Inorg. Chem. **2004**, *43*, 6130–6132

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

Miki Kassai, R. Gnana Ravi, Sarah J. Shealy, and Kathryn B. Grant*

Department of Chemistry, Center for Biotechnology and Drug Design, Georgia State University, P.O. Box 4098, Atlanta, Georgia 30302-4098

Received April 30, 2004

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 °C).

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,k,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 envisaged 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 pK_a values of Zr^{IV}-bound water molecules are ≤ 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 ≤ 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

* To whom correspondence should be addressed. E-mail: kbgrant@gsu.edu.

(1) (a) 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.

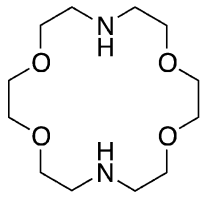
10.1021/ic049433j CCC: $27.50
Published on Web 09/02/2004

Table 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]

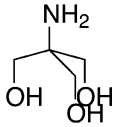
		Zr^{IV}		azacrown ether		Zr^{IV} + azacrown ether		
	peptide	pH	yield (%)	pH	yield (%)	pH	yield (%)	ether increase (%)
1	KG	7.4	12	7.2	0	7.1	17	42
2	GK	7.1	16	7.0	0	7.1	17	6
3	GL	7.0	5	7.2	0	7.2	35	600
4	LG	7.0	3	7.2	1	7.2	54	1700
5	GH	7.3	15	7.2	0	7.1	56	273
6	PG	7.2	7	7.2	1	7.1	63	800
7	HG	7.0	10	7.2	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}] × 100. Reported pH values are an average of pre- and postreaction measurements. Average pH drifts were 1.3 ± 0.4, 0.1 ± 0.1, and 0.1 ± 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.

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.



1



2

A series of 16 dipeptides was studied first (Table 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 azacrown 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 °C,[10] we utilized the azacrown ether to buffer the reaction pH. As expected, pre- and postreaction measurements revealed minimal pH drift; Table 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 reverse-phase HPLC analysis (Supporting Information).

Whereas zirconium(IV)-assisted 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 190−1775% upon addition of the azacrown ether (Table 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 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 1). It is also important to note that insoluble Zr^{IV} precipitates were formed in all of the $ZrCl_4$ 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 azacrown ether (60 °C, 2 mM Gly-Gly, 10 mM $ZrCl_4$, 20 h). Because the pK_a of the Tris ligand is 8.1 at 25 °C, the reaction pH was adjusted to 7.0 by direct addition of **2**.[9] Interestingly, although **2** helped to reduce Zr^{IV} precipitation, peptide hydrolysis was slightly decreased under the experimental conditions we employed (Table 1, entry 17). 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.[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 by 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 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.

(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.

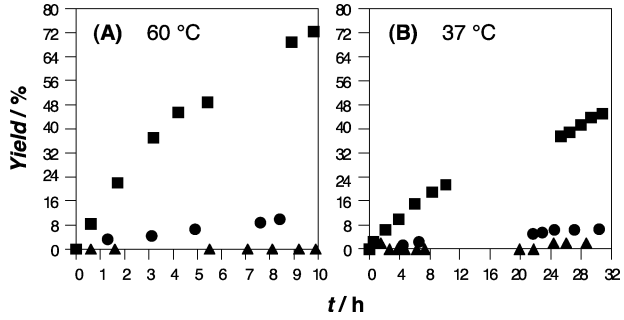


Figure 1. Time course plots for hydrolysis of 2 mM Gly-Glu. Percent yield = (mM free Gly released/2 mM) × 100. (a) 60 °C: (■) 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, pH 7.1; (●) 10 mM $ZrCl_4$, pH 7.1; (▲) 20 mM 4,13-diaza-18-crown-6, pH 7.0. (b) 37 °C: (■) 10 mM $ZrCl_4$, 20 mM 4,13-diaza-18-crown-6, pH 7.3; (●) 10 mM $ZrCl_4$, pH 7.0; (▲) 20 mM 4,13-diaza-18-crown-6, pH 7.0.

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 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 4 mM 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 1, entry 20). Time course experiments were then conducted at 37 and 60 °C to monitor reaction kinetics. Products obtained at individual time points were derivatized with dabsyl chloride, and hydrolysis yields were determined by subsequent reverse-phase HPLC analysis (Figure 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 to be 36.6 ± 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).[12] At pH 7.1 and 60 °C, $t_{1/2}$ was 69.3 ± 5.5 and 5.3 ± 0.1 h for Zr^{IV} hydrolysis of Gly-Glu without and with 4,13-diaza-18-crown-6, respectively. (As shown in Figure 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 4,13-diaza-18-crown-6 were reacted at 60 °C, pH 7. Yields of free glycine were 56%, 75%, and 83% after 45, 94, 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.

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 $ZrCl_4$ (pH 7, 23.5 °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 $ZrOCl_2 \cdot H_2O$ to substitute for 10 mM $ZrCl_4$ and reacted the oxide chloride with 2 mM Gly-Glu in the absence and presence of 4,13-diaza-18-crown-6 (pH 7.0−7.2, 60 °C, 20 h). Hydrolysis yields were 16% and 77%, respectively (Table 1, entry 21). In addition, only 7 mM 4,13-diaza-18-crown-6 was required to achieve a final pH of 7.2, indicating that $ZrOCl_2 \cdot H_2O$ 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.

Acknowledgment. We thank the National Science Foundation for financial support under CAREER Award CHE-9984772 and the American Chemical Society Petroleum Research Fund for Type G Grant 32897-G3.

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.

IC049433J

(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.