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Tuning Zr(IV)-assisted peptide hydrolysis at near-neutral pH

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Abstract

The present study has compared the effects of a total of 17 ligands on Zr(IV)-assisted hydrolysis of the dipeptide Gly–Gly (60 °C, pH 6.8–7.4, $t = 4$ h and $t = 10$ h). The macrocyclic azacrown ether ligands 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane and 1,4,10-trioxa-7,13-diazacyclopentadecane produced the overall highest amounts of hydrolysis, followed by the open-chain ligand 2-(2-aminoethoxy)-ethanol. While it was not necessary to have a ring structure to enhance Zr(IV) reactivity, the structural feature "$ROCH_2CH_2OCH_2CH_2NR$" appeared to contribute to increased levels of peptide cleavage.

Keywords: Amide bond; Cleavage; 4,13-diaza-18-crown-6; Ligand; Metal

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,1c,1d,1e,1k,1m,1n,1p,1q,1r,1s,1t]. 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 [1j]. A new bidentate thioether 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 [1p]. 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 conditions [1t]. It was found that 25% of the dipeptide was cleaved at 60 °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 [1n,1q,1r]. 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 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 ≈15.7 to ⩽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

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efficient catalytic turnover. At pH values above 5.0–5.2, Zr(IV) forms insoluble gels and precipitates [7].

In a previous paper, we showed that the macrocyclic ligand 1,4,10,13-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 [1n]. Insoluble Zr(IV) precipitates were formed in both of the reactions and 4,13-diaza-18-crown-6 did not reduce the extent of the precipitation. 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). When 78 mM of HEPES was added to a typical hydrolysis reaction (60 °C and pH 6.6–7.1, 2 mM Gly–Gly, 10 mM $ZrCl_4$, 20 h), Gly–Gly was hydrolyzed in high yield (80%), similar to the cleavage produced using 20 mM of 4,13-diaza-18-crown-6 (90%), and comparable levels of Zr(IV) precipitation were observed [1r]. However, in the presence of 40 mM of Tris, hydrolysis was only 22% complete, similar to Zr(IV)-assisted hydrolysis of Gly–Gly when 4,13-diaza-18-crown-6, HEPES, and Tris were omitted (26%) [1n]. The second effect of Tris was to significantly reduce Zr(IV) precipitation. Thus, 4,13-diaza-18-crown-6 and HEPES were show to facilitate Zr(IV)-assisted peptide hydrolysis, while Tris produced a slight inhibitory effect. 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. 1 and Supplementary Figs. S3 and S4 in Supporting Information). 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 Zr(IV)-assisted peptide hydrolysis.

Individual solutions containing 2 mM of the dipeptide Gly–Gly, 10 mM of $ZrCl_4$, and 20 mM of ligand were reacted at 60 °C and near-neutral pH (Supporting Information). Aliquots were removed at 0 h, 4 h, and 10 h time points, after which unreacted dipeptide and amino acid monomers released upon peptide amide bond hydrolysis were derivatized with 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride) and then identified by reversed-phase HPLC. Thus, the amounts of hydrolysis produced in the presence of each of the 17 ligands were ranked by calculating ratios of the HPLC peak height of the dabsylated hydrolysis product glycine to the peak height of dabsylated Gly–Gly (Supporting Information).

In our first experiment, we compared Tris (**1**) to its component parts 2-amino-1,3-propanediol (**1a**) and ethanolamine (**1b**) (Fig. 1a and Supplementary Fig. S3a). We again observed that ligand **1** was successful in substantially reducing the formation of insoluble Zr(IV) precipitates. In addition, **1** 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** was shown to significantly reduce precipitation, but increase cleavage yields [2b,2c].) Ligand **1a** produced intermediate levels of hydrolysis and precipitation, followed by **1b**, which produced the most precipitation and the highest amounts of peptide cleavage. Ligands **1**, **1a**, and **1b** form complexes with a wide variety of metals including Eu(III) (**1**), Cu(II) (**1** and **1a**), and Zr(IV) (**1b**) [8]. In acidic solutions, Zr(IV) forms a weak bis complex in which each ethanolamine ligand **(1b)** is bidentate and coordinates through its N and O donor atoms [8a,8c]. Alternatively, the 3:1 complex formed between **1** and Cu(II) is relatively strong ($\log K_f = 9.55$ in H_2O) [8b]. We therefore hypothesized that levels of Zr(IV) precipitation and peptide hydrolysis might be reduced according to the ability of individual ligands to form stable complexes containing multiple 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. 1b and Supplementary Fig. S3b). It had 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 metals such as Pr(III), Ni(II), and Cu(II) [10]. While ligand **2c** has not been studied, **2b** forms a ternary complex with [Cu(II)(Gly–Gly)] in which **2b** 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. Notwithstanding, significant Zr(IV) precipitation and reasonable levels of hydrolysis were observed in all of the 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. 1c and Supplementary Fig. 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** [1n]. While **3a** has not been studied, acidic solutions of **3b** form a weak mono complex in which the ligand produces a single, eight-membered chelate ring by coordinating to Zr(IV) through its two oxygen donor atoms [8a,8c]. Interestingly, the 1:1 complex formed between ligand **3c** and Cu(II) is strong ($\log K_1 = 18.4$, in H_2O at pH 7.0). The nitrogen and three oxygen atoms of the ligand interact with Cu(II) to form a total of three five-membered chelate rings [12]. In the presence of Zr(IV), ligands **3** and **3a** generated approximately the same levels of peptide hydrolysis within experimental error at the