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Communications

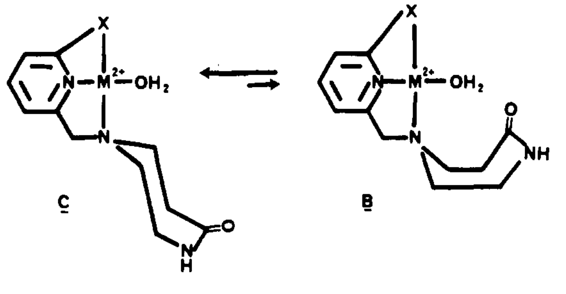
Models of Zinc-Containing Proteases. Rapid Amide Hydrolysis by an Unusually Acidic Zn^{2+}-OH_2 Complex[1]

Sir:

The elucidation of the mechanism of peptide hydrolysis by the zinc metalloenzyme carboxypeptidase A (CPA) has been hampered by an ambiguity in the catalytic role of the Zn^{2+}. The primary function could involve either carbonyl polarization, in which the Zn^{2+} serves as a Lewis acid to bind the carbonyl and to make it more susceptible to nucleophilic attack, or the formation of a zinc-bound hydroxide, which would allow a more potent hydroxide nucleophile to be present at neutral pH.[2] Zinc–carbonyl mechanisms for CPA have been in favor, since it is this geometry that is present in the 2.0 Å resolution X-ray structure of CPA with the poor substrate Gly-Tyr.[3] Further, the kinetic pK_a of 6.1–7.0 observed for CPA[4] has been associated with the deprotonation of Glu-270 rather than the ionization of a zinc-bound water.[5]

We describe here the synthesis and characterization of a rigid zinc–amide complex in which a zinc-bound water is positioned perpendicular to the plane of an amide bond. Zinc-promoted hydrolysis of the amide bond was observed to occur in this complex with a rate enhancement of 10^7. Furthermore, both titrimetric and kinetic data indicate a pK_a near 7 for the zinc-bound water.

To assess the viability of a metal-bound hydroxide in amide hydrolysis, we have chosen the specific alignment between the amide and metal-bound hydroxide shown in Figure 1 since this geometry allows a favorable interaction of the reactant groups. Previous Cu^{2+} complexes of 1[6] and 2,[7] which incorporate this geometry in conformationally mobile systems, have led to rate enhancements of up to 10^6 for amide hydrolysis at neutral pH and 50 °C. More modest accelerations of 10^3 and no acidic protons were observed for Zn^{2+}. The reaction pathway in these complexes requires a chair–boat equilibrium before the rate-determining step, since an X-ray crystal structure of **1**-Cu^{2+}-Br·H_2O has shown the azalactam moiety in a chair (*C*) rather than a boat (*B*) conformation.[8]



1-M^{2+}-OH_2: X = $-CO_2^-$

2-M^{2+}-OH_2: X = $-CH_2N(CH_3)_2$

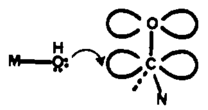
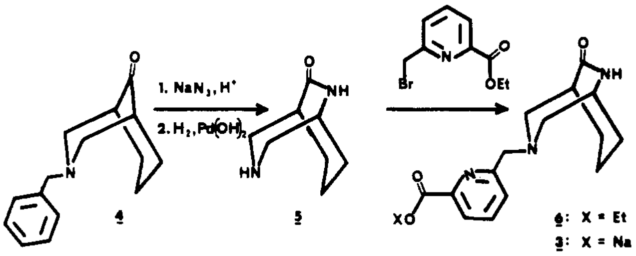


Figure 1. Optimum geometry for nucleophilic attack by M-OH on an amide acyl carbon.

Scheme I



We have synthesized the tridentate ligand **3**, as shown in Scheme I,[9] to eliminate the chair–boat equilibrium from the reaction pathway. The bicyclic azalactam portion of the free ligand is expected to prefer a chair–chair conformation, in analogy with known bicyclo[3.3.1]nonane derivatives.[10] Although **3** can potentially bind a metal ion in a number of geometries, coordination as shown in **3**-M^{2+}-OH_2 forces the adoption of a boat–chair conformation (*B*,*C*) to alleviate severe transannular steric interactions in **3**-M^{2+}-OH_2(*C*,*C*).[11] We have compared the selective ^{13}C line-broadening effects of added Cu^{2+} to solutions of **1** and **3**[12] and have found that the amide carbonyl carbon of **3** is much

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(1) (a) Presented in part at the 186th National Meeting of the American Chemical Society, Washington, DC, 1983. (b) Abstracted from: Olson, J. R. Ph.D. Thesis, The University of Michigan, Ann Arbor, MI, 1983.

(2) Galdes, A. *Inorg. Biochem.* **1982**, *3*, 268–313.

(3) Lipscomb, W. N.; Hartsuck, J. A.; Reeke, G. N., Jr.; Quiocho, F. A.; Bethge, P. H.; Ludwig, M. L.; Steitz, T. A.; Muirhead, H.; Coppola, J. C. *Brookhaven Symp. Biol.* **1968**, *No. 21*, 24–90.

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(5) Lipscomb, W. N. *Tetrahedron* **1974**, *30*, 1725–1732.

(6) Groves, J. T.; Dias, R. M. *J. Am. Chem. Soc.* **1979**, *101*, 1033–1035.

(7) Groves, J. T.; Chambers, R. R. *J. Am. Chem. Soc.* **1984**, *106*, 630–638.

(8) Olson, J. R. Ph.D. Thesis, The University of Mchigan, Ann Arbor, MI, 1983.

(9) Intermediates **4**, **5**, and **6** all gave satisfactory elemental analyses (C,H,N) and spectral properties. Samples of **3** had satisfactory ^1H and ^{13}C NMR spectra.

(10) (a) Peters, J. A.; Baas, J. M. A.; van de Graff, B.; van Bekkem, H. *Tetrahedron* **1978**, *34*, 3313–3323. (b) Jeyaraman, R.; Jawaharshingh, C. B.; Avila, S.; Ganapathy, K.; Eliel, E. L.; Manoharan, M.; Morris-Natscke, S. *J. Heterocycl. Chem.* **1982**, *19*, 449–458.

(11) Approximate values for the stability constants of Zn^{2+} and Cu^{2+} complexes with **3** were graphically determined from potentiometric data to be 1.5×10^4 and 7.1×10^5, respectively.

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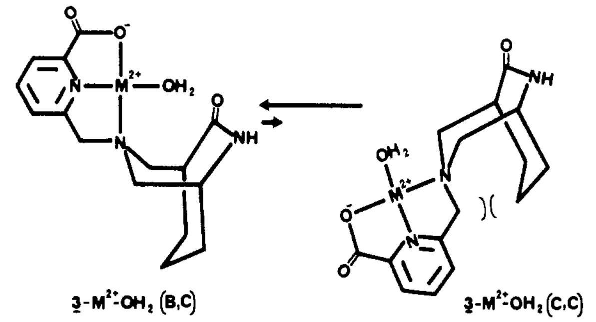

3-M^{2+}-OH$_2$ (B,C) **3**-M^{2+}-OH$_2$ (C,C)

more sensitive to added Cu^{2+} than the respective carbon of **1** (Figure 2). This is consistent with the proposed solution structures for **1**-M^{2+}-OH and **3**-M^{2+}-OH$_2$, since the distance between the paramagnetic Cu^{2+} and the amide carbonyl is significantly shorter in the latter.

A pH–rate profile for the amide hydrolysis of **3**-Zn^{2+}-OH$_2$ at 50 °C over the pH range 5.2–7.4 is shown in Figure 3.[13] The sigmoidal pH–rate curve is similar to that of **1**-Cu^{2+}-OH$_2$ and **2**-Cu^{2+}-OH$_2$[6,7] and can be understood in terms of hydrolysis occurring through both a zinc-bound water form (k_1) and a zinc-bound hydroxide form (k_2) in which **3**-Zn^{2+}-OH reacts 10 times faster than **3**-Zn^{2+}-OH$_2$. The base-catalyzed process (k_3) slightly improves the fit at high pH values[6,7] although its contribution is small. These processes take the form of eq 1, in which K_w is the

$$k_{\mathrm{obsd}} = k_1\left[\frac{a_{\mathrm{H}}}{K_{\mathrm{app}} + a_{\mathrm{H}}}\right] + k_2\left[\frac{K_{\mathrm{app}}}{K_{\mathrm{app}} + a_{\mathrm{H}}}\right] + k_3\frac{K_{\mathrm{w}}}{a_{\mathrm{H}}}\left[\frac{K_{\mathrm{app}}}{K_{\mathrm{app}} + a_{\mathrm{H}}}\right] \tag{1}$$

autopyrotolysis constant for water (pK_w = 12.97, 50 °C, 0.5 M $NaClO_4$) and K_{app} is the apparent ionization constant required to fit the data. The ratios in brackets represent the mole fractions of the respective zinc species. A least-squares fit of the data to eq 1 gave $k_1 = 6.48 \times 10^{-7}$ s^{-1}, $k_2 = 7.14 \times 10^{-6}$ s^{-1}, $k_3 = 1.10 \times 10^{-4}$ M^{-1} s^{-1}, and pK_{app} = 7.01. Comparison of k_{obsd} at pH 7 with the estimated rate constant for the base hydrolysis of **3** at pH 7 ($k = 2.8 \times 10^{-13}$ s^{-1})[14] indicated a *rate enhancement of 1.4 × 10^7 for amide hydrolysis by Zn^{2+}* at 50 °C. This is in marked contrast to the small or even inhibitory rate enhancement of model systems that emulate a "carbonyl polarization" geometry between metal ion and amide carbonyl.[15]

Titrations of the Zn^{2+} and Cu^{2+} complexes of **3** at 50 °C showed two equivalence points, attributable to first the neutralization of two ligand protons and then to the titration of a metal-bound water. The pK_a values for the metal-bound water in **3**-Zn^{2+}-OH$_2$ and **3**-Cu^{2+}-OH$_2$ were found to be extraordinarily low, 7.1 and 6.6, respectively. Furthermore, both titrations were completely reversible. The pK_a of the zinc-bound water must be considered an estimate, however, since the amount of metal-bound water titrated normally corresponded to 0.6–0.8 equiv of base.[16]

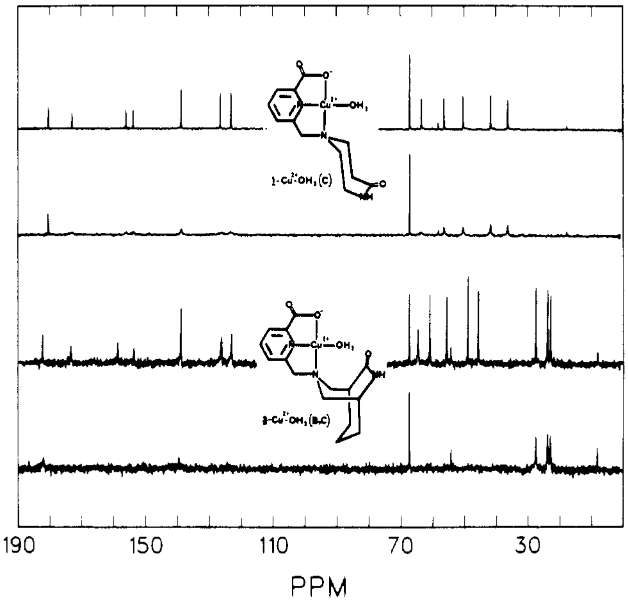

Figure 2. ^{13}C relaxation in **1** (top) and **3** (bottom) by addition of Cu^{2+}. Ligand to Cu^{2+} ratios of 1000 and 25 are shown for each ligand. All spectra were determined in D_2O with dioxane (67 ppm) as an internal standard.

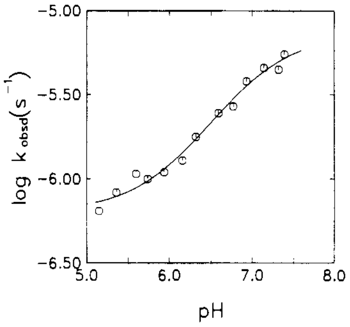

Figure 3. Plot of log k_{obsd} vs. pH for the amide hydrolysis of **3**-Zn^{2+}-OH$_2$ at 50 °C. Reaction solutions were 0.4 M in buffer (MES or HEPES) and adjusted to μ = 0.5 M by the addition of $NaClO_4$. The hydrolyses were followed by monitoring the production of primary amine as determined with an *o*-phthalaldehyde reagent[6,7] at 335 nm with a Cary 219 spectrophotometer. Suitable first-order plots were obtained over the entire pH range with correlation coefficients ≥0.98.

The low pK_a values for the metal-bound water in Zn^{2+} and Cu^{2+} complexes of **3** are due in part to the coordination environment of the metal ion, which is anticipated to have a five-coordinate geometry[17] comprised of the tridentate **3**, an equatorial water, and an axial water. Such a reduction in coordination number is expected to have a pK_a-lowering effect on a metal-bound water.[18] Furthermore, on the basis of estimated individual ligand field of the Cu^{2+} complex,[19] only one strong donor ligand is present. Thus, these complexes have the advantage of tridentate chelation that discourages dimerization and disproportionation reactions,[20] yet losses of Lewis acidity at the metal ion are minimized due to a five-coordinate geometry that has only one strong donor group.

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(12) For discussion of Cu^{2+} broadening effects, cf: (a) Dillon, K. B.; Rossotti, F. J. C. *J. Chem. Soc., Dalton Trans.* **1973**, *1005*–1013. (b) Voelter, W.; Sokolowski, G.; Weber, U.; Weser, U. *Eur. J. Biochem.* **1975**, *58*, 159–166. (c) Espersen, W. G.; Martin, R. B. *J. Am. Chem. Soc.* **1976**, *98*, 40–44.

(13) Amide hydrolysis was also found to occur in homogeneous solutions from pH 7.4 to 7.8. However, unambiguous rate constants could not be calculated in this region due to problems in obtaining reasonable A_∞ values.

(14) The pseudo-first-order rate constant k, for the base hydrolysis of **3** at pH 7, was estimated from the second-order base hydrolysis constant ($k_{\mathrm{OH}} = 2.6 \times 10^{-7}$ M^{-1} s^{-1}) and hydroxide concentration ([OH$^-$] = $1.0 \times 10^{-5.97}$ M, pK_w = 12.97, 50 °C, 0.5 M $NaClO_4$); i.e., $k = k_{\mathrm{OH}}[\mathrm{OH}^-]$.

(15) (a) Fife, T. H.; Squillacote, V. L. *J. Am. Chem. Soc.* **1977**, *99*, 3762–3769. (b) Fife, T. H.; Squillactoe, V. L. *Ibid.* **1978**, *100*, 4787–4793. (c) Tang, C. C. Ph.D. Thesis, Columbia University, 1978. (d) Breslow, R.; Fairweather, R.; Keana, J. *J. Am. Chem. Soc.* **1967**, *89*, 2135–2138.

(16) Titration of less than a full equivalent of M-OH$_2$ has also been observed by: (a) Hay, R. W.; Morris, P. J. *J. Chem. Soc. A* **1971**, 1518–1523. (b) Brown, R. S.; Huguet, J. *Can. J. Chem.* **1980**, *58*, 889–901. (c) Bertini, I.; Canti, G.; Luchinat, C.; Mani, F. *Inorg. Chem.* **1981**, *20*, 1670–1673.

(17) For clarity, all of the metal complexes in this report have been drawn without the expected water molecule in the fifth coordination site.

(18) (a) Courtney, R. C.; Gustafson, R. L.; Chaberek, S., Jr.; Martell, A. E. *J. Am. Chem. Soc.* **1958**, *80*, 2121–2128. (b) Wooley, P. *Nature (London)* **1975**, *258*, 677–682. (c) Billo, E. J. *Inorg. Nucl. Chem. Lett.* **1975**, *11*, 491–496.

(19) Billo, E. J. *Inorg. Nucl. Chem. Lett.* **1974**, *10*, 613–617.

(20) Martell, A. E.; Chaberek, S., Jr.; Courtney, R. C.; Westerback, S.; Hyytiainen, H. *J. Am. Chem. Soc.* **1957**, *79*, 3036–3041.

Comparison of the titration data for Zn^{2+} and Cu^{2+} complexes of **1** and **3** reveal an intriguing phenomenon. Although a similar coordination environment is expected in the metal complexes of **1** and **3**, a large difference exists in the pK_a of the metal-bound waters. In particular, titrations at 50 °C have previously shown a pK_a of 7.1 for **1**-Cu^{2+}-OH_2, while a pK_a for **1**-Zn^{2+}-OH_2 could not be observed due to precipitation.[6,7] That **1**-Cu^{2+}-OH_2 and **3**-Cu^{2+}-OH_2 have similar coordination geometries is evidenced by the fact that λ_{max} for each complex is about 735 nm.[19] Thus, the lower pK_a values for the metal-bound water in Zn^{2+} and Cu^{2+} complexes of **3** must be due in part to factors other than the net Lewis acidity of the metal ion. Differential solvation effects in **1**-M^{2+} and **3**-M^{2+} is one attractive explanation since the metal-bound water in **3**-M^{2+}-OH_2 may lie in a more hydrophobic environment in the boat conformation of the azalactam than in the chair. Hydrophobic effects have been shown to affect metal-bound water acidity in Zn^{2+} and Cu^{2+} complexes[21] and have been proposed to account for lowered pK_a for a zinc-bound water in the zinc metalloenzyme carbonic anhydrase.[22] An electrophilic interaction of the nearby amide carbonyl in **3**-M^{2+}-OH_2 with the metal-bound water could also lower the pK_a.

In summary, the development here of a Zn^{2+} model complex that exhibits a rate enhancement of 10^7 for Zn^{2+}-promoted amide hydrolysis and a pK_a of 7 for a zinc-bound water offers compelling evidence that a "zinc hydroxide" mechanism is viable in this model system. A similar mechanism should be considered for CPA. Indications that the Glu-270 may assist in the deprotonation of the zinc-bound water in CPA[23,24] are consistent with this suggestion.

Acknowledgment. Partial support of this work by the National Science Foundation is gratefully acknowledged.

Registry No. **1**-Cu^{2+}-OH_2, 69896-33-9; **3**, 97278-41-6; **3**-Cu^{2+}-OH_2, 97278-39-2; **3**-Zn^{2+}-OH_2, 97278-40-5; **4**, 81879-64-3; **5**, 97278-42-7; **6**, 97278-43-8; 6-(bromomethyl)-2-(ethoxycarbonyl)pyridine, 97278-44-9; proteinase, 9001-92-7.

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(22) (a) Coleman, J. E. *Ann. N.Y. Acad. Sci.* **1984**, *429*, 26–48. (b) Lindskog, S. E. In "Zinc Enzymes"; Spiro, T. G., Ed.; Wiley: New York, 1983; Vol. 5. (c) Prince, R. H.; Woolley, P. R. *Angew. Chem., Int. Ed. Engl.* **1972**, *11*, 408–417.

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Articles

Contribution from the Department of Chemistry,
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Kinetic Study of the Complexing of 2-Aminophenol Derivatives by Nickel(II)

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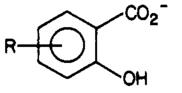
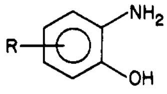
Received November 19, 1984

The systems 2-aminophenol + $Ni(OH_2)_6^{2+}$ and 2-aminophenol-4-sulfonic acid have been investigated by measuring the ligand acid dissociation constants, monoligand complex formation constants, and the kinetics of complexing for pH 6.4–7.0 in 0.30 M $LiClO_4$ at 25 °C. The kinetic results indicate that first-bond formation at the OH function is rate limiting with a rate constant of 2.7×10^3 M^{-1} s^{-1} and 2.9×10^3 M^{-1} s^{-1} for 2-aminophenol and 2-aminophenol-4-sulfonate, respectively. The results are compared and rationalized with respect to earlier studies of salicylate derivatives and acetohydroxamic acid.

A recent kinetic study[1] of the reaction of $Ni(OH_2)_6^{2+}$ with salicylate derivatives (I) provided information on the reactivity of these systems and the acidity of the coordinated OH. This information should be useful in the interpretation of results on 2-aminophenol derivatives (II), where the initial reaction site (OH or NH_2) is less certain and intramolecular hydrogen bonding might reduce the reactivity of II. The dissociative ion-pair mechanism[2] would lead one to predict that the anion I should react more rapidly than the neutral ligand II. As will be seen, the simple formation rate constants are actually in the opposite order because the rate-limiting step is chelate ring closing with I but first-bond formation with II.



I



II

The complex formation constants with 2-aminophenol and 2-aminophenol-4-sulfonic acid have been measured because the earlier values of Sims[3] and Perrin[4] have been put into question by recent results.[5]

Experimental Section

Materials. 2-Aminophenol (Aldrich Chemical Co.) and 2-aminophenol-4-sulfonic acid (Riedel-de Haenag-Seelze) were recrystallized twice by dissolution in aqueous $NaHCO_3$ followed by addition of HCl to pH 4, under an argon atmosphere. The solid was collected and dried under vacuum.

Anal. Calcd for 2-aminophenol (C_6H_7NO): C, 66.04; H, 6.47; N, 12.84. Found: C, 65.90; H, 6.46; N, 12.73. Calcd for 2-aminophenol-4-sulfonic acid hemihydrate ($C_6H_7NO_4S\cdot{}^1/_2H_2O$): C, 36.40; H, 4.04; N, 7.06. Found: C, 36.16; H, 4.08; N, 6.93.

Aqueous nickel(II) perchlorate was prepared from nickel carbonate and perchloric acid and standardized as described previously.[6] The buffers PIPES (Aldrich) and MES (Sigma) were used as supplied.

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