Models of Zinc-Containing Proteases. Catalysis of Cobalt(III)-Mediated Amide Hydrolysis by a Pendant Carboxylate

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Abstract: The rate of intramolecular amide hydrolysis in substitutionally inert Co(III) complexes has been investigated. In these models for zinc-containing peptidases, such as carboxypeptidase A, thermolysin, and angiotensin-converting enzyme, the Co(III) is oriented perpendicularly to the amide plane in such a way that neither the lone pairs of the carboxyl oxygen nor the amide nitrogen can coordinate to the metal. A metal-bound water or hydroxide, however, has facile access to the acyl carbon in these complexes. Large rate enhancements of 10^7–5×10^8 were observed for Co(III)-mediated amide hydrolysis. A buffer effect was observed in the neutral region of the pH rate profile where a significant portion of the reaction proceeded through the metal-bound hydroxide species. In the same pH region, amide hydrolysis was 20 times faster in the presence of a bifunctional buffer such as phosphate. These results suggest a buffer-assisted breakdown of a tetrahedral intermediate. The introduction of a carboxylic acid function in close proximity to the reactive hydroxo cobalt(III) center of the complex enhanced the rate of metal-promoted amide hydrolysis by more than 30 times over that due to the metal alone. The initial product of amide hydrolysis was shown to have a free amino group, which subsequently coordinated to the metal center. Intramolecular amide hydrolysis by a metal-bound hydroxide, a process shown to benefit from advantageous stereoelectronic effects, is the only mechanism consistent with the results. A similar zinc–hydroxide mechanism can be advanced for carboxypeptidase in which Glu-270 serves to deprotonate a metal-bound water and protonate the departing amine.

The mechanism of peptide bond hydrolysis catalyzed by the zinc-containing proteases such as carboxypeptidase A, thermolysin, and angiotensin-converting enzyme has been under intense investigation for many years.[1] The first high-resolution X-ray crystal structures of carboxypeptidase A with the slow-reacting substrate glycyltyrosine at the active site revealed coordination of the substrate amide carbonyl oxygen to zinc which, in turn, was coordinated to the enzyme through two histidines and a glutamic acid. Also apparent were a nearby tyrosine hydroxyl (Tyr-248), a glutamic acid carboxylate (Glu-270), and an arginine (Arg-145).[2] This arrangement suggested a mechanism for proteolysis in which the Glu-270 carboxylate served either as a nucleophile to afford an acyl enzyme intermediate[3] or as a general base to facilitate the addition of hydroxide to the carbonyl.[4] Tyr-248 had been ascribed a role as proton donor to the incipient amino nitrogen. Interestingly, thermolysin, though evolutionarily unrelated to carboxypeptidase A, was found to have a nearly identical array of active site functional groups and metal ligands.[5]

Several lines of evidence now suggest a mechanism for amide hydrolysis in which a zinc-bound hydroxide serves as the nucleophile for the acyl carbon.[6] A zinc–hydroxide mechanism is generally accepted for the mechanism for carbonic anhydrase.[7] Carboxypeptidase A has been shown by site-specific mutagenesis not to require Tyr-248 for protease activity;[8] accordingly, some other active site functional group must perform that function. Further, thermolysin has been shown not to have an active site counterpart to Tyr-248 in carboxypeptidase A.[5] Finally, the only model systems that have shown large rate enhancements for zinc-mediated amide hydrolysis have been those from our laboratory[9] and that of Fife,[10] which have been shown to involve a zinc–hydroxide pathway.

That the zinc–hydroxide mechanism may now be preferred for the zinc-containing proteases is further supported by the series of enzyme–inhibitor complexes reported by Christianson and Lipscomb[11] and by Matthews.[5c] The most revealing of these structures shows a hydrated ketone coordinated in a bidentate fashion to the active site zinc of carboxypeptidase A, one of the *gem*-diol oxygens being hydrogen bonded to Glu-270, the other hydrogen bonded to Arg-127. A zinc–hydroxide mechanism, in which Glu-270 serves as the proton-shifting functionality and polarization of the amide carbonyl derives from a hydrogen bond from Arg-127, has been suggested by these authors to accommodate these structures. It has been pointed out by Breslow, however, that a general-base mechanism with zinc polarization of the amide carbonyl can also use Glu-270 as the proton switch.[12] Significant bifunctional catalysis, which has been reported in a

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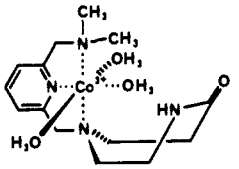
model system involving Co(III)–carbonyl coordination, supports this view.[12]

The model systems we have developed[9] were designed to limit the range of considerable mechanisms for metal-assisted amide hydrolysis by enforcing selective interactions between the amide bond and the metal centers. Specifically, by preventing coordination of the amide carbonyl oxygen to the metal, we have been able to focus attention on the mechanism and reactivity of metal–hydroxides with amides. In one system for which an aquo–zinc moiety is suspended above the plane of the amide linkage,[9c] the $Zn-OH_2$ was found to have an extraordinarily low pK_a, 7.01, and the metal-induced rate enhancement was found to be 1.4×10^7.

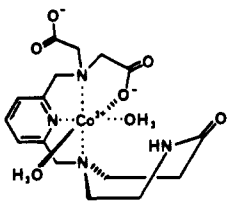
We describe here the examination of the mechanism of the Co(III)-mediated hydrolysis of three similar metal coordinating lactams. This model system has several attractive features; (1) the choice of Co(III) has allowed unambiguous assignment of Co(III)–OH as the reactive nucleophile, (2) the lactam provides an amide functionality of typical peptide reactivity, and (3) a pendant carboxylate as well as phosphate buffer has been observed to provide a significant rate acceleration in accord with the suggested roles of Glu-270 and Glu-143 in carboxypeptidase A and thermolysin.

Results

The azalactam ligands **1** and **2** were prepared by modification of previously described procedures.[9] The corresponding cobalt(III) complexes, (Co^{3+}–**1** and Co^{3+}–**2**), were generated by the addition



Co^{3+}-1



Co^{3+}-2

of hydrogen peroxide to a solution of the corresponding 1:1 Co^{2+} complex. That no Co^{2+} complex remained after this oxidation was confirmed by inspection of the ^1H NMR spectrum. The reactions of both Co^{3+}–**1** and Co^{3+}–**2** were accompanied by changes in the visible spectrum. These observed changes were pH dependent and, upon analysis, represented biphasic kinetics. Therefore, the data were treated as resulting from two consecutive first-order reactions.[13]

A representative plot of ln $(A - A_\infty)$ versus time at pH 5.15 for Co^{3+}–**1** is presented in Figure 1. Subtraction of the effect of the second process resulted in the first-order ln A versus time plot shown in Figure 1 (inset). That the initial process represented the hydrolysis of the amide in these complexes was established by three different methods.

In the first method, replacement of Co^{3+} with Cu^{2+} in aliquots of the reaction mixture led to the appearance of the absorption spectrum of the known amino acid complex,[14] derived independently from the hydrolysis of Cu^{2+}–**1**. The rate constant (k_{obsd}) of amide hydrolysis for Co^{3+}–**1** was determined to be $(2.0 \pm 0.05) \times 10^{-5}$ s^{-1} at pH 7.2 by this method. The appearance of the product could also be monitored colorimetrically by detection of the amino group with ninhydrin. The time dependence of these changes was consistent with the rate of amide hydrolysis deter-

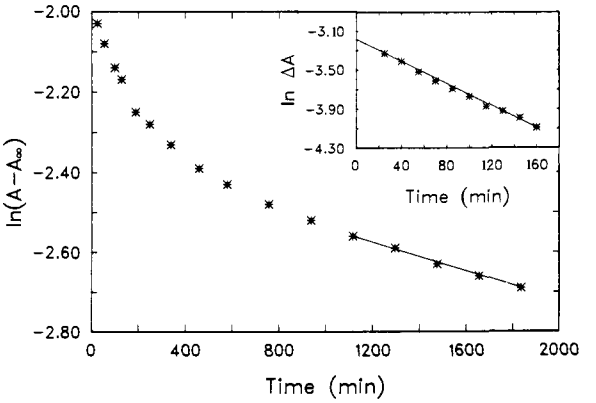


Figure 1. Ln $(A - A_\infty)$ values (*) vs time for the amide hydrolysis and subsequent rearrangement of Co^{3+}–**1** at pH 5.15 in 0.2 M MES buffer at 25 °C and $\mu = 0.5$ ($NaClO_4$). The solid line (—) is the least-squares best fit for the rearrangement process. Inset: Ln ΔA values (*) vs time for the amide hydrolysis of Co^{3+}–**1** at pH 5.15 in 0.2 M MES at 25 °C and $\mu = 0.5$ ($NaClO_4$). The solid line (—) is the least-squares best fit for this process.

mined by treating the data as two consecutive first-order reactions at this pH.

The production of free amine was also monitored by the use of *o*-phthalaldehyde (OPA) and 2-mercaptoethanol. This reagent is known to react rapidly and selectively with primary amines and ammonia to produce a chromophore with λ_{max} 330 nm.[15] The adducts formed have been identified as 1-(alkylthio)-2-alkylisoindoles.[16]

The production of uncoordinated amine was monitored under acidic, neutral, and basic reaction conditions. In all three cases, the concentration of free amine as determined by OPA initially increased due to the release of a primary amine formed upon amide hydrolysis of Co^{3+}–**1** or Co^{3+}–**2**. The rate of this increase coincided with the rates for amide hydrolysis obtained by considering the data as two consecutive first-order reactions. The concentration of free amine reached a maximum and, then, began to decrease. That this decrease in free amine was due to coordination with the Co^{3+} was confirmed by removal of the Co^{3+}. At the completion of the reaction at pH 5.41, the absorbance at 333 nm was 0.06. After removal of the metal from this sample the absorbance at 333 nm was 0.16. Therefore, the reaction with OPA did not occur with the primary amine coordinated to Co^{3+}. The production of an uncoordinated free amine during the hydrolysis of Co^{3+}–**1** was also supported by the appearance of the 3200-cm^{-1} band in the Raman spectrum. Amide hydrolysis was also confirmed by comparison of the 360-MHz ^1H NMR of the recovered reaction product with authentic amino acid. The second linear portion in the plot of ln $(A - A_\infty)$ versus time (Figure 1) represents the coordination of the amine produced upon amide hydrolysis with Co^{3+}.

The rates at which these post amide hydrolysis rearrangements occurred were pH dependent. The appearance of the visible spectrum of the amino acid–metal complexes of **1** and **2** changed with pH. At pH 5.15, the amino acid–metal complex of **1** has a broad visible absorption band (λ_{max} 512 nm, $\epsilon = 15.9$ M^{-1} cm^{-1}). The shape, intensity, and position of the absorption band were all changed at pH 7.91. At this pH, the absorption band was narrower with a λ_{max} at 525 nm ($\epsilon = 323.5$ M^{-1} cm^{-1}).

The infrared spectrum of the hydrolysis products in both acidic and basic solution showed a carbonyl stretching frequency at 1640 cm^{-1}. This frequency is consistent with those observed for associated carboxylates (C(O)–O–M), typically between 1628 and 1658 cm^{-1}.[17] Unassociated, ionized carboxylates have a C–O

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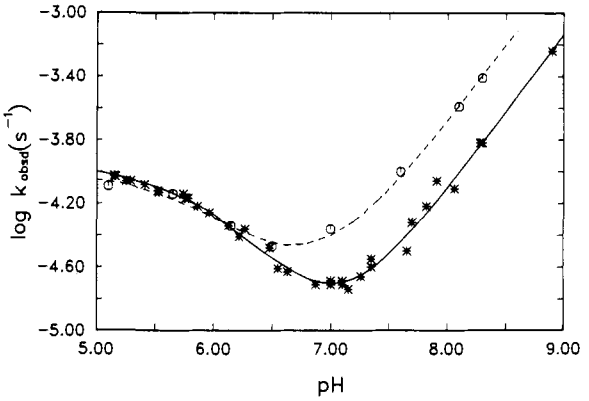

Figure 2. Log k_{obsd} values vs pH for the amide hydrolysis of Co^{3+}–**1** (*) and Co^{3+}–**2** (O) at 25 °C and μ = 0.5 ($NaClO_4$). The solid line (—) is the best-fit curve calculated according to the rate expression given in eq 1 and the rate constants for Co^{3+}–**1** given in Table 1. The broken line (---) is the best-fit calculated curve obtained by using the rate expression of eq 6 and the rate constants for Co^{3+}–**2** given in Table I.

Table I. Comparison of the Rate Constants for the Amide Hydrolysis of Co^{3+}–**1** and Co^{3+}–**2** at 25 °C[d]

complex	pK_a^{titr}	pK_a^{app}	k_1, s^{-1}	k_2, s^{-1}	k_{OH}, M^{-1} s^{-1}
				5.0×10^{-6}	
Co^{3+}–**1**[a]	5.94 ± 0.4	5.92	1.1×10^{-4}	7.1×10^{-7}[c]	57.8
Co^{3+}–**2**[b]	6.05 ± 0.3	5.83	9.8×10^{-5}	1.6×10^{-5}	150.0

[a] r^2 = 0.9967 for fit of rate data to eq 1. [b] r^2 = 0.9989 for fit of rate data to eq 1. [c] Extrapolated to zero buffer. [d] All data determined at μ = 0.5 M ($NaClO_4$).

stretching frequency around 1600 cm^{-1}.[15]

pH Dependence of Amide Hydrolysis for Co^{3+}–1 and Co^{3+}–2. The rates of amide hydrolysis in Co^{3+}–**1** and Co^{3+}–**2** were investigated in detail between pH 5.10 and 8.9. The experiments were carried out at 25 °C at constant ionic strength in 0.2 M noncomplexing buffers such as 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), and 2-(cyclohexylamino)ethanesulfonic acid (CHES).[18] The pH rate profiles for the amide hydrolyses of Co^{3+}–**1** and Co^{3+}–**2** are illustrated in Figure 2. Both hydrolyses could best be accommodated by first-order reactions of an aqua–Co^{3+} lactam complex and the corresponding hydroxo–Co^{3+} complex. The overall rate expression took the form of eq 1 for the amide hydrolysis depicted in Schemes I and II.

$$k_{obsd} = k_1[a_H/(K_a + a_H)] + k_2[K_a/(K_a + a_H)] + k_{OH}[K_a/(K_a + a_H)][K_w/a_H] \quad (1)$$

The curves in Figure 2 were calculated from the experimental data by using eq 1, where K_w was the autoprotolysis constant for water (pK_w = 13.9, 25 °C, 0.5 M $NaClO_4$[19]) and K_a was the ionization constant for the metal-bound water required to fit the data. The titrametric pK_a^{titr} for the metal-bound water species of Co^{3+}–**1** was experimentally determined to be 5.94 ± 0.04. The pK_a value used to obtain the best-fit curve in Figure 2, pK_a^{app}, was 5.92. For Co^{3+}–**2** the corresponding values were pK_a^{titr} = 6.05 ± 0.03 and pK_a^{app} = 5.83.

The excellent agreement between the calculated and the titrimetrically determined pK_a values confirmed that the equilibrium involved in the rate process is indeed the deprotonation of the metal-bound water. The values calculated from the least-squares fit of the data to eq 1 gave values of k_1 (1.1×10^{-4} s^{-1}), k_2 (5.04×10^{-6} s^{-1}), and k_{OH} (57.8 m^{-1} s^{-1}) for Co^{3+}–**1**. However, the value for the unassisted metal-bound hydroxide term (k_2) included the effect of the buffer on the reaction. The uncatalyzed k_2 term was determined to be 7.1×10^{-7} s^{-1} by extrapolation to zero buffer.

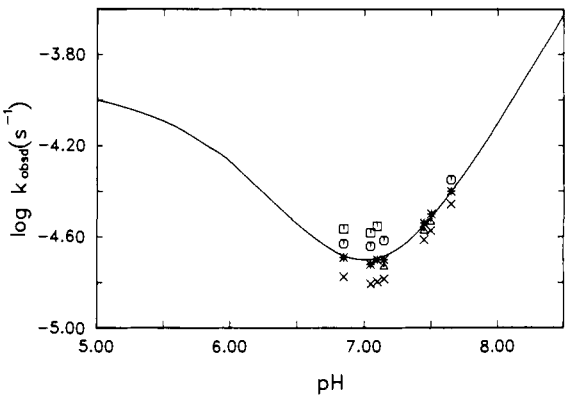

Figure 3. Log k_{obsd} values vs pH for the amide hydrolysis of Co^{3+}–**1** in 0.0 (×), 0.1 (Δ), 0.2 (*), 0.4 M (O), and 0.6 M HEPES buffer (□) at 25 °C and μ = 0.5 ($NaClO_4$). The solid line (—) is the best-fit curve calculated as in Figure 2.

The rate constants k_1 (9.8×10^{-5} s^{-1}), k_2 (1.6×10^{-5} s^{-1}), and k_{OH} (150.0 M^{-1} s^{-1}) were calculated for Co^{3+}–**2**. The results are presented in Table I.

Effect of Buffers and Ionic Strength Variations on the Amide Hydrolysis of Co^{3+}–1. In acidic regimes below pH 5.74, in which the amide hydrolysis proceeded through the aqua–Co^{3+} species (k_1), or at high pH (>8.4), where the second-order base-catalyzed metal-bound hydroxide process (k_{OH}) dominated, the rate of amide hydrolysis showed no effet due to the buffer. Effects of buffer concentration on the amide hydrolysis of Co^{3+}–**1** were evident between pH 6.85 and 7.65, however. In this region the buffer concentration was varied between 0.1 and 0.6 M at constant pH, temperature, and ionic strength (Figure 3). The results from these experiments showed the largest buffer effect around neutrality where the largest portion of the reaction was proceeding through the second term (k_2) of the rate expression.

Whether this buffer catalysis resulted from the acidic or basic form of the buffer was investigated by analyzing the change in apparent rate constants for the buffer-catalyzed reaction (k_2') as the buffer composition was changed (supplementary material). The *y* intercept of this line (k_{BH}) had a value of 1.53×10^{-5} M^{-1} s^{-1} while k_B was 3.31×10^{-5} M^{-1} s^{-1}. When the data were analyzed by the method described by Benkovic and Bruice,[20] similar values of $k_{BH} = 1.64 \times 10^{-5}$ M^{-1} s^{-1} and $k_B = 3.57 \times 10^{-5}$ M^{-1} s^{-1} were obtained. Therefore, it appears that both forms of the buffer participated in the catalysis of the amide hydrolysis of Co^{3+}–**1**, with k_B being slightly larger than k_{BH}.

Since both acid and base forms of the buffer catalyzed the amide hydrolysis of the unassisted metal-bound hydroxide species (k_2), it was of interest to determine if a bifunctional buffer such as phosphate would catalyze the rate. The rate of amide hydrolysis increased with increasing phosphate buffer concentration. In fact, the rate of amide hydrolysis was approximately 20 times faster at pH 6.78 in the presence of 0.2 M phosphate buffer. The rate of amide hydrolysis was also dependent on the ionic strength.

Discussion

The cobalt(III)-assisted amide hydrolysis of **1** and **2** is proposed to proceed through two proton-related forms, the metal-bound water species L–Co^{3+}–OH_2 and the metal-bound hydroxide species L–Co^{3+}–OH. Under acidic conditions, the hydrolysis involves the reaction of the metal-bound water (k_1) with the amide bond or a kinetically equivalent process (e.g., H^+ + L–Co^{3+}–OH). That this process is faster than that observed for the unassisted reaction of metal-bound hydroxide (k_2) is completely analogous to the behavior of Co^{3+}–glycinamide studied in detail by Buckingham.[21]

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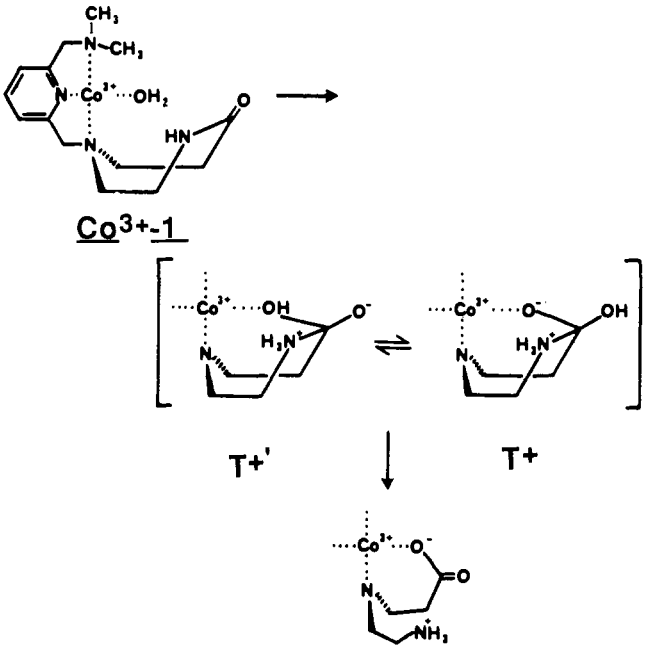
Scheme I

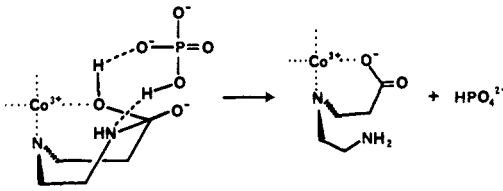


However, water bound to cobalt(III) is unable to add to easily hydrolyzable substrates in analogous bimolecular reactions of $[(NH_3)_5Co(OH_2)]^{3+}$ with CO_2, anhydrides, or labile esters.[22] Further, we have shown that for Cu^{2+}–**1** and Zn^{2+}–**1** the M–OH species is ca. 10-fold more reactive than M–OH_2.[9]

Space-filling models of these complexes indicate that the metal-bound hydroxide is in the proper position for amide hydrolysis, nearly in contact with the acyl carbon of the amide when the azalactam is in the required boat conformation. Buckingham et al. have studied both lactonization and amide hydrolysis of cobalt(III) complexes. In both cases it was shown through ^{18}O-labeling experiments that these processes were intramolecular.[21] The first-order (in metal complex) kinetic behavior observed for the cobalt(III) complexes of **1** and **2** indicates that the amide hydrolysis in Co^{3+}–**1** and Co^{3+}–**2** is intramolecular as well.

A likely mechanism for amide hydrolysis through the Co^{3+}–OH_2 species is presented in Scheme I. The initial proton transfer would result in the tetrahedral intermediates $T^{+\prime}$ and T^{+}. These are similar to the intermediates observed with simple amides under acidic and neutral conditions where the rate-limiting step is considered to be addition to the carbonyl. These intermediates would be expected to cleave the C–N bond rapidly. This process could proceed through discrete intermediates like $T^{+\prime}$ and T^{+} or in a concerted manner as suggested by Buckingham.[21a]

Under neutral or basic conditions, Co^{3+}–**1** and Co^{3+}–**2** are proposed to undergo amide hydrolysis through a metal-bound hydroxide species, L–Co^{3+}–OH. Possible mechanistic pathways for the amide hydrolysis of Co^{3+}–**1** at neutral pH are presented in Scheme II. Intramolecular attack of the metal-bound hydroxide would initially result in the formation of T′. This species can be converted to either T or T^- by proton transfer. An intermediate such as $T^{\pm}$ would be expected to cleave the C–N bond rapidly, while for T, expulsion of RHN^- would be unfavorable until it is converted to $T^{\pm}$ through another proton transfer.[23] In studies of *endo*-6-hydroxybicyclo[2.2.1]heptane-*endo*-2-carboxamides, the T^- intermediate was proposed to hydrolyze directly to product in a concerted step.[24] Therefore, it is reasonable to suggest a concerted process through which T^- could be converted directly to product (k_8) without the formation of intermediates T or $T^{\pm}$. In such a process, the amine leaving group would be protonated by either solvent or buffer simultaneously with the breaking of the amide bond. The breakdown of T^- would be catalyzed by the acidic form of buffers (k_3, k_8). Bifunctional buffers such as phosphate would be expected to convert T′ directly to product by catalyzing the necessary proton transfer in the concerted process presented in below eq 2.



(2)

The above discussion has avoided the question of whether addition of the hydroxide group to the carbonyl (k_2) to form a tetrahedral intermediate or some other step along the reaction coordinate is rate limiting. Buckingham has proposed that the amide hydrolysis of *cis*-$[Co(en)_2(OH)(glyNHR)]^{2+}$ involved rate-determining attack to form the tetrahedral intermediate T′ under neutral conditions and T^- under basic conditions.[21a] In these systems, buffer species acted as general bases, catalyzing the conversion of starting material directly to T^- by deprotonation of M–OH. However, general-base catalysis of the formation of T^- is kinetically indistinguishable with general acid–specific base catalysis of the breakdown of the tetrahedral intermediate T^-.[24] Studies involving imidates have been interpreted as involving rate-determining breakdown of the tetrahedral intermediate under neutral or basic conditions.[25,26] The alcoholysis of amides is the microscopic reverse of the aminolysis of esters. The general-base-catalyzed aminolysis of esters is a stepwise process for which the rate-limiting step is the reaction of T′ and the base necessary for its conversion to T^-.[27] The microscopic reverse of this process would be the acid-catalyzed breakdown of the tetrahedral intermediate T^-. Accordingly, conversion of T^- to the product is implicated as the rate-limiting step.

The data presented here for the hydrolysis of Co^{3+}–**1** could be explained in terms of rate-determining breakdown of T′ for the reaction of the metal-bound hydroxide species. Both the basic and the acidic form of the buffer appear in the rate expression. This suggests that both forms of the buffer can catalyze the necessary proton transfers in order to convert T′ to product (k_2–k_4). In the case of the unassisted metal-bound hydroxide species, this proton transfer would be the result of T′ interacting with the solvent to produce T. In the presence of buffers, T′ could be converted to T′ directly, perhaps accounting for the catalysis seen by buffers around neutrality. In the case of a bifunctional buffer, such as phosphate, T′ could be directly converted to the product by the necessary proton transfer in a concerted process. This would account for the large rate enhancements observed with phosphate buffer. (This same conversion could be accelerated by the free carboxylate ligand in Co^{3+}–**2**, as discussed below.) The rapid hydrolysis in the presence of external hydroxide would result from its ability to catalyze the conversion of T′ to T^- (k_2).

An important mechanistic question concerning these systems is at what point does the amide nitrogen interact with the metal. While this question could not be addressed in our previous investigation of Cu^{2+}–**1** and Zn^{2+}–**2**, the slow rate of ligand exchange at Co^{3+} has allowed this point to be pursued. In Co^{3+}–**1**, the amide nitrogen cannot be bound to the metal in any reasonable molecular conformation. The Raman spectroscopy and OPA measurements described above have shown that an uncoordinated amine was produced initially upon hydrolysis of the amide. If the amide

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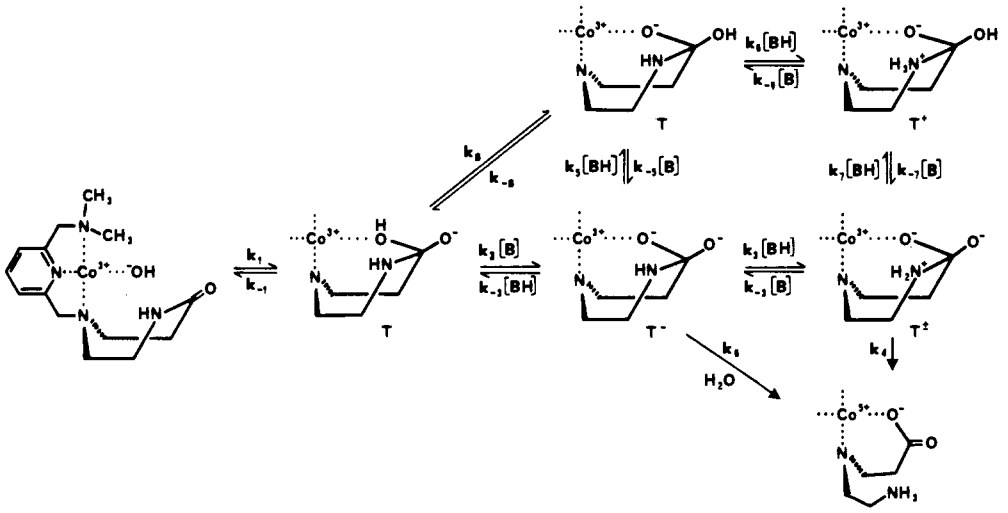
(27) Satterthwait, A. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 7031–7044.

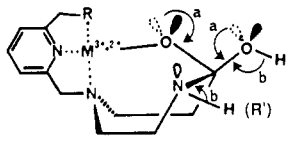
Scheme II



nitrogen was bound to the substitutionally inert cobalt(III) in the starting material, then upon hydrolysis the cobalt(III)–nitrogen bond would not be expected to cleave to produce free amine. In fact, cobalt(III) is known to show a particular affinity for amine ligands.[28-30] Thus, since production of free amine from the Co^{3+}–**1** complex occurred concurrently with amide hydrolysis, the metal cannot be coordinated to the amide in the starting complex nor in the transition state leading to hydrolysis.

Another mechanistic question addressed by these studies is whether the metal-bound nucleophile is actually responsible for the amide hydrolysis. That the observed rate of amide hydrolysis is faster than the subsequent ligand exchange at Co^{3+} requires an intramolecular hydrolysis pathway rather than the kinetically equivalent pathway of external hydroxide attack.

Stereoelectronic considerations suggested by Deslongchamps indicate that cleavage of a carbon–oxygen or a carbon–nitrogen bond is facilitated only when two heteroatoms (oxygen or nitrogen) of the tetrahedral intermediate each have a lone pair (or exchangeable proton) oriented antiperiplanar to the departing *O*-alkyl or *N*-alkyl group.[31] As we have suggested,[9] this situation is extant in the tetrahedral intermediate for the amine leaving group (pathway a in **3**) in the metal complexes of **1** and **2**.[32]

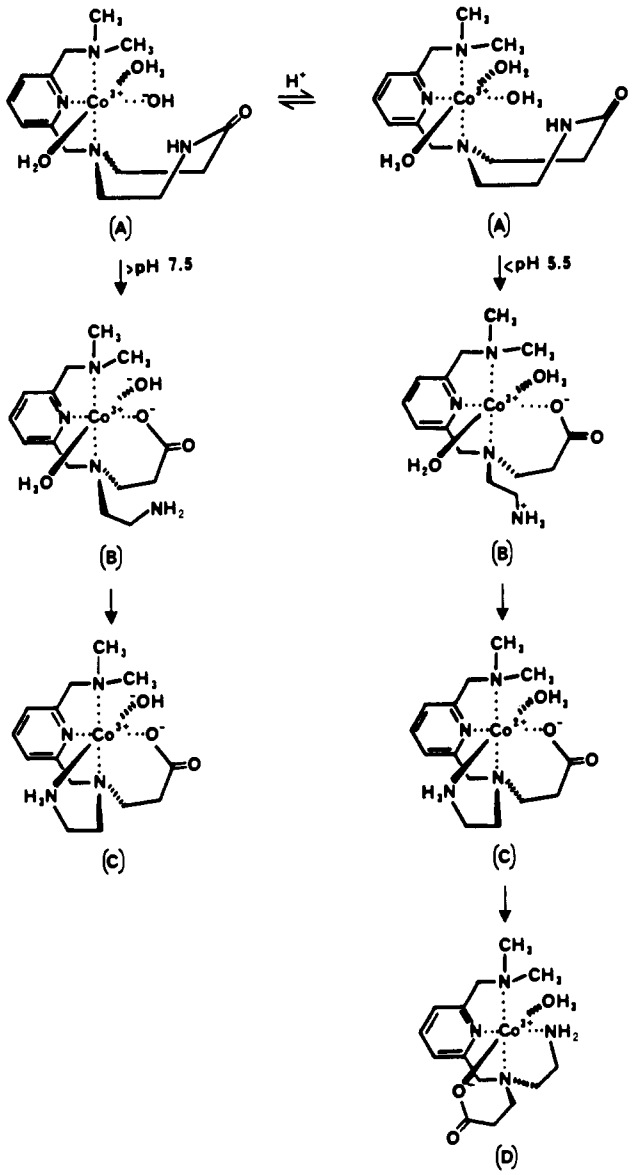


3: R = $N(CH_3)_2$ or $N(CH_2COOH)_2$

4: R = $N(CH_3)_2$; R' = CH_2Ph

The return to starting material by C–O bond cleavage along path b in **3** is also stereoelectronically allowed. Accordingly, the precise geometry of **3** allows amide hydrolysis to proceed via two successive processes, each of which would be facilitated by stereoelectronic assistance. By contrast, inspection of Co^{3+}–**1** or Co^{3+}–**2** reveals that external attack of a hydroxide at the acyl carbon to form **3** will not be facilitated by these effects.

Scheme III



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(31) (a) Deslongchamps, P. *Tetrahedron* **1975**, *31*, 2463–2490. (b) Deslongchamps, P. *Heterocycles* **1977**, *7*, 1271–1317. (c) Deslongchamps, P.; Beaulieu, N.; Chênevert, R.; Dickinson, R. A. *Can. J. Chem.* **1980**, *58*, 1051–1058. (d) Deslongchamps, P.; Caron, M. *Can. J. Chem.* **1980**, *58*, 2061–2068.

(32) (a) The importance of stereoelectronic control for the hydrolysis of cyclic amidines has been questioned recently. However, the dominant role of the oxy*anion* substituent is absent in **3** and **4**. Cf.: Perrin, C. L.; and Nuñeg, O. *J. Am. Chem. Soc.* **1986**, *108*, 5997–6003. (b) Also see: Dugas, H. *Bioorganic Chemistry, A Chemical Approach to Enzyme Action*, 2nd ed.; Springer Verlag: New York, 1989; pp 224–229.

Table II. Relative Rate Enhancements for Co(III)-Mediated Amide Hydrolysis at pH 7.0 and 25 °C

amide	catalyst	k_{obsd}	k_{rel}
1	OH^-	9.4×10^{-13} [a]	1
	Co^{3+}	1.6×10^{-5} s^{-1} [d]	1.7×10^7
	Cu^{2+}	3.4×10^{-5} s^{-1} [b]	
1	Co^{3+} + 0.1 M phosphate[c]	1.5×10^{-4}	
	Co^{3+} + 0.2 M phosphate[c]	3.0×10^{-4}	
	Co^{3+} + 0.4 M phosphate[c]	5.6×10^{-4}	
2	OH^-	6.4×10^{-14} [a]	1
	Co^{3+}	3.4×10^{-5} s^{-1} [d]	5.3×10^8
4	Cu^{2+}	9.0×10^{-7} s^{-1} [e]	

[a] Calculated by the method described in the text. [b] At pH 7.0, 50 °C, μ = 0.5 ($NaClO_4$), $r^2 \geq 0.99$. [c] At pH 6.78, 25 °C, μ = 1.0 ($NaClO_4$), $r^2 \geq 0.97$. [d] Estimated value for k_{obsd} at zero buffer concentration. [e] At pH 7.0, 50 °C, μ = 0.5 ($NaClO_4$), $r^2 \geq 0.99$.

That stereoelectronic control could be important in these systems was discerned from the rate of amide hydrolysis in the Cu^{2+} complex of **4**, the *N*-benzyl derivative of **1**. Studies on the rate of amide hydrolysis would predict that a tertiary amide should hydrolyze faster than the corresponding secondary amide.[33] However, Cu^{2+}–**4** was found to hydrolyze 38 times more slowly than the Cu^{2+} complex of the unsubstituted compound, Cu^{2+}–**1**. In order for stereoelectronic effects to facilitate formation of the tetrahedral intermediate in **4**, the *N*-benzyl group must be in the more sterically demanding pseudoaxial position. Direct access to the more stable conformer shown (**4**) would block the requisite lone pair on nitrogen.

Post Amide Hydrolysis Rearrangements. The amide hydrolyses of Co^{3+}–**1** and Co^{3+}–**2** and subsequent coordination of the amino group led to the formation of a *β-cis*-tetraamine–Co^{3+} complex, which was found to undergo isomerization to form the corresponding trans isomer.[34] These processes are illustrated in Scheme III. The starting complex, Co^{3+}–**1**, (A), upon hydrolysis will produce the Co^{3+} complex (B), with the new carboxylate ligand trans to the pyridine nitrogen and the amino group uncoordinated. Displacement of water by the pendant primary amine produced complex C.[34] This new *β-cis*-tetraamine–Co^{3+} complex (C) appeared to be stable in basic solutions, but isomerized to the corresponding trans isomer under acidic conditions (D). The visible spectrum of a basic reaction mixture could be converted almost immediately to the one observed for a reaction run in acidic medium by the addition of perchloric acid. However, the addition of base to an acidic system did not produce any change in the visible spectra. Therefore the difference between the two final products is not simply the titration of a proton. Similar rearrangements have been observed for Co^{3+} trien complexes.[35]

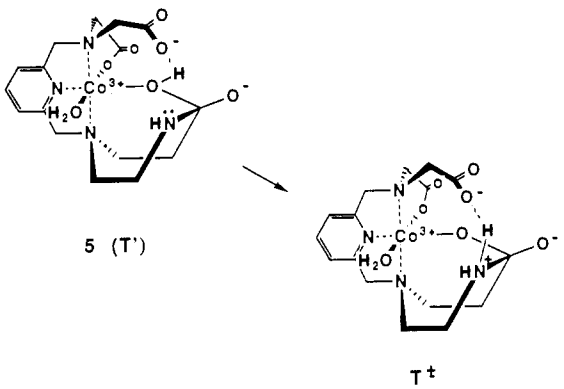
Relative Rate Enhancements and Comparison of Co^{3+}–1 and Co^{3+}–2. The relative rate enhancements for the cobalt(III)-promoted amide hydrolysis of Co^{3+}–**1** and Co^{3+}–**2** at pH 7.0 and 25 °C were found to be 1.7×10^7 and 5.3×10^8, respectively. These values were determined by dividing the k_{obsd} for the metal-promoted amide hydrolysis reaction by estimated k_{obsd} values for the base hydrolysis reaction, which were calculated from the second-order rate constants (k_{OH}) for these complexes reported previously. A summary is presented in Table II.

The cobalt(III) complexes of **1** and **2** are structurally very similar. The major difference appears to be the substitution of one of the axial metal-bound water molecules in Co^{3+}–**1** for a carboxylate ligand in Co^{3+}–**2**. Titration of Co^{3+}–**2** showed a pK_a at ca. 2.5, which we ascribe to the free carboxyl.

The pH rate profiles of Co^{3+}–**1** and Co^{3+}–**2** are compared in Figure 2. Upon examination of these two curves it is apparent that both complexes react at approximately the same rate in the aquo form (k_1). However, the presence of the neighboring carboxylate in Co^{3+}–**2** resulted in a 2-fold increase in the rate constant for amide hydrolysis for the Co^{3+}–OH form (k_2). This result is surprising since neutralization of positive charge at cobalt by coordination of one carboxylate and the field effect of the free carboxylate anion might be expected a priori to retard the rate of amide hydrolysis. These considerations probably account for the *depressed* reactivity of **2** toward hydroxide and, accordingly, the larger relative rate enhancement of Co^{3+}–**2** over Co^{3+}–**1**.

In the acidic regime, formation of the tetrahedral intermediate (T) is certainly rate determining. However, the free carboxylate, which would be deprotonated above pH 2.5, apparently cannot assist the formation of T. The 31-fold increase in k_{rel} for Co^{3+}–**2** vs Co^{3+}–**1** (23-fold in k_2) suggests a unique function of the pendant carboxylate at neutral pH. Expulsion of the amino group from T is expected to be the rate-limiting step at this pH. We suggest that the role of the carboxylate is to remove the proton from the initially formed tetrahedral intermediate (T′ in Scheme II) and to transfer it to the amine nitrogen to afford T±. This role has been suggested for Glu-143 of thermolysin by Matthews[5c] and by Christianson and Lipscomb for carboxypeptidase A.[11d] The pronounced acceleration due to phosphate on k_2 gives more evidence for the support for this proton-transfer role.[12]

Consideration of the molecular conformation of the tetrahedral intermediate derived from Co^{3+}–**2** at neutral pH (**5**) helps to define



the nature of this carboxylate catalysis. The free carboxylate in **5** is in a position favorable for the deprotonation of the cobalt-bound hydroxyl group but not the exocyclic hydroxyl group. The amino nitrogen is also favorably disposed for protonation from the same carboxylate. Thus, in the terms of Scheme II, the carboxylate in Co^{3+}–**2** can catalyze decomposition of the tetrahedral intermediate T′ by converting it to T±.

Interestingly, no intramolecular carboxylate catalysis was observed in the cobalt(III) model system recently described by Schepartz and Breslow.[12] This system did show significant catalysis by phosphate and acetate buffers, however. Accordingly, the precise placement of the pendant carboxylate in **5** appears to be critical for the acceleration effect we have observed here. It is possible, in addition, that the effect of the pendant carboxylate in **5** is dependent on the presence of the water molecule present in the coordination sphere of cobalt in Co^{3+}–**2**.

Our earlier model studies with this metal-coordinating lactam system have shown very large rate enhancements for zinc (>10^7) and a low pK_a for a zinc-bound water (p$K_a \cong 7$).[9b,c] The pendant carboxylate in Co^{3+}–**2** has revealed another 30-fold effect on k_{rel} (23-fold in k_2), and the 38-fold retardation in the rate of Co^{3+}–**4** has been attributed to a stereoelectronic effect. Taken together, these three effects begin to provide the degree of acceleration that is required to understand the enzymic rate.

Conclusions

We have shown that amide hydrolysis is facilitated by intramolecular addition of Co(III)–OH to the amide carbonyl. Breakdown of the tetrahedral intermediate has been shown to expel the free amino group prior to metal–nitrogen coordination. Ex-

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pulsion of the free amine was facilitated by bifunctional buffer catalysis. A pendant carboxylate group has been shown to enhance the rate of amide hydrolysis. This effect can be attributed to a proton switching function, which implicates a similar role for Glu-230 in carboxypeptidase A and Glu-143 in thermolysin. In addition, stereoelectronic effects can be discerned in this model system which may lead to another order of magnitude in the rate.

Experimental Section

A. General Procedures. All chemicals used were of reagent-grade quality. *o*-Phthalic dicarboxaldehyde (OPA) purchased from the Aldrich Chemical Co. was purified by two crystallizations from petroleum ether (bp 38–46 °C). Cuprous chloride was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Palladium on powdered charcoal (5% catalyst) was purchased from Matheson, Coleman, and Bell (Norwood, OH). Sodium perchlorate and cobalt(II) perchlorate were obtained from G. Frederick Smith and Co. (Columbus, OH). Stock cobalt(II) perchlorate solutions were standardized gravimetrically by established procedures.[36] The buffers 2-(*N*-morpholino)ethanesulfonic acid (MES, pK_a 6.15), *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES, pK_a 7.55), 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS, pK_a 8.4), and 2-(cyclohexylamino)ethanesulfonic acid (CHES, pK_a 9.50) were purchased from Calbiochem (La Jolla, CA) or Sigma Chemical Co. (St. Louis, MO).

Thin-layer chromatography (TLC) was conducted on Uniplate silica gel GF precoated TLC plates from Analtech (Newark, DE). Preparative column chromatography was carried out with Woelm silica (63–200, activity III) or G-10 Sephadex from Sigma.

Distilled water was redistilled over potassium permanganate and then freshly boiled before use in preparation of buffer solutions. Buffer solutions were prepared just prior to use in most cases, but never stored for more than 1 week.

Measurements of pH were made with a GK 2321C (25 °C) combination electrode attached to a Radiometer TTT-2b titrator. The electrode was frequently standardized with standard buffer solutions. The titrator was also equipped with a PHA943 Titrigraph Module, a SBR3 Titrigraph, an ABU12T Autoburette, and a TTA3 Titration Assembly to obtain automatic titration recordings.

Nuclear magnetic resonance spectra were obtained on Varian T60, JEOL FX90Q, JEOL HA100, or Bruker WM-360 spectrometers. Infrared spectra were recorded on a Perkin-Elmer Model 457 spectrophotometer. A Cary Model 219 was used to obtain UV–visible spectral data. Constant temperature for these spectra was maintained with the Forma Scientific Model 2095 bath and circulator (±0.5 °C).

4-Benzyl-1-[[6-[(dimethylamino)methyl]-2-pyridyl]methyl]hexahydro-5*H*-1,4-diazepin-5-one (4). 1-[[6-(Dimethylamino)methyl]-2-pyridyl]-methyl]hexahydro-5*H*-1,4-diazepin-5-one (0.3 g, 1.14 mM) was refluxed with 150 mL of dry benzene and approximately 0.075 g (3.1 mM) of NaH at 89 °C for 36 h under a nitrogen atmosphere. The reaction mixture was then allowed to cool to room temperature. Benzyl chloride (0.14 g, 1.11 mM) in 3 mL of dry benzene was added in 0.5-mL aliquots every 15 min. The reaction mixture was then stirred over the course of 22 h. After rotary evaporation the resulting solid was triturated with 5 × 25 mL portions of hexane. The remaining solid was dissolved in methylene chloride, washed with a saturated sodium chloride solution, and dried over magnesium sulfate. The organic layer was then gently warmed (50 °C) to increase the percentage of N-benzylated material and then rotary evaporated to dryness. The resulting solid (0.4 g, 1.14 mM, 100%) was a mixture of the desired N-benzylated and the corresponding O-benzylated compounds. The desired amide was isolated by preparative TLC (2.5 mL of $CHCl_3$/2.5 mL of MeOH/5 mL of concentrated EtOAc/5 drops of concentrated NH_4OH), followed by HPLC on a Lobar A LiChroprep Si 60 column using a solvent system of 2 mL of $(CH_3)_2CO$/2 mL of $CHCl_3$/4 drops of concentrated NH_4OH. Removal of the solvent under vacuum gave 0.24 g (0.67 mM, 58.5%) of **4** as a very hygroscopic light yellow solid, which by ^1H NMR contained only traces of impurity. ^1H NMR ($CDCl_3$): δ 7.54 (tr, 1 H), 7.27, 7.21 (s with sh, 7 H), 4.5 (s, 2 H), 3.7 (s, 2 H), 3.56 (s, 2 H), 3.35 (m, 2 H), 3.2 (s, 0.5 H), 2.75 (tr, 4 H), 2.4 (m, 2 H), 2.27 (s, 6 H), 1.25 (s, 1 H). MS: *m/e* (rel. intensity) 353 (34.3), 309 (30.1), 218 (1.8), 203 (4.6), 161 (10.1), 150 (100), 133 (22.1), 107 (84), 106 (79.1), 91 (89.7), 58 (95). ^{13}C NMR ($CDCl_3$): ppm 177.0 (1 C), 158.9 (1 C), 148.5 (1 C), 137.7 (1 C), 136.3 (1 C), 133.0 (1 C), 130.1 (1 C), 128.6 (1 C), 127.8 (1 C), 127.4 (1 C), 121.0 (1 C), 120.6 (1 C), 65.2 (1 C), 64.0 (1 C), 56.9 (1 C), 50.3 (1 C), 49.5 (1 C), 45.1 (2 C), 42.0 (1 C), 37.6 (1 C). Anal. Calcd for $C_{21}H_{28}H_{28}N_4O\cdot 3H_2O$: C, 62.07; H, 8.37; N, 13.79. Found: C, 61.84; H, 8.06; N, 13.88.

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B. Kinetic Measurements. *o*-Phthalic Dicarboxaldehyde (OPA) Reagent for Monitoring Amide Hydrolysis. Boric acid (3.1 g, 50.0 mM), EDTA disodium salt dihydrate (1.41 g, 3.79 mM), and 37.5 mL (38.3 mM) of 1.02 N sodium hydroxide were combined and diluted in sufficient water to give 250 mL total volume. The pH was adjusted to 9.75 with sodium hydroxide, and the recrystallized OPA (23 mg, 0.17 mM) and β-mercaptoethanol (0.61 mL, 8.7 mM) were added. This solution was always made fresh each day.

Co^{3+}-Promoted Amide Hydrolysis of 1 Monitored by OPA. Typically, cobalt(II) perchlorate and compound **1** were combined in a one to one ratio. The mixture was cooled with ice and treated with 30% hydrogen peroxide. After 1 min, excess peroxide was quenched with Pd/C (4 min), filtered, and added to 1 mL of the appropriate buffer. For the first hour, 50 μL of the solution was added to 3 mL of the OPA reagent in a cuvette and the absorbance at 333 nm at 25 °C was recorded. Subsequent aliquots were determined at appropriate intervals.

The Co^{3+}-promoted amide hydrolysis of **1**, **2**, and **4** was accompanied by a significant blue shift in the visible spectrum. The course of the reaction was followed by monitoring the changes in absorbance at 530, 530, and 490 nm, respectively. These changes were pH dependent.

All Co^{3+}-promoted amide hydrolysis reactions run to generate the pH–rate profile were carried out in 0.2 M buffer solutions, which were adjusted to an ionic strength of 0.5 M with added sodium perchlorate. In all reactions, Co^{3+} was generated from Co^{2+} after complexation with the ligand by the addition of 30% hydrogen peroxide. Excess peroxide was quenched by treatment with palladium on powdered charcoal, which was removed by Millipore filtration. The solution was then added to 3 mL of the appropriate buffer in sample cuvettes. The cuvettes were maintained at 25 ± 0.01 °C (μ = 0.5 M, $NaClO_4$) throughout the experiment.

In a typical experiment 1 equiv of cobalt(II) perchlorate was complexed with 0.072 mM of the appropriate lactam. This mixture was chilled in an ice bath and then treated with 30 μL of 30% hydrogen peroxide for 1 min. The reaction was quenched with 20 mg (5% catalyst) of Pd/C and allowed to react for 4 min. The solution was then filtered and divided between four sample cuvettes each containing 3 mL of buffer solution. The reactions were monitored by visible spectroscopy over the course of up to 4 days at 25 °C.

Rate constants were calculated by the "slog" method from a linear least-squares regression of ln ΔA vs time. Correlation coefficients (r^2) were generally greater than 0.99.

Isolation of 6 from Base Hydrolysis of 1. Compound **1** (200 mg, 0.76 mM) was dissolved in 4.5 mL of 0.5 N lithium hydroxide, and the mixture was maintained at 100 °C under nitrogen for 26 h. The pH was adjusted to 7 with perchloric acid and the solution lyophilized. The resulting solid was purified by gel filtration (Sephadex G-10; eluent, water) to give **6** as the mono perchloric acid salt whose purity was confirmed by its ^1H NMR spectrum.[14]

Isolation of 6 from Co^{3+}-Promoted Amide Hydrolysis of 1. Compound **1** (100 mg, 0.38 mM) and 1.0 mL (0.39 mM) of 0.385 M cobalt(II) perchlorate were combined at 0 °C with 1 mL of 30% hydrogen peroxide. After 60 s, excess hydrogen peroxide was quenched by treatment with 75 mg (5% catalyst) of palladium on powdered charcoal. After 4 min, the reaction mixture was filtered and the pH was adjusted to 7.5 with lithium hydroxide. After 48 h (approximately 5 half-lives), the solution was treated with activated zinc dust (5 g) and allowed to stir for 4 h. The solution was filtered and saturated with hydrogen sulfide, and then the pH was adjusted to 10 with lithium hydroxide. After removal of the metal sulfides by filtration, perchloric acid was added (pH 7), and then the solution was lyophilized. The resulting solid was purified by gel filtration (Sephadex G-10; eluent, water) to give **6**, which was pure as judged by its ^1H NMR spectrum.

Acknowledgment. Support for this research by the National Science Foundation is gratefully acknowledged. This research was carried out at the University of Michigan.

Registry No. 1, 87828-76-0; Co^{3+}-**1**, 120944-04-9; **2**, 87828-77-1; Co^{3+}-**2**, 120944-05-0; **4**, 120944-07-2; Cu^{2+}-**4**, 120944-06-1; PO_4^{3-}, 14265-44-2; protease, 9001-92-7; carboxypeptidase A, 11075-17-5; thermolysin, 9073-78-3; angiotensin-convertin enzyme, 9015-82-1; peptidase, 9031-96-3.

Supplementary Material Available: Tables of *A* values vs time for the amide hydrolysis and ligand rearrangement at varying pH (appendixes A and C), ionic strengths, and buffers (appendix C) and dependence of amide hydrolysis on total buffer concentration (appendix B) of Co^{3+}–**1** (20 pages). Ordering information is given on any current masthead page.