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Model Studies for Molecular Recognition of Carbonic Anhydrase and Carboxypeptidase

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ABSTRACT

Model studies using Zn^{2+} complexes of various derivatives of macrocyclic triamines ([12]aneN_3) and tetraamines (cyclen) have been found to be useful in elucidating and understanding the intrinsic properties of substrate or inhibitor recognition by zinc ions at the active centers of carbonic anhydrase and carboxypeptidase.

Introduction

The active center of carbonic anhydrase II (CA II) consists, in essence, of a zinc-binding site (His 94, His 96, and His 119), an adjacent hydrophobic pocket (Val 143, Val 121, Trp 209, Leu 198) for precatalytic substrate association site, and a proton-transfer path (Thr 199, His 64).[1] Carboxypeptidase A (CPA) contains a Zn^{2+} ion ligated by His 69, His 196, and Glu 72 at the active center, flanked by collaborating catalysis residues and substrate-binding residues, including Glu 270, Arg 71, Arg 127, Asn 144, Arg 145, and Tyr 248.[2]

The role of molecular recognition remains a central issue in these zinc enzymes. CA recognizes not only its regular substrates (and products) CO_2 and HCO_3^-, but also irregular substrates, such as phosphoesters, carboxyesters, and aldehydes. It also binds halide anions, SCN^-, acetate anion, sulfonamides, carboxamides, phenol, imidazole, and alcohols as inhibitors. Meanwhile, CPA recognizes substrate peptides and carboxyesters, as well as resulting substrate-hydrolyzed products and peptide-mimic (e.g., phosphonamidate) inhibitors. During our study of macrocyclic polyamines, we have recognized some interesting properties of coordinated Zn^{2+} cations, namely their ability to interact with OH^-,[3] HCO_3^-,[4] halide anions,[3] SCN^-,[3,5] acetate,[3] sulfonamides,[4] carboxamides,[6] phenol,[7,8] imidazole,[9] alchohls,[10−12] carboxylate anions,[13] and phosphoesters.[12, 14] These findings led us to consider whether such complexation might provide a rudimentary mimic of the molecular recognition features displayed by CA and CPA.

Needless to say, model studies using simple Zn^{2+} macrocyclic polyamine complexes cannot serve to explain all aspects of the complex enzymatic process. Still, they could prove useful in elucidating some of the intrinsic chemical roles played by zinc. Although there have been quite a number of enzymatic studies focused on the role of Zn^{2+}, including studies of Co^{2+}-substituted enzymes[15] and amino acid variants,[1] many key questions regarding the fundamental function of the coordinated zinc cation remain unanswered. In this context, Zn^{2+} macrocyclic polyamines offer a substantial advantage as zinc enzyme models. This is because they are kinetically and thermodynamically stable, at least to a large degree, in aqueous solution at physiological pH. By contrast, many other Zn^{2+} model systems have proved useful only in nonaqueous solvents, proving specifically unstable in aqueous media. Since the molecular recognition properties of the enzymes are known to be modulated as a function of pH in aqueous solution, this special feature of Zn^{2+} macrocyclic complexes is viewed as being extremely useful.

Recognition of Hydroxide Anion

To a first approximation, in both carbonic anhydrases and carboxypeptidases, zinc-bound hydroxide anions act as nucleophiles and attack bound substrates. Toward this end, water molecules are activated by zinc. The acidity of the zinc center in these enzymes is thus of fundamental concern. In CA II,[1] a pK_a value of 6.8 for the Zn^{2+}-bound H_2O was determined indirectly by measuring the pH-dependent *p*-nitrophenyl acetate (NA) hydrolysis activity. Substitution of one of the histidine ligands by a negatively charged Asp or Glu residue greatly raises the pK_a value (to more than 8.5). In the native system, the stability of the Zn^{2+}-bound hydroxide is augmented by a hydrogen bond network consisting of the Thr 199 hydroxyl group and the Glu 106 CO_2^- group. Substitution of Thr 199 by Ala or Val increases the pK_a values to 8.3 and 8.7, respectively. In CPA, a pK_a value of 6.2 was assigned to the Zn^{2+}-bound H_2O, as judged from a kinetic study involving substrate peptide hydrolysis.[16] However, this pK_a value was found to be influenced greatly by the adjacent Glu 270. It may also be affected by initial substrate complexation. As a consequence of this potential variability, it is probably safe to say that the intrinsic acidity of zinc in not only CPA, but also CA, remains incompletely assigned at present.

The Zn^{2+}−1,5,9-triazacyclododecane (abbreviated as [12]aneN_3) complex **1a** is a potentially attractive model system. It possesses a tetrahedral structure with a H_2O at the fourth coordination sites. This latter deprotonates (to **1b**) with a pK_a value of 7.3 at 25 °C and $I = 0.1$ ($NaClO_4$), as determined by pH−potentiometric titration measurements (Scheme 1).[3] In other words, **1a** recognizes hydroxide anion very strongly and does so in a 1:1 manner with a log K_{assoc} value of 6.4 (at $I = 0.2$), where K_{assoc} is the affinity constant. A trimeric complex [(**1b**)$_3$·$HClO_4$·$(ClO_4)_3$] was isolated from pH 8 aqueous solution. The X-ray crystal

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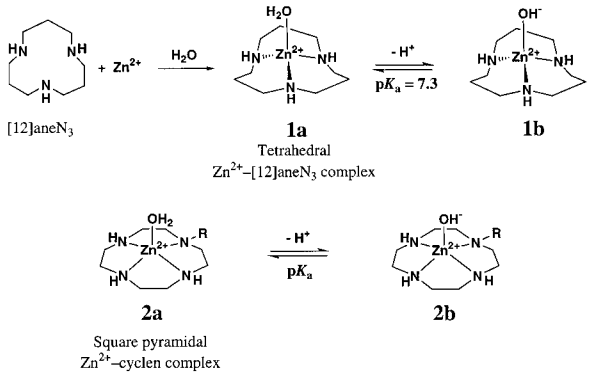
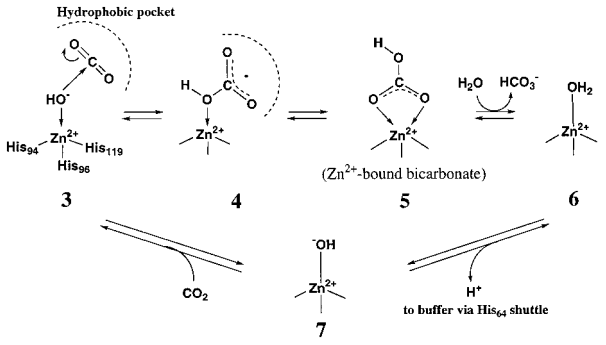
Scheme 1



Scheme 2



structure of this complex, containing three units **1b**, revealed tetrahedral coordination environment about the zinc center with a Zn^{2+}−OH bond length (1.94 Å) that is shorter than the various Zn^{2+}−N bonds (average 2.02 Å). When dissolved in aqueous solution, two basic Zn^{2+}−OH^- species (**1b**) and one neutral Zn^{2+}−H_2O species (**1a**) are produced, as evidenced by pH−potentiometric titrations. These Zn^{2+}−OH species (**1b**) turned out to be good nucleophiles in the CA-like catalyzed hydration of acetaldehyde (at 0 °C) as well as in the hydrolysis of an unactivated ester, methyl acetate (at 25 °C). The second-order rate constant−pH profiles for both were sigmoidal, yielding pK_a values of 7.9 (at 0 °C) and 7.3 (at 25 °C), which are identical to the thermodynamic pK_a values of 7.9 (at 0 °C) and 7.3 (at 25 °C). These findings thus provide an important demonstration that simple zinc complexes, if appropriately chosen, can activate H_2O at physiological pH for catalytic reactions analogous to those of zinc-containing enzymes such as CA and CPA. A square pyramidal Zn^{2+}−1,4,7,10-tetraazacyclododecane (abbreviated as cyclen) complex **2a** (R = H) also coordinates waters to produce a Zn^{2+}−OH_2 complex that deprotonates (to **2b**) with a pK_a of 7.9 (at 25 °C).[14] This latter hydroxyl complex, again a Zn^{2+}−OH species, is also a strong nucleophile. With a long alkyl chain $C_{16}H_{33}$ attached to the cyclen ligand (R = $C_{16}H_{33}$) in **2**, the Zn^{2+}−OH is also generated with a pK_a of 7.6 in comicellar solution with Triton X-100 surfactant, which shows higher nucleophilicity in general.[17]

Recognition of HCO_3^-

In the CA catalysis of CO_2 hydration,[1] the hydrophobic pocket (consisting of Val 143, Val 121, Trp 209, and Leu 198) adjacent to the zinc-bound hydroxide serves as the substrate CO_2 association site. The product HCO_3^- is formed by the direct nucleophilic attack of the Zn^{2+}-bound hydroxide anion on the immobilized CO_2 substrate contained in this hydrophobic pocket. The resulting product, a Zn^{2+}−HCO_3^- species **4**, is rapidly displaced by H_2O, thus completing the catalytic cycle (Scheme 2).

Questions may be raised: What is the affinity constant for Zn^{2+}−HCO_3^-, and how does H_2O displace the Zn^{2+}−HCO_3^-? The bicarbonate binding to CA was determined to occur with a log *K* of 1.6, as judged by inhibition studies involving the kinetics of CA-promoted NA hydrolysis at pH 8.5.[18] The bicarbonate binding affinity of **1a** was similarly determined by inhibition kinetic studies involving NA hydrolysis (by **1b**) at pH 8.4 to have a log K_{app} value of 2.8 at 25 °C.[4] The stronger binding of HCO_3^- to Zn^{2+} bound to **1** as compared to that for CA is worth consideration. In CA, Thr 199, standing as a "doorkeeper" in the hydrophobic region, may destabilize the product Zn^{2+}−HCO_3^- complex **5** but stabilize the catalytically competent Zn^{2+}−HCO_3^- complex **4**.[19] The substitution of Thr 199 by Ala 199 increases the HCO_3^- binding affinity of CA by 20 times,[20] producing a value more closely in line to what is seen for our ZnL−HCO_3^- complex. A weaker Zn^{2+}−HCO_3^- interaction should prove useful in the subsequent substitution by H_2O (**5** → **6**), a process that is otherwise very unfeasible. Subsequent H^+ removal through a His 64 "shuttle" (**6** → **7**) may lower the energy barrier for the HCO_3^- displacement (**5** → **6**), since the resulting Zn−OH^- species (**7**) is likely to be more stable, at least in terms of metal−ligand interactions, than a corresponding Zn−HCO_3^- species (**5**). It is understood that the proton-transfer step needed to regenerate the active catalyst, Zn−OH^- (**7**), is rate-determining. The hydration of CO_2 is thus thermodynamically and kinetically more favorable at alkaline pH.

Our CA model complex **1** provides for the first time a convincing chemical mechanism that illustrates the important role zinc(II) plays in the reversible hydration of CO_2 and the dehydration of HCO_3^- catalyzed by CA.[21] In particular, the fast hydration of CO_2 catalyzed by **1** was seen to be followed by measuring H^+ production (CO_2 + H_2O → HCO_3^- + H^+) at 25 °C. The kinetics, in fact, demonstrated the catalytic nature of the zinc(II) complex **1** at various pH values. The second-order rate constants $(k^h_{cat})_{obs}$ (first order in [**1**] and [CO_2]) are plotted as a function of pH in Figure 1a. The sigmoidal curve indicates a kinetic process controlled by an acid−base equilibrium. It exhibits, in particular, an inflection point (pK_{kin}) at about pH 7.4, which is almost the same as the potentiometrically determined pK_a value of 7.3 for **1a** ⇄ **1b** + H^+.[3] Thus, **1b** must represent the actual active species in the catalytic hydration of CO_2. The CO_2 hydration mechanism is summarized in eq 1, wherein the acid−base equibrilium **1a** → **1b** + H^+ is expected to occur very quickly. A similar

$$CO_2 + ZnL{-}OH^- \,(\mathbf{1b}) \xrightarrow{k^h_{cat}} [ZnL{-}{}^-OCO_2H] \rightarrow HCO_3^- + ZnL{-}OH_2\,(\mathbf{1a}) \quad (1)$$

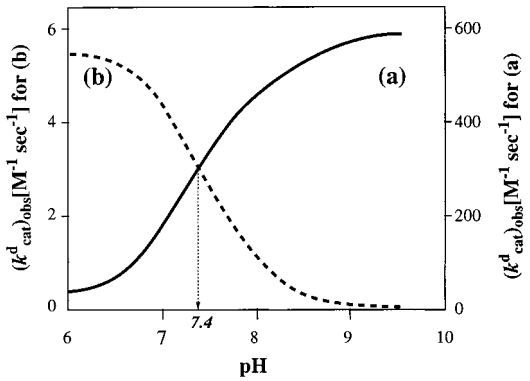


FIGURE 1. Rate−pH profile by reaction for (a) CO_2 hydration and (b) HCO_3^- dehydration catalyzed by zinc(II)−[12]aneN$_3$ **1**

sigmoidal pH dependence in the CO_2 hydration rate (k_{cat}/K_m) of human CA II (its pK_a is 6.8)[22] can also be accounted for by assuming that the Zn^{2+}−OH complex at the active center of CA II reacts with CO_2. In the real enzyme catalytic reaction, the rate-determining step involves proton transfer from the product Zn−OH_2 species to solvent with the nearby His 64 as a proton shuttle. This regenerates the active Zn−OH intermediates. In our model system, aquation of the product bicarbonate complex [ZnL−$^-$$OCO_2H$] gives off HCO_3^- and **1a**, and the following deprotonation to **1b** is relatively fast under the reaction conditions.

The reverse HCO_3^- dehydration has been catalyzed for the first time in a model system via the use of **1**. The rate was followed by measuring the evolution of OH^- for the reaction ($HCO_3^- \rightarrow CO_2 + OH^-$). The rates sigmoidally increased with lowering pH (see Figure 1b). The kinetic data were fit to the reaction shown in eq 2, and the pK_a value for **1a** $\rightleftarrows$ **1b** + H^+ obtained in this kinetic-based manner was found to be 7.3 at 25 °C. The most important

$$HCO_3^- + ZnL{-}OH_2\ (\mathbf{1a}) \xrightarrow{k^d_{cat}} [ZnL{-}{}^-OCO_2H] \rightarrow CO_2 + ZnL{-}OH^-\ (\mathbf{1b}) \quad (2)$$

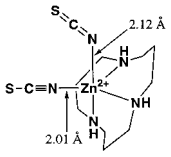
conclusion from this model HCO_3^- dehydration study is that the reactive species is the zinc(II)−OH_2 complex, which undergoes substitution by HCO_3^- followed by decarboxylation as shown in eq 2.

Although the magnitudes of the second-order rate constants k^h_{cat} and k^d_{cat} differ in the two curves (Figure 1a,b), they have an inflection point at almost the same pH (ca. 7.4) and are symmetrical (with scales adjusted). Thus, it was concluded that the model complex **1** is endowed with the same essential zinc functions as CA and thus acts to both mirror and elucidate the pH-dependent hydration and dehydration reactions catalyzed by CA. A similar CA model study with the Zn^{2+} complex **2** gave rise to an analogous mechanistic conclusion.[23]

Recognition of Anionic Inhibitors N_3^- and NCS^-

Monovalent anions such as thiocyanate SCN^- and azide N_3^- comprise an important class of CA II inhibitors. These

Scheme 3



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anions are competitive inhibitors of the bicarbonate dehydration process.[24] An X-ray crystallographic analysis of the enzyme azide complex revealed a direct Zn^{2+}-bound N_3^- entity (at pH 8.0) with no hydrogen-bonding contact between this inhibitor and functionality present on nearby amino acid residues such as the hydroxyl group of Thr 199.[25] Thiocyanate similarly inhibits CA with a log K_i value of 3.2 at pH 8.5 (as determined by inhibition kinetics in CA-promoted NA hydrolysis reactions).[18] Here, an X-ray crystallographic analysis (to 1.9 Å resolution), involving SCN^- bound to CA II at pH 8.5, showed that the Zn^{2+} center is in an ill-defined five-coordinate complex that contains coordinated SCN^- anion with an almost linear Zn^{2+}−NCS geometry and a Zn^{2+}−N distance of 1.9 Å as well as a bound water molecule (Zn−O 2.2 Å).[26] It was suspected that N_3^- and SCN^- act as competitive inhibitors by occupying the coordination site normally taken by HCO_3^-, thereby precluding bicarbonate dehydration.

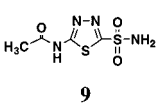
An X-ray crystallographic analysis of Zn^{2+}−[12]aneN$_3$-$(NCS)_2$ **8** revealed a five-coordinate, trigonal bipyramidal structure with one presumably stronger equatorial Zn^{2+}−NCS^- bond [the Zn^{2+}−N bond length is 2.012 Å, and the Zn^{2+}−N−C angle is nearly linear (171.3°)] and one apparently weaker axial Zn^{2+}−NCS bond [the relevant Zn^{2+}−N bond length is 2.119 Å, and the Zn^{2+}−N−C angle is more acute at 152.2°] (Scheme 3).[5] With our CA model **1**, the 1:1 Zn^{2+}−NCS^- affinity (i.e., the first) was determined by pH−potentiometric titration methods to have a log K of 2.4 at 25 °C and $I = 0.20$).[3] Like HCO_3^-,[4] thiocyanate and azide anions were found to inhibit competitively the catalytic activity of NA hydrolysis by **1**. Thus, complex **1**, acting as a model, lends support to the notion that the competitive inhibition of HCO_3^- dehydration by NCS^- and N_3^- in CA originates from competitive coordination to Zn^{2+}.

Recognition of Sulfonamides

The neutral inhibitors of CA comprise weak acids such as sulfonamides, carboxamides, imidazole, and alcohols with high pK_a values. Such inhibitors may bind either as neutral molecules or as deprotonated anions to the active center Zn^{2+}. Typical examples are medicinally important aromatic sulfonamides such as acetazolamide **9** (pK_a = 7.5) that have been recognized to bind to CA II through their deprotonated sulfonamide nitrogen. The apparent affinity of **9** is defined by its K_i values of 0.1 μM (wild type), 7 μM (mutant Ala 199), and 2.2 μM (mutant Gln 106) at pH 8.8.[27]

Acetatazolamide **9** was found to bind to Zn^{2+}−[12]-aneN$_3$ **1** in a 1:1 stoichiometry and with a log K_i of 3.6,

determined from inhibition of NA hydrolysis in 10% (v/v) CH_3CN aqueous solution at pH 8.40.[4] Like HCO_3^- (log K_i = 2.8), **9** was also found to act as a competitive inhibitor of NA hydrolysis by **1**. Accordingly, we propose that the intrinsic Zn^{2+}−sulfonamide affinity constants in CA contribute roughly 4 log units to the overall K_i values. According to molecular mechanism studies involving the binding of benzenesulfonamides to CA, roughly 60% of the CA−sulfonamide interaction energy comes from the direct coordination of sulfonamides to the Zn^{2+} centers.[28] Our proposal is in accord with such an estimate. The interaction between the sulfonamide anion and **1** was directly and quantitatively detected by monitoring UV absorption shifts. This gave almost the same K_i values as those determined kinetically. This supports the conclusion that sulfonamide coordinates to **1** only in its deprotonated form.



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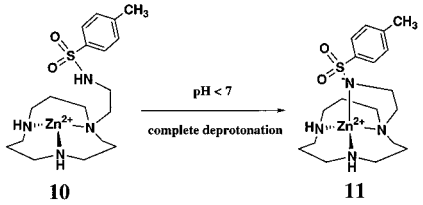
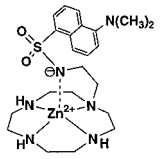
acetazolamide

Scheme 4



10 **11**

The first unequivocal model in support of the proposed Zn^{2+}−sulfonamide coordination was constructed by synthesizing a sulfonamide-pendant [12]aneN$_3$ **10**. Using this system, it proved possible to show that the Zn^{2+} center would, indeed, act to deprotonate sulfonamides (p$K_a \approx$ 10), yielding **11** at pH 7 (Scheme 4).[4] This model study also led to development of a selective fluorescent dansylamide-pendant cyclen probe for Zn^{2+} (**12**) that was to work at neutral pH in aqueous solution.[29, 30]



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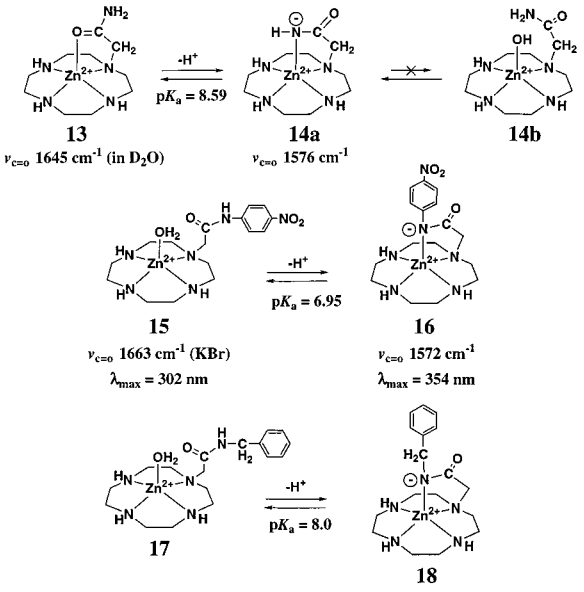
Zn^{2+}–dansylamide-pendant-cyclen

Molecular Rocognition of Carboxamides

Another class of reversible CA inhibitors, carboxamides, with larger pK_a values (ca. 14−15), were long thought to bind to the Zn^{2+} centers in the active sites of CA and CPA as neutral species. However, a pH-dependent inhibition and affinity study of iodoacetamide (K_i = 40 mM at pH 8, by spectrophotometry and kinetics) and Co(II)-substituted CA I gave data that could be better fit by assuming the amide bound either in its anionic form (−CONH$^-$) to CA in its acid form (Zn^{2+}−OH_2) or as a neutral species

Scheme 5

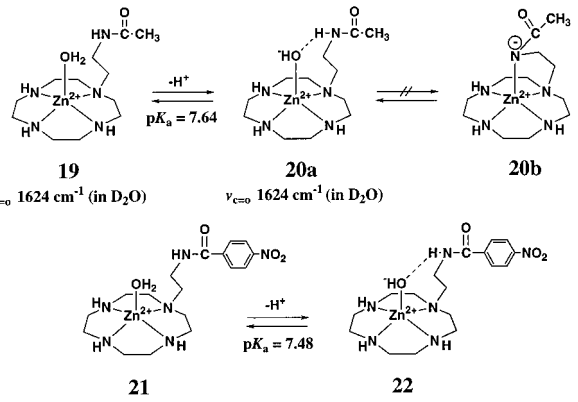


(−$CONH_2$) interacting with the basic form of CA (Zn^{2+}−OH).[31] The association rates of the amides were extremely slow (1−10 M^{-1} s^{-1}), compared with the values of 10^6−10^9 M^{-1} s^{-1} for the sulfonamides. It was proposed that the slow association kinetics displayed by amide-type inhibitors could reflect an energetically unfavorable deprotonation of the amides before final coordination to the Zn^{2+} centers.[32]

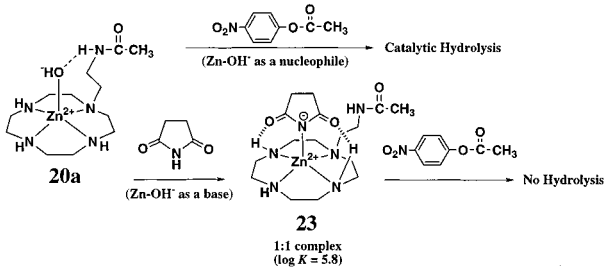
Unfortunately at present, no concrete evidence, such as that from X-ray diffraction studies, is available to support the proposed amide−CA interactions. Unlike aromatic sulfonamides, the binding of acetamides and other small amides would have little assistance from adjacent hydrophobic amino acid residues. This leads to the conclusion that direct Zn^{2+}-deprotonated amide bonding would be responsible for amide−CA complex formation. In a binding study involving the interaction between the potentially "ambivalant" substrate oxamate (^-O_2C−$CONH_2$) and CA I, deprotonation of the amide group (within the enzyme−ligand complex) was found to occur with a pK_a of 9.4.[31] The binding of deprotonated amides to metal ions such as Cu^{2+}, Ni^{2+}, or Co^{3+} is well known.[33] However, except for some macrocyclic polyamine systems, there are few Zn^{2+} complexes of deprotonated amides in the literature.[34] Therefore, a most essential question is whether Zn^{2+} can intrinsically deprotonate amides (pK_a ≈ 14) at physiological pH.

As in the previous models of the aromatic sulfonamide inhibitors **10** and **12**,[4] we designed and synthesized various carboxamide-pendant cyclens. Using these, we were able to isolate Zn^{2+} complexes of neutral carboxamides, wherein either the carbonyl oxygen (**13**) or a water molecule was found to be bound to Zn^{2+} center (**15** and **17**) at neutral pH (Scheme 5).[6] Potentiometric pH titrations of **13**, **15**, and **17** (and also UV spectrophotometric titrations of **15**) established the deprotonation constants (pK_a) to be 8.59, 6.95, and 8.0, respectively, at 25 °C and I = 0.1 ($NaClO_4$). These deprotonated species were identi-

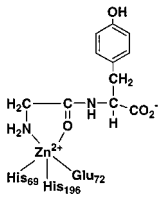
Scheme 6



Scheme 7



Scheme 8



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fied as being amidate N^--coordinating complexes (i.e., **14a**, **16**, and **18**), rather than the electrostatically equivalent Zn^{2+}−OH species (e.g., **14b**). An X-ray crystallographic analysis of **16** confirmed this assignment. Other useful characterization data were also obtained. For instance, a shift in the infrared frequencies of $\nu_{C=O}$ was found specifically at 1645 (in D_2O) or 1686 cm^{-1} (KBr pellet) for **13** vs 1576 (in D_2O) or 1584 cm^{-1} (KBr) for **14a**, and 1663 cm^{-1} (KBr) for **15** vs 1572 cm^{-1} (KBr) for **16**. Likewise, the UV absorption spectral maximum was seen to change from λ_{max} = 302 nm (ϵ = 14 500) for **15** to 354 nm (ϵ = 14 800) for **16**, a change we consider to be consistent with the proposed structural changes.

Taken in concert, these experiments served to demonstrate that amides in acidic to neutral conditions may bind to Zn^{2+} through these carbonyl oxygens, whereas, with the pK_a values of the coordinated amides being lowered from 14−15 to as low as 8.6 (for unactivated amides) or from ∼13 to 7.0 (for activated amides) at higher (but still near neutral) pH, the resulting deprotonated amide nitrogens can bind to Zn^{2+} directly. To the extent that this is true, species **13**, **15**, and **17** represent the first models that are useful for demonstrating Zn^{2+}-facilitated carboxamide deprotonation and the first to allow a determination of pK_a values. Indeed, this work allows us to suggest with confidence that deprotonation of carboxamide inhibitors is as feasible as the deprotonation of sulfonamides at CA and CPA active sites. Consistent with such thinking is the finding that none of the amidate complexes we produced were able to catalyze NA hydrolysis; this implies the lack of a kinetically active Zn−OH species.

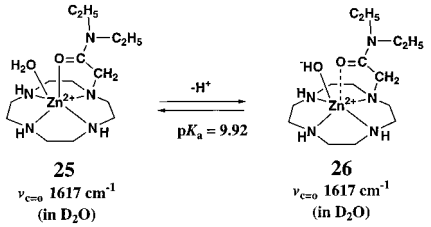
In another type of amide-pendant cyclen complexes **19** and **21**, monodeprotonation also occurred with pK_a values of 7.64 and 7.48, respectively (Scheme 6).[6] However, the products were not the amidate complexes (e.g., **20b**), but rather the electrostatically equivalent Zn^{2+}−OH species **20a** and **22**. The assignment of the Zn^{2+}−OH structures came from (1) the similarity of their pK_a values to the pK_a of 7.7 for (Zn^{2+}−*N*-methylcyclen) **2** (R = CH_3), (2) the similarity in $\nu_{C=O}$ (in D_2O) before (1624 cm^{-1}) and after deprotonation (1624 cm^{-1}), and (3) catalytic activties in NA hydrolysis analogous to that of **2**. The observed catalytic NA hydrolysis by **20a** was found to be inhibited competitively by succinimide; this is due to consumption of Zn^{2+}−OH acting (as a base) to yield a very stable 1:1 succinimide anion complex **23** (Scheme 7). The 1:1 affinity constant log *K* (*K* = [**23**]/[**19**][succinimide anion], determined by pH-metric titration) was 5.8. By way of comparison, the Zn^{2+}−cyclen complex **2a** was found to display a similar log *K* value of 5.6 when it was allowed to interact with succinimide anion.

The contrasting behavior of the two different pendant amide Zn^{2+} complexes **13** and **19** is instructive. As demonstrated by **13**, the favorable interaction of the amide oxygen with Zn^{2+} (presumably due to the formation of a stable five-membered chelate) acts to inhibit the formation of a Zn^{2+}−OH complex at neutral pH. On the other hand, this same kind of interaction is expected to favor deprotonation of the amide at alkaline pH. Such a rationalization may provide an explanation for why the amide is not hydrolyzed by CA.[31] To the extent that this is true, it becomes of interest to compare our model **13** to what is thought to occur when the poor and nonproductive substrate Gly-Tyr binds to CPA.[35] In this latter instance, the zinc ion of the active site is found to be coordinated to the carbonyl oxygen and the amino terminus. The catalytically critical important Zn^{2+}-bound water was found to be absent from the active site (see **24** in Scheme 8).

On the other hand, if the interaction between the amide oxygen and the Zn^{2+} center is weaker, as it is in the case of **19** (possibly because any putative seven-membered chelate would be unfavorable), the Zn^{2+}−OH species is more favorably formed at neutral pH. This latter species, in turn, may not be basic enough to promote proton transfer from the (unactivated) amide NH to yield **20b**. Still, this kind of Zn^{2+}−OH species (e.g., in **20a** and **22**) is expected to be sufficiently reactive to react with acidic substrates such as succinimide or with activated electrophiles such as NA.

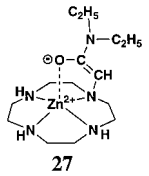
The mechanistic role of zinc in CPA, according to the Zn^{2+}−hydroxide mechanism, is to activate a water molecule with the assistance of Glu270, thereby providing a

Scheme 9



nucleophile capable of attacking at the peptide bond polarized by Arg127. Our second amide-pendant complex **19** may present such a CPA model. Specifically, the Zn^{2+}−OH species **20a** is generated with a pK_a of 7.6 and with a scissile amide held in the vicinity. Still, another assisting functionality, such as that provided by Arg127 in CPA, is likely to prove necessary before a model can be made that can hydrolyze an otherwise inactive amide bond.

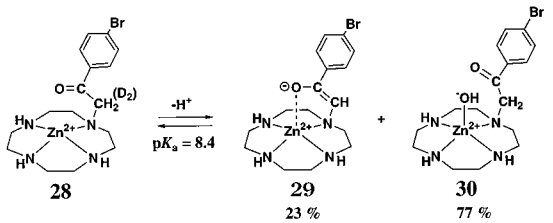
Given the above, we synthesized a *N*,*N*-dimethylcarbamoyl-pendant cyclen complex **25**, which, in contrast to **13**, has no dissociable amide hydrogens. Interestingly, the Zn^{2+}−OH species **26** was formed with a pK_a value of 9.92, which is about 2 orders higher than the pK_a values for Zn^{2+}−(*N*-methylcyclen) **2a** ⇄ **2b** (where R = CH_3), due to the inhibitory binding of the carbonyl oxygen (Scheme 9). We have observed that this Zn^{2+}−OH species, **27**, although generated only at high pH, acts as a nucleophile and hydrolyzes NA. Moreover, we saw H/D exchange for



the methylene adjacent to the carbonyl in alkaline D_2O solution, implying an intermediate Zn^{2+}−enolate complex **27** formation.

Recently, we reported as a model for zinc-containing aldolase, a Zn^{2+}−enolate complex **29**, along with an equilibrating Zn^{2+}−OH species **30** (in the ratio of 23%: 77%). Both were prepared from the carbonyl-pendant complex **28** with a pK_a value of 8.4 (Scheme 10).[36] Like **25**, H/D exchange occurred at the methylene adjacent to the carbonyl.

Scheme 10



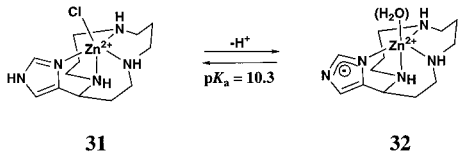
When a very reactive β-lactam carboxamide such as benzylpenicillin is in the vicinity of Zn^{2+}−cyclen **2** in physiological pH solution, the carboxamide is subject to rapid hydrolysis by the Zn^{2+}−OH species, **2b**.[37]

Recognition of Imidazole

The similar coordinating properties and pK_a values observed for amides (p$K_a \approx 14-15$) and the "pyrrole-like" nitrogens of imidazoles (p$K_a = 14.2$) led us to suggest an "ambivalent" mode for imidazole binding, wherein the imidazole binds to CA in its neutral form at low pH but binds as an anionic imidazolate at high pH.[32] Imidazole is a competitive inhibitor for CA at low pH but a noncompetitive inhibitor at high pH, at least for CO_2 hydration by the human CA I. On the basis of the pH-dependent binding and spectral changes observed for the interaction of imidazole with Co^{2+}-substituted CA I, a pK_a value of 8.0 was assigned to the transition from a bound (near the zinc) neutral imidazole Im to a bound (at the zinc) imidazolate anion Im^-. The imidazolate might compete with OH^- at the zinc center of this enzyme.[38] Consistent with this, Bertini and Luchinat assigned a four- to five-coordinate Co^{2+}−CA structure with imidazole at low pH (i.e., a loose Co^{2+}−Im bond) and a four-coordinate structure at high pH (i.e., a tightly bound Co^{2+}−Im^- complex) on the basis of absorption spectral analyses.[15]

To prove further the conditions under which Zn^{2+} may act to deprotonate imidazole, an imidazole-pendant [12]aneN_3 was synthesized.[9] The X-ray crystal structure of the resulting zinc complex **31** revealed a five-coordinate trigonal bipyramid structure with the imidazole N at an equatorial site (Zn^{2+}−N distance 2.025 Å) and a Cl^- counteranion at an apical position (Zn−Cl distance 2.361 Å). A pH−potentiometric titration, in conjunction with an NMR spectroscopic study of the monodeprotonated complex **32**, led us to the suggestion that deprotonation of the Zn^{2+}-bound imidazole occurred with a pK_a value of 10.3 (Scheme 11). Neither the imidazole complex **31** (at pH 8) nor the imidazolate complex **32** (studied at pH 10−11 in aqueous solution) acted to hydrolyze NA.

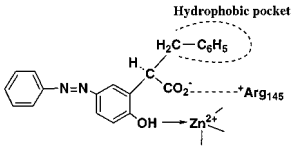
Scheme 11



Recognition of Phenol

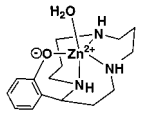
Phenol (p$K_a = 10$) is another competitive inhibitor of CA II with regard to its signature CO_2 hydration function.[15,38,39] Like the case for imidazole, an ambiguity existed in the literature as to whether the phenolate anion binds to the acid (Zn^{2+}−OH_2) form or whether the phenol binds to the basic (Zn^{2+}−OH) form. Kinetic arguments favored the binding of neutral phenol to the acid (Zn−OH_2) form of the enzyme.[40] On the other hand, an NMR spectroscopic study of Co(II)−CA II led us to suggest that (1) phenol is largely ionized, (2) phenolate binding does not displace H_2O from the metal center, and (3) phenolate may not be directly coordinated.[15] The crystal structure of the CA II−phenol complex showed that the phenol binds in the

Scheme 12



33

Scheme 13



34

hydrophobic pocket but does not coordinate directly to the Zn center. Rather, it is found to be ligated to the Zn-bound H_2O through hydrogen bonds.[39]

The acidity of the active site zinc ion in CPA was studied by a UV spectral measurement of a substrate analogue, L-2-(1-carboxy-2-phenylethyl)-4-phenylazophenol **33**.[41] Upon ligation to the CPA active site (Scheme 12), the azophenol moiety underwent a shift of pK_a from a value of 8.76 to 4.9, which provided a direct measurement of the Lewis acidity of the active site zinc ion.

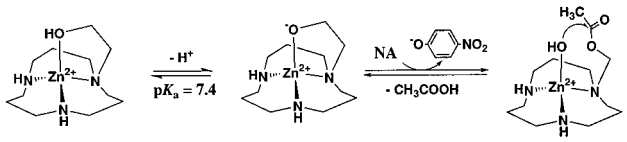
The zinc-induced deprotonation of phenol was demonstrated by using a phenol-pendant [12]aneN_3.[7] The crystal structure of the Zn^{2+} complex **34** is trigonal bipyramidal with an extremely short equatorial phenolate−Zn^{2+} bond distance (1.93 Å) (Scheme 13).[8] A water molecule is also seen to be bound to Zn^{2+} at an apical site and with a longer bond distance (2.22 Å). Deprotonation of the phenol was promoted by coordination with the pK_a shifting from 9.2 (without zinc) to 6.8 with zinc.

Recognition of Alcohols and Alkoxide Anions

Alcohols, whose pK_a values (∼15) are nearly the same as those of water, imidazole, and carboxamides moieties, are also inhibitors of CA albeit rather weak ones. It is thus of interest to ascertain whether alcoholic OH can also be deprotonated by the zinc active center of the enzyme or suitable model systems. We have prepared Zn^{2+} complexes of the ethanol-pendant [12]aneN_3 **35**[10] and an ethanol-pendant cyclen **37**.[11] Their pH-dependent behavior in the presence of Zn^{2+} is depicted in Schemes 14 and 15, respectively.

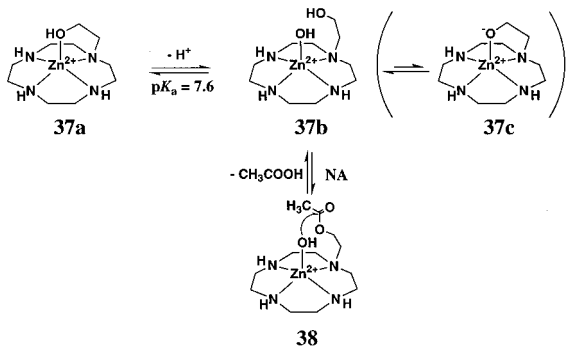
The pendant alcohol OH in **35a** was found to undergo deprotonation with a pK_a of 7.4 (at 25 °C). This yielded an alkoxide complex **35b**, which was isolated as a dimer and characterized by X-ray diffraction means.[10] On the other hand, deprotonation of **37a** (with a pK_a of 7.6)yielded a hydroxide complex **37b** (rather than an alkoxide complex **37c**), as judged from NMR spectroscopic and competitive anion binding studies.[11] Interestingly, the Zn−OH complex **37b** was found to be kinetically equivalent to the alkoxide complex **37c**, a species that catalytically hydrolyzed NA. Both **35b** and **37b** exclusively yielded the "acyl intermediate" **36** and **38**, respectively, in the rate-determining steps.

Scheme 14



35a **35b** **36**

Scheme 15



37a **37b** **37c**

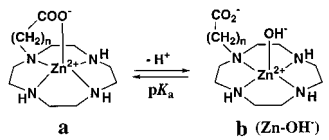
38

In both cases, these intermediate species were subject to rapid intramolecular attack by Zn−OH to complete the NA hydrolysis cycle. The dynamic equivalency (or equilibrium) as seen for **37b** and **37c** might be possible for phenol binding to the zinc active center of CA.

Recognition of Acetate

Acetate is another inhibitor of CA.[15] In CPA, the carboxylate anion of L-phenylalanine (produced by cleavage of *N*-benzoyl-L-Phe) binds to Zn^{2+}.[42] Conversely, Glu270$^-$ in the vicinity of Zn^{2+} in CPA may act as a nucleophile (at least in the classic "acid anhydride" mechanism), attacking the zinc-activated amide carbonyl. To characterize the properties of a carboxylate anion held near the Zn^{2+} center, we prepared Zn^{2+} complexes of carboxymethyl-**39**, carboxyethyl-**40**, and carboxypropyl-pendant cyclen **41** (Scheme 16).[13] In acidic aqueous solution, all these Zn^{2+} complexes should exist in equilibrium between the CO_2^--bound and the CO_2^--unbound forms.

Scheme 16



a **b** (Zn-OH^-)

39: n = 1 (pK_a = 10.6)
40: n = 2 (pK_a = 10.0)
41: n = 3 (pK_a = 8.7)

As the methylene chain was lengthened in these models, the relevant pK_a values were seen to decrease (see Scheme 16). Since the seven-membered carboxylate chelate in **41a** would be unstable, OH^- most easily displaces CO_2^- (to give **41b**) with a pK_a value of 8.7. On the other hand, the five- and six-membered carboxylate chelates in **39a** and **40a** are more stable, thus giving rise to higher pK_a values of 10.6 and 10.0, respectively. The X-ray crystallographic study of **41a** revealed a dimeric structure

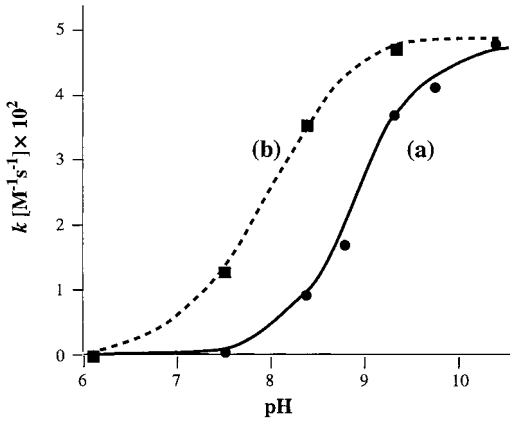
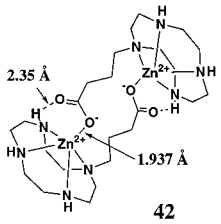


FIGURE 2. pH−rate profile for the Zn^{2+} complexes-catalyzed NA hydrolysis at 25 °C, $I = 0.10$ ($NaNO_3$) in 10% (v/v) CH_3CN: (a) with **41** and (b) with **2** (where R = CH_3).

42, which is in equibrilium with the monomeric species **41** in aqueous solution.



42

The nucleophilicity of these complexes was measured in NA hydrolysis in pH 6.1−10.4 buffers at 25 °C. The reactivity order at pH 9.3 was found to be **41** ≫ **40** > **39**. Plots of the second-order rate constants against pH gave a sigmoidal curve for **41** (Figure 2a). The half k_{max} (=4.9 × 10^{-2} M^{-1} s^{-1}) occurred at pH 8.9, a value concordant with the measured pK_a of 8.9. This is consistent with the reactive form being the Zn−OH species **41b**. The rate−pH profile for the reference *N*-methyl cyclen compound **2** (R = CH_3) is shown in Figure 2b. The half k_{max} (=4.7 × 10^{-2} M^{-1} s^{-1}) occurred at pH 7.8, again almost equal to the pK_a value for this species. Since the k_{max} values were almost the same for **41** and **2** (R = CH_3), it is concluded that the adjacent CO_2^- group present in **41b** does not contribute as a reactive species. Moreover, we found a complete loss of the reactivity in the presence of 5 mM succinimide for both **41** and **2** (R = CH_3). Presumably, this reflects the fact that the succinimidate anion ligand effectively "plugs" both of the zinc active sites, as in **23**.

Recognition of Phosphoesters

Phosphate anions are very often good inhibitors for zinc enzymes including CPA. Zinc ion in model complex **1** is found to bind strongly with dianionic (at neutral pH, the second deprotonation constant pK_{a2} = 5.8) phosphomonoesters such as monohenyl phosphate (PP^{2-}) with a 1:1 affinity constant log *K* of 3.5, which is higher than 2.6 for acetate.[14] This is due to the −2 charge and bidentate nature of PP^{2-}. As we go on to monoanionic phosphodiesters and neutral phosphotriesters, the affinity becomes successively weaker. This is compatible with the common phosphate inhibition trend of zinc enzymes. It is of interest to see that phosphomonoesters act as inhibitors, while phosphodiesters and phosphotriesters act as substrates for the Zn^{2+}−OH nucleophile in **1b**.

Conclusion

The present model studies provide important support for the contention that the molecular recognition properties of CA and CPA are governed in large measure by the intrinsic properties of the zinc ion. The pH dependence of substrate or inhibitor (X) binding affinity to the enzymes arises essentially from a competition between OH^- and X for the zinc sites. The affinity of X for Zn^{2+}−OH_2 + X ⇄ Zn^{2+}−X + H_2O and the affinity of OH^- for Zn^{2+}−OH_2 ⇄ Zn^{2+}−OH (pK_a = 7.3 and 7.9 at 25 °C for Zn^{2+}-[12]aneN_3 **1** and Zn^{2+}-cyclen **2** complexes, respectively) can be accurately determined by pH−potentiometric titration methods using appropriate model complexes. Thus, in acidic media, Zn^{2+}−X (e.g., HCO_3^-) interactions occur, while in neutral to alkaline media, Zn^{2+}−OH interactions dominate. The Zn^{2+}−OH species, when formed with our models, are found to be nucleophiles capable of catalyzing the hydration of CO_2 and aldehydes, as well as the hydrolysis of carboxyesters. As known for these reactions by CA, the rate−pH profiles all gave sigmoidal curves, which permitted an estimation of the kinetic pK_a values. In all our cases, the latter were found to be coincident with the thermodynamic pK_a values. CA-inhibiting weak acids such as sulfonamides, carboxamide, phenol, and imidazole were demonstrated to be deprotonated and to bind strongly to our Zn^{2+} complexes at physiological pH. This binding eliminates the catalytically requisite Zn^{2+}−OH species. As a consequence, catalytic events are obviated. Some carboxamides remain un-deprotonated near Zn^{2+}. In this case, the key Zn^{2+}−OH species is formed at neutral pH, and it acts as a nucleophile. The two different interactions of carboxamides with Zn^{2+} may reflect the different recognition behavior of carboxamides noted for CA and CPA. Another weak CA inhibitor alcohol also deprotonates and binds to Zn^{2+} with pK_a values similar to those of water. The resulting Zn^{2+}-bound alkoxides are strong nucleophiles to carboxyesters. Carboxylates bound to Zn^{2+} are found to act merely as inhibitors of Zn^{2+}−OH, possessing no nuleophilicity in NA hydrolysis. Phosphate anions also bind to Zn^{2+} in our model; the higher the anionic charge, higher the affinity. The results from the present model studies could prove useful in elucidating the underlying chemistry of CA, CPA, and other zinc enzymes as well as in the design of new inibitors of zinc enzymes.

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