaprapuk, A. Cho, A. Chaudhari, J. Chem. Soc. Chem. Commun. (2000) 597;

(j) T. Takarada, M. Yashiro, M. Komiyama, Chem. Eur. J. 6 (2000) 3906;

(k) N.M. Milovic, N.M. Kostić, J. Am. Chem. Soc. 124 (2002) 4759;

(l) J.W. Jeon, S.J. Son, C.E. Yoo, I..S. Hong, J. Suh, Bioorg. Med. Chem. 11 (2003) 2901;

(m) M. Yashiro, Y. Sonobe, A. Yamamura, T. Takarada, M. Komiyama, Y. Fuji, Org. Biomol. Chem. 1 (2003) 629;

(n) C.E Yoo, P.S Chae, J.E Kim, E.J. Jeong, J. Suh, J. Am. Chem. Soc. 125 (2003) 14580;

(o) M. Kassai, R. Ravi, S.J. Shealy, K.B. Grant, Inorg. Chem. 43 (2004) 6130;

(p) A. Erxleben, Inorg. Chem. 44 (2005) 1082;

(q) S.A. Stoffregen, A.K. Griffin, N.M. Kostić, Inorg. Chem. 44 (2005) 8899;

(r) K.B. Grant, M. Kassai, Curr. Org. Chem. 10 (2006) 1035;

(s) K.B. Grant, M. Kassai, Abstracts, 58th Southeast Regional Meeting of the American Chemical Society, Augusta, Georgia, November 1-4, 2006, Abstract 404;

(t) Q. Pan, W. Jiang, Z. Liao, T. Zhang, C. Liu, Inorg. Chem. 45 (2006) 490;

(u) M.D. Zivkovic, S. Rajkovic, U. Rychlewska, B. Warzajtis, M.I. Djuran, Polyhedron 26 (2007) 1541.

[2] (a) R.A. Moss, J. Zhang, K.G. Ragunathan, Tetrahedron Lett. 39 (1998) 1529;

(b) R. Ott, R. Krämer, Angew. Chem. Int. Ed. 37 (1998) 1957;

(c) M. Jagoda, R. Krämer, Inorg. Chem. Commun. 8 (2005) 697.

[3] R.C. Fay, in: G. Wilkinson, R.D. Gillard, J.A. McCleverty (Eds.), Comprehensive Coordination Chemistry, vol. 3, Pergamon, Oxford, 1987, pp. 364, 384-387.

[4] J. Burgess, in: Metal Ions in Solution, Halsted Press (Wiley), New York, 1978, p. 259f.

[5] S. Ghosh, A. Sharma, G. Talukder, Biol. Trace Elem. Res. 35 (1992) 247.

[6] A. Singhal, L.M. Toth, J.S. Lin, K. Affholter, J. Am. Chem. Soc. 118 (1996) 11529.

[7] A. Clearfield, J. Mater. Res. 5 (1990) 161.

[8] (a) E.I. Toma, Poverkh. Yavleniya Adsorbtsiya, Koord. Vzaimodeistvie (1972) 16 From: Ref. Zh., Khim. 1973, Abstr. No. 2V105;

(b) J.M. Pfefferle, J.C.G. Buenzli, Helv. Chim. Acta 72 (1989) 1487;

(c) S. Canepari, V. Carunchio, R. Schina, Polyhedron 18 (1999) 3263.

[9] K. Hegetschweiler, P. Saltman, Inorg. Chem. 25 (1986) 107.

[10] V. Hietapelto, R.S. Laitinen, J. Pursiainen, E. Rahkamaa, Acta Chem. Scand. 53 (1999) 7.

[11] E.C. O'Brien, E. Farkas, A. Rockenbauer, K.B. Nolan, J. Inorg. Biochem. 77 (1999) 135.

[12] D.C. Crans, I. Boukhobza, J. Am. Chem. Soc. 120 (1998), 8069.

[13] (a) F. Arnaud-Neu, E.L. Loufouilou, M.-J. Schwing-Weill, J. Chem. Soc. Dalton Trans. 12 (1986) 2629;

(b) P.J. Cragg, S.G. Bott, G. Simon, A.L. Atwood, Lanthanide Actinide Res. 2 (1988) 265;

(c) J.C.G. Buenzli, F. Pilloud, Inorg. Chem. 28 (1989) 2638.

[14] A. Kajimura, J. Sumaoka, M. Komiyama, Carbohydr. Res. 309 (1998) 345.

Supporting Information

Materials and Methods.

Reagents:

All reagents were of the highest available purity. Distilled, deionized water was utilized in the preparation of all buffers. Metal solutions were freshly prepared in all cases. Sodium bicarbonate, sodium phosphate monobasic and dibasic salts, dimethylformamide (DMF), glycine (Gly), glycylglycine (Gly-Gly), glycylglutamate (Gly-Glu), dimethylaminoazobenzenesulfonyl chloride (dabsyl chloride, DABS-Cl), iminodiacetic acid, and 37% formaldehyde were purchased from Sigma. HPLC grade acetone was from EMD Biosciences, Inc. and Optima grade acetonitrile was from Caledon Labs. Molecular biology grade ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from the Eastman Kodak Company. D-glucamine was from TGI America and NaOH was from J.T. Baker. Ligand **7** was prepared according to a reported procedure [1]: to a 100 mL aqueous solution of iminodiacetic acid (16.7 g, 0.125 mol) and 6.8 mL of *p*-cresol (6.75 g, 0.063 mol) were added 40 mL of an aqueous solution of NaOH (10.5 g, 0.25 mol)

cooled in an ice-water bath. Then, a total of 15 mL of 37% formaldehyde was added dropwise at 0 ^{o}C. The solution was stirred for 30 min at room temperature, stirred for 4 h at 70 ^{o}C, and then concentrated to dryness. The resulting pale yellow solid was recrystallized from methanol (90% yield). ^{1}H NMR (300 MHz, D_2O): δ 2.21 (s, 3H), 3.20 (s, 8H), 3.76 (s, 4H), and 6.99 (s, 2H). All other chemicals including 4,13-diaza-18-crown-6, $ZrCl_4$ (purity > 99.99%), and *p*-cresol were purchased from the Aldrich Chemical Company.

Peptide Hydrolysis Reactions:

A total of 16 μL of Gly-Gly (100 mM stock concentration in ddH_2O) was reacted at 60 °C in the presence of ddH_2O, 80 μL of $ZrCl_4$ (100 mM stock concentration in ddH_2O), and 80 μL of ligand (100 mM stock concentration in ddH_2O). Prior to each reaction, the pH was adjusted at 25 °C to 7.0 by direct addition of NaOH and/or HCl to a final volume of 800 μL. An Eppendorf Thermomixer R was then used to maintain temperature at 60 °C $\pm$ 0.1 °C. At 0 h, 4 h, and 10 h time points, 140 μL aliquots were removed. Reaction pH was then determined by averaging pre and post reaction measurements, after which the aliquots were quenched by adding 28 μL of a stock solution of 0.5 M EDTA pH 8.0 (1 h, at 25 °C; 83.3 mM final EDTA concentration).

HPLC Analysis of Peptide Hydrolysis Reaction Products:

Amino acid produced in Zr(IV)-assisted peptide hydrolysis reactions were identified by HPLC analysis of their dabsylated derivatives (Fig. 4.S1). In a total volume of 120 μL, a 45 μL aliquot of each reaction was treated with 60 μL of 25 mM dabsyl chloride in acetone in the presence of 15 μL of 1 M sodium bicarbonate buffer pH 10 [2]. Solutions were reacted at 70 °C for 15 min, chilled on ice, and diluted with 120 μL of ethanol. Twenty μL of each sample were

then analyzed on a Beckman System Gold High Performance Liquid Chromatograph (HPLC) system equipped with a Varian MICROSORB-MV™ C_{18} 5 µm, 100 Å, 4.6 x 250 mm reversed-phase column. Using the gradient elution scheme shown in Table 4.S1, separations were conducted at 50 °C with a 1 mL/min flow rate of mobile phase A (2% DMF in 20 mM sodium phosphate buffer pH 6.5), and of mobile phase B (6% DMF in acetonitrile). Dabsylated hydrolysis products were detected by UV absorption at 466 nm (Fig. 4.S2) and their identities were confirmed by comparison of retention times to corresponding dabsylated amino acid standards. Appropriate dabsylated standards were then added to the reaction mixtures and were shown to enhance peaks corresponding to hydrolysis products. The peak heights of amino acid product and of dipeptide starting material were measured at each time point. Relative product yields were then estimated by calculating the ratio of the peak height of the amino acid to the peak height of the unreacted dipeptide.

Figures.

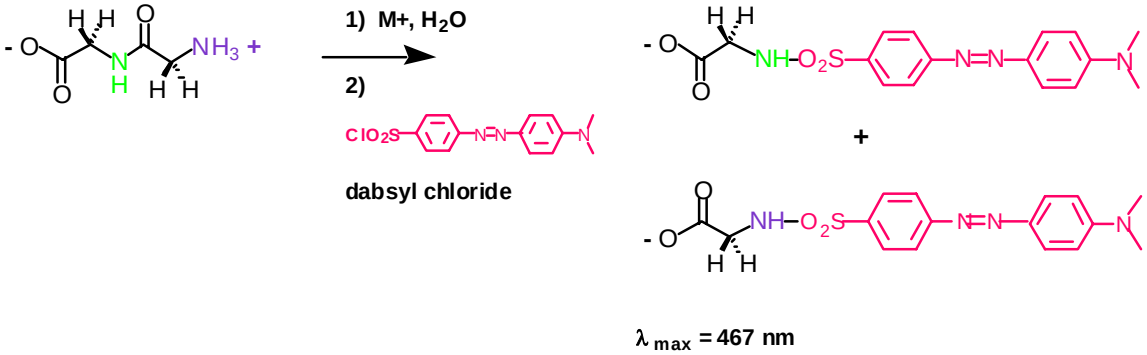

Fig. 4.S1. Dabsyl chloride reacts with the primary amino groups of two glycine residues released upon metal-assisted hydrolysis of the peptide amide bond of Gly-Gly. Two dabsylated derivatives of glycine are produced.

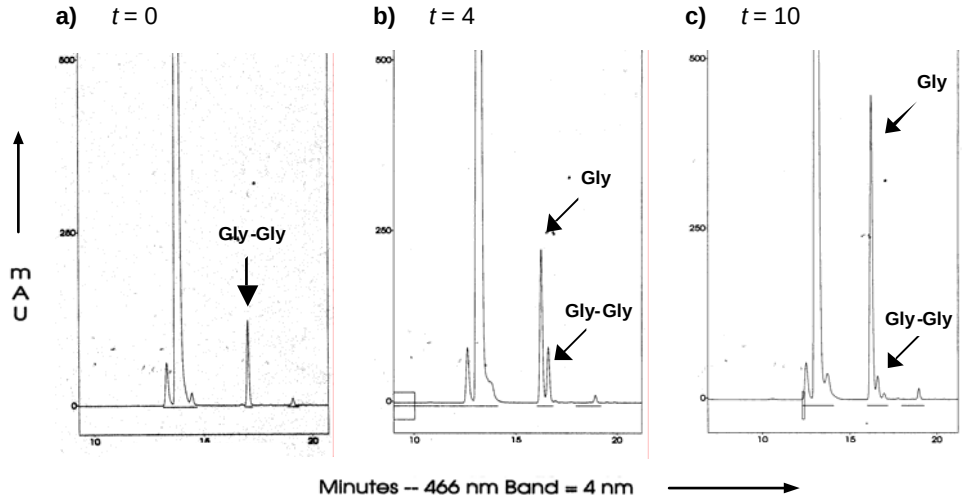

Fig. 4.S2. Representative HPLC chromatograms of a peptide hydrolysis reaction (60 °C, pH 7.1, 2 mM Gly-Gly, 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6) dabsylated at three time points: **A)** t = 0 h, **B)** t = 4 h, **C)** t = 10 h. The peaks corresponding to dabsylated hydrolysis product Gly and dabsylated starting material Gly-Gly are identified.

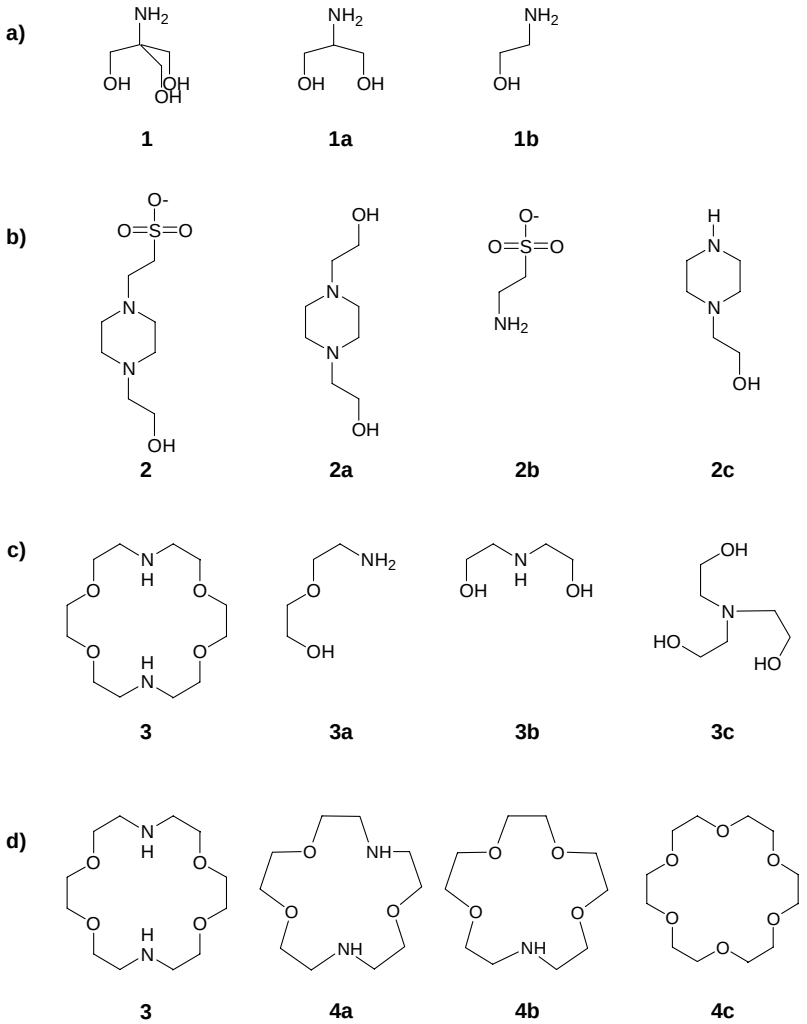

Fig. 4.S3. **A) Tris** (**1**) and component parts 2-amino-1,3-propanediol (**1a**) and ethanolamine (**1b**). **B) HEPES** (**2**) and component parts 2-amino-ethanesulfonic acid (**2b**), 1-piperazineethanol (**2c**) and derivative 1,4-piperazinediethanol (**2a**). **C) 4,13-diaza-18-crown-6** (**3**) and component parts 2-(2-aminoethoxy)-ethanol (**3a**) and 2,2'-iminobis-ethanol (**3b**) and derivative 2,2',2"-nitrilotris-ethanol (**3c**). **D) 4,13-diaza-18-crown-6** (**3**) and similar macrocyclic ligands: 4,10-trioxa-7,13-diazacyclopentadecane (**4a**), 1,4,7,10-tetraoxa-13-azacyclopentadecane (**4b**), and 18-crown-6 (**4c**).

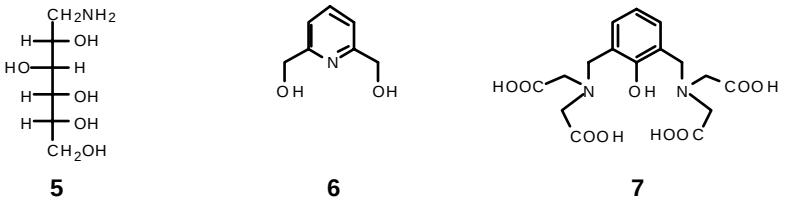

Fig. 4.S4. Ligands which facilitate Ce(IV)- or Zr(IV)-assisted phosphodiester bond hydrolysis [3]: D-glucamine (**5**), 2,6-pyridinedimethanol (**6**), and *N,N'*-[(2-hydroxy-1,3-phenylene)bis(methylene)]bis[*N*-(carboxymethyl)-glycine] (**7**).

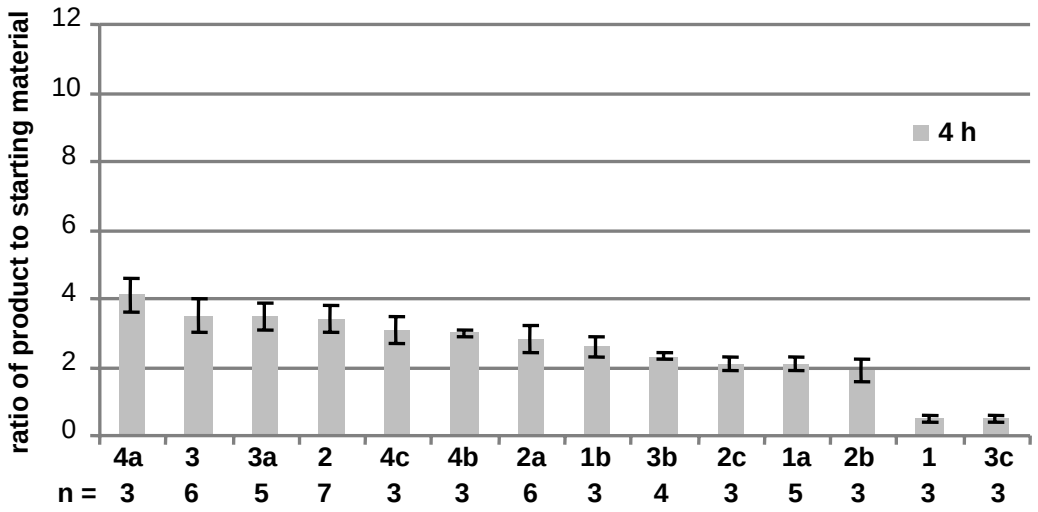

Fig. 4.S5. The ratio of the peak height of the hydrolysis product glycine to the peak height of unreacted Gly-Gly plotted at **t = 4 h** for hydrolysis reactions containing 2 mM of Gly-Gly, 10 mM of $ZrCl_4$, and 20 of ligand at 60 °C and pH 6.8-7.4. The letter **n** indicates the number of trials. Error bars indicate standard deviation.

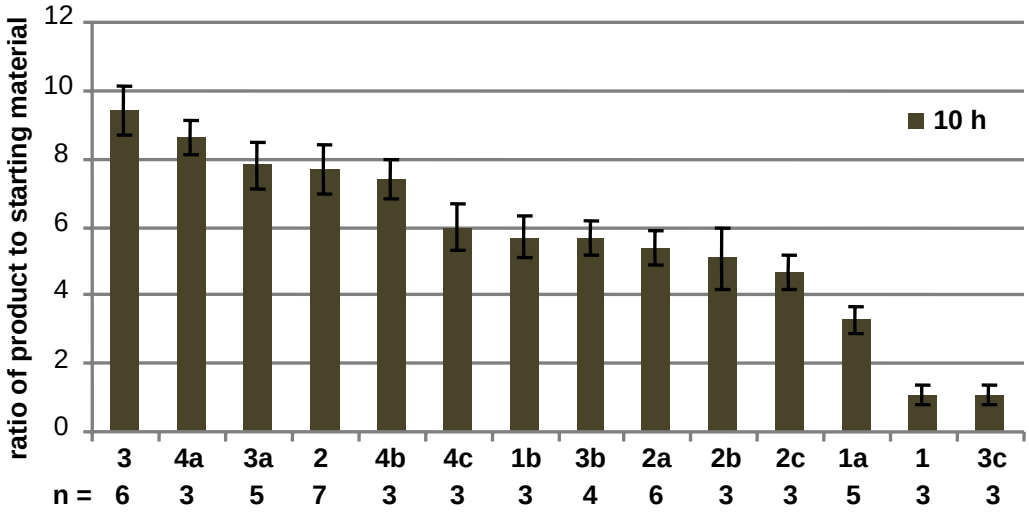

Fig. 4.S6. The ratio of the peak height of the hydrolysis product glycine to the peak height of unreacted Gly-Gly plotted at **t = 10 h** for hydrolysis reactions containing 2 mM of Gly-Gly, 10 mM of $ZrCl_4$, and 20 of ligand at 60 °C and pH 6.8-7.4. The letter **n** indicates the number of trials. Error bars indicate standard deviation.

Table.

Table 4.S1: Reversed-phase HPLC gradient elution scheme

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	80	20
25	55	45
35	20	80
40	80	20

References.

[1] M.E. Branum, A.K. Tipton, S. Zhu, L. Que Jr., J. Am. Chem. Soc. 123 (2001) 1898.

[2] J.-Y. Chang, R. Knecht, D.G. Braun, Methods Enzymol. 91 (1983) 41.

[3] (a) A. Kajimura, J. Sumaoka, M. Komiyama, Carbohydr. Res. 309 (1998) 345;

(b) R. Ott, R. Krämer, Angew. Chem. Int. Ed. 37 (1998) 1957;

(c) M. Jagoda, R. Krämer, Inorg. Chem. Commun. 8 (2005) 697.

CHAPTER V

Conclusion

(This chapter was written by Miki Kassai with assistance from Prof. Kathryn B. Grant.)

We first described Zr(IV)-assisted peptide hydrolysis of the dipeptide Gly-Gly and of its blocked dipeptide analogs at acidic to neutral pH. The hydrolysis reactions were analyzed using a fluorescent microplate reader and the hydrolysis products were identified by HPLC and ESI mass spectrometry. In our first experiment, AcGly-Gly reacted in the presence of $ZrCl_4$ was treated with the fluorophore fluorescamine. The highest fluorescence intensities were observed at 60 ^{o}C and at pH values between 4.4 and 4.7. Thus, hydrolysis yields were the highest under these conditions. The fluorescence intensities (and hence hydrolysis yields) were diminished as the pH was increased above pH 5.0 because of the formation of insoluble Zr(IV) precipitates. (The hydrolysis reactions became more and more turbid as the reaction pH was raised.) We then conducted hydrolysis experiments in which we studied Gly-Gly and its blocked *N*- and *C*-terminal dipeptide analogs AcGly-Gly, AcGly-GlyOMe and Gly-Gly-OMe. Although Zr(IV) was capable of cleaving the *N*- and *C*- terminal blocked dipeptides, our results showed that the free dipeptide Gly-Gly was hydrolyzed more efficiently. According to our HPLC and ESI mass spectrometry data, all of the amide bonds and/or ester linkages in the dipeptide Gly-Gly, and in its blocked dipeptide analogs were hydrolyzed by Zr(IV).

In order to enhance the reactivity of Zr(IV) under near physiological conditions of pH and temperature, we employed the macrocyclic ligand 4,13-diaza-18-crown-6. At 60 °C and near neutral pH, $ZrCl_4$ in the presence of 4,13-diaza-18-crown-6 hydrolyzed Gly-Leu in 35% yield

and Gly-Glu in 97% yield. In a study of 16 dipeptides, we showed that Zr(IV) preferentially hydrolyzed dipeptides containing glycine and both neutral and negatively charged amino acids with side chains containing oxygen. Then we conducted time course experiments at 37 ^{o}C and 60 ^{o}C to monitor reaction kinetics. The half-life of Zr(IV)/4,13-diaza-18-crown-6 – assisted hydrolysis of Gly-Glu at pH 7.3 and 37 ^{o}C was 36.6 ± 2.7 h, which represents a significant rate enhancement compared to the average half-life of ~ 200 years estimated for spontaneous hydrolysis of unactivated peptide amide bonds under nearly identical conditions (pH 6.8 – 7.0, 37 ^{o}C). At pH 7.1 and 60 ^{o}C, half-lives were 69.3 ± 5.5 h and 5.3 ± 0.1 h for Zr(IV)-assisted hydrolysis of Gly-Glu in the absence and presence of 4,13-diaza-18-crown-6, respectively. In ^{1}H NMR experiments, we observed weak interaction between Zr(IV) and 4,13-diaza-18-crown-6. We next compared the effects of 4,13-diaza-18-crown-6 to Tris and HEPES ligands. In the presence of HEPES, Zr(IV)-assisted peptide hydrolysis was almost as efficient as when the hydrolysis reaction was conducted in the presence of 4,13-diaza-18-crown-6. However, when Tris was used, hydrolysis yields were decreased from 90% to 22%. This indicated that 4,13-diaza-18-crown-6 and HEPES could be employed to facilitate Zr(IV)-assisted peptide hydrolysis, while Tris would be expected to produce an inhibitory effect.

We next reacted a total 17 compounds in the presence of Gly-Gly and $ZrCl_4$. Our goal was to identify ligands capable of further enhancing the reactivity of Zr(IV) at near neutral pH. A second aim was to identify key structural features that might be responsible for either promoting or inhibiting Zr(IV)-assisted peptide hydrolysis. We examined: Tris, HEPES, and 4,13-diaza-18-crown-6 derivatives; macrocyclic ligands similar to 4,13-diaza-18-crown-6; and ligands that were used by other groups to facilitate meal-assisted hydrolysis of phosphodiester bonds. Among the Tris derivatives, ethanolamine showed the high levels of hydrolysis followed by 2-amino-

1,3-propanediol and then Tris. We observed that turbidity and hydrolysis levels were reduced in the same order, according to the potential ability each ligand to form multiple chelate rings. Among the HEPES series, HEPES produced the highest hydrolysis yields followed by 1,4-piperazinediethanol, 2-amino-ethanesulfonic acid, and 1-piperazineethanol. Thus, any change made to the HEPES framework had a negative impact on cleavage efficiency. Among the 4,13-diaza-18-crown-6 derivatives, 4,13-diaza-18-crown-6 showed the highest hydrolysis yields followed by 2-(2-aminoethoxy)-ethanol, 2,2'-iminobis-ethanol, and then the multidentate chelating ligand 2,2',2"-nitrilotris-ethanol. In addition to producing extremely low levels of peptide hydrolysis, 2,2',2"-nitrilotris-ethanol substantially reduced Zr(IV) precipitation. Both 4,13-diaza-18-crown-6 and 2-(2-aminoethoxy)-ethanol produced more cleavage compared to 2,2'-iminobis-ethanol, leading us to hypothesize that the structural feature "$ROCH_2CH_2OCH_2CH_2NR$" might contribute to efficient peptide hydrolysis by Zr(IV). In our comparison of macrocyclic 18-menbered and 15-membered crown ether ring systems containing the hard donor atoms N and/or O, hydrolysis yields were reduced in the following order: 4,13-diaza-18-crown-6 ~ 4,10-trioxa-7,13-diazacyclopentadecane > 1,4,7,10-tetraoxa-13-azacyclopentadecane ≥ 18-crown-6. The ligands 4,13-diaza-18-crown-6 and 4,10-trioxa-7,13-diazacyclopentadecane possess two nitrogen donor ligands, while 1,4,7,10-tetraoxa-13-azacyclopentadecane, and 18-crown-6 have 1 and 0, respectively. We therefore concluded that the presence of nitrogen atoms might have had a positive impact on Zr(IV)-assisted peptide hydrolysis yields.

Finally, we investigated three open-chain ligands which facilitate either Ce(IV)- or Zr(IV)-assisted phosphodiester bond hydrolysis: D-glucamine, 2,6-pyridinedimethanol, and *N*,*N'*-[(2-hydroxy-1,3-phenylene)bis(methylene)]bis[*N*-(carboxymethyl)-glycine]. Zirconium(IV)

precipitation was dramatically reduced in each case and all three ligands produced negligible levels of peptide hydrolysis.

Overall, we demonstrated that Zr(IV)/4,13-diaza-18-crown-6 is capable of efficiently hydrolyzing peptides under near physiological conditions of temperature and pH. The kinetics of Zr(IV) hydrolysis in the presence of 4,13-diaza-crown-6 are much faster compared to spontaneous hydrolysis of the unactivated peptide amide bond. We also showed that it was not necessary for a ligand to have a ring structure to enhance the reactivity of Zr(IV), and that ligands with a macrocyclic framework appeared to be slightly more effective. Finally, we observed that the strong ligands capable of forming multiple chelate rings simultaneously reduced levels of Zr(IV) precipitation and levels of peptide hydrolysis. We therefore speculated that, at neutral pH, effective Zr(IV)-assisted peptide hydrolysis might be heterogeneous in nature.

In the future, we aspire to develop new Zr(IV) complexes which substantially increase the efficiency and specificity of peptide hydrolysis under physiological conditions of temperature and pH. These complexes could one day be utilized as new therapeutic agents designed to target disease-related proteins.