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A promising structural zinc enzyme model for CO_2 fixation and calcification

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ABSTRACT

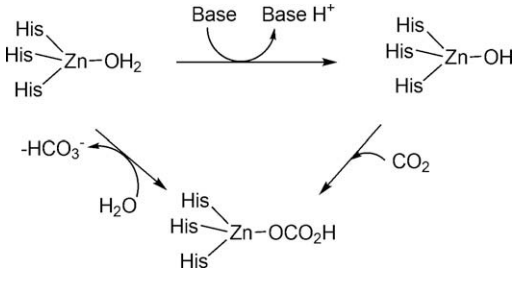
A new ligand, namely, *N*-{tris([2-[(1-methylbenzimidazol-2-yl)ethyl]methyl]amino)-2-oxoethyl}iminodiacetic acid is synthesized and characterized and used to prepare a zinc complex as a promising model for the active site of the nacreous protein in mollusc shells. Preliminary investigation of CO_2 fixation and calcification is studied with regard to the influence of the pK_a value of the coordinated water molecule and the carboxylate groups.

A great deal of interest has been focused on the role of metal ions in the active centers of hydrolytic metalloenzymes.[1] In *Carbonic anhydrase* CA, the active site of the enzyme consists of a zinc center coordinated to three histidine imidazole groups and a water molecule in a distorted tetrahedral environment.[2] The arrangement of the donors, together with an adjacent imidazole and a hydrophobic environment around the Zn(II) ion, determines the considerably high acidity of the metal-coordinated water molecule (pK_a, ca. 7). Mollusc shells are generally composed of $CaCO_3$ crystals enclosed in calcitic shell layers. Analysis of the deduced amino acid residue sequence[3] revealed that the nacrein protein contains two functional domains; one is a *Carbonic anhydrase* active site that catalyzes HCO_3^- formation from CO_2 (Scheme 1), while the other is a Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) repeated domain which binds Ca^{2+}. These domains may function as a template upon which calcification occurs.

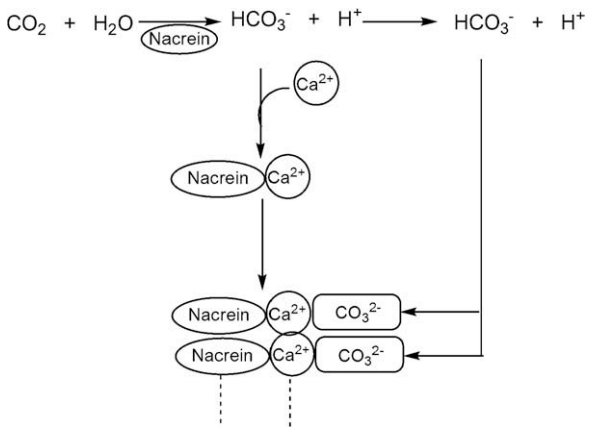
As one of the approaches adopted to resolve the nature of the active site in CA, a variety of Zn(II) complexes with synthetic receptors have been used as model systems for the enzyme.[5–7] Recently, we have reported simulation of CO_2 hydration and phosphoester hydrolysis using structural zinc enzyme model complexes.[8–17]

As a measure to cope with the possible global warming due to the greenhouse effect of CO_2, our aim is to clarify how to fix and calcify CO_2 as $CaCO_3$ by synthesizing a zinc complex, which functionally mimics the active site of the nacrein protein in molluscs (Scheme 2).

We have synthesized and characterized a new ligand, namely, *N*-{tris([2-[(1-methylbenzimidazol-2-yl)ethyl]-methyl]amino)-2-oxoethyl}iminodiacetic acid **L** (Scheme 3). The ligand was used to prepare a complex in which a Zn(II) ion is coordinated to three benzimidazole nitrogens. In addition, a water molecule is bound to complete the distorted tetrahedral coordination sphere around Zn(II), and Ca(II) is bound to the carboxylate groups. These two functional domains may provide a model mimicking the active site of the nacrein protein in mollusc shells.



Scheme 1. A proposed mechanism for the action of the *Carbonic anhydrase* active site toward the hydration of CO_2.[4]



Scheme 2. A model for the nacreous layer formation by nacrein.[3]

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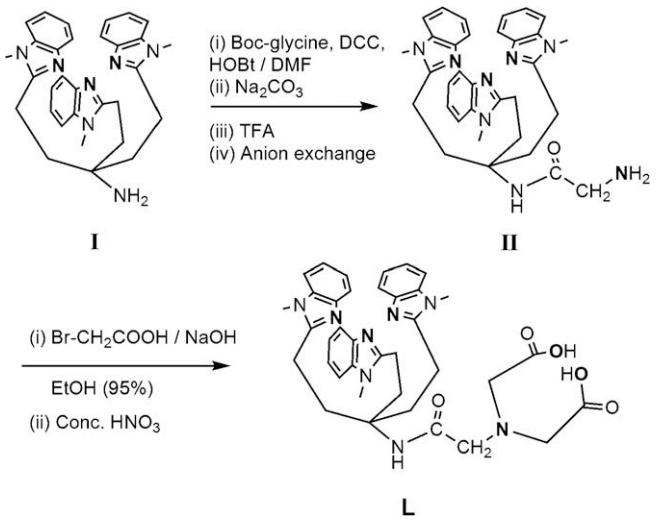


Scheme 3. Synthesis of ligand L.

α-Tris[2-(1-methylbenzimidazol-2-yl)]ethyl]methyl-aminoglycine **II** was synthesized from **I**.[18] Boc-glycine (0.78 mmol, 0.137 g) was dissolved in anhydrous DMF (10 ml), and 1-hydroxybenzotriazole (0.78 mmol, 0.12 g) was added. After 0.5 h, dicyclohexylcarbodiimide (0.885 mmol, 0.177 g) was added to the solution under stirring. The mixture was allowed to react at 0 °C for 1 h. Then tris[2-(1-methylbenzimidazol-2-yl)ethyl]methylamine **I**[18] (0.78 mmol, 0.394 g) was added. The pH was adjusted to 7 with *N*-methylmorpholine, and the mixture was allowed to react at 0 °C for 4 h, followed by 48 h at room temperature, and then filtered to remove dicyclohexylurea, and evaporated to give a solid which was suspended in dichloromethane (30 ml), washed with sodium carbonate (10%, 10 ml), dried, and dissolved in ethyl acetate (5 ml) and left aside at 4 °C overnight. A mixture of **II** and dicyclohexylurea was formed (yield 50%). The mixture was dissolved in trifluoroacetic acid (5 ml) and stirred for 0.5 h at room temperature. Afterwards, the trifluoroacetic acid was evaporated and the remaining solid was dissolved in water (5 ml) and extracted with ethyl acetate (3 × 10 ml). The aqueous layer was evaporated to give the acid form. The aqueous solution of the acid form was neutralized using aqueous NaOH (4 M) to give the free amino compound **II**.[19]

The ligand **L**[20] was synthesized as follows. A solution of **II** (0.5 mmol, 0.28 g) in ethanol (5 ml) was added dropwise to a stirred solution of bromoacetic acid (1.5 mmol, 0.21 g) in ethanol (5 ml). During the addition, the pH of the mixture was maintained above 7 with 4 N NaOH. The resulting mixture was heated to reflux for 24 h. The solvent was evaporated and the residue was recrystallized from concentrated HNO_3 to give the acid form $[H_3L][NO_3]_3$.

The acid–base behavior of the ligand in both aqueous and methanolic (wt 33%) solutions was investigated by means of pH titration (Fig. 1). In the pH range investigated (2–12), the ligand can bind four protons, presumably one at the tertiary amino group and three at the benzimidazole nitrogens. These step-wise protonation constants were determined, and are listed in Table 1.

The ^{1}H NMR spectra of the ligand **L** at different pH values based on the measured pK_a values are shown in Figure 2. The positions of resonances A and B (Scheme 4) are expected to be shifted according to changes in the electronic environment around these protons. Such changes can be brought about through modifications in the bonding at the donor sites owing to deprotonation in the solution. This of course depends on the site of deprotonation as well as on the degree of deprotonation. Thus, (i) if the deprotonation occurs solely at the carboxylate groups, only the A protons would be

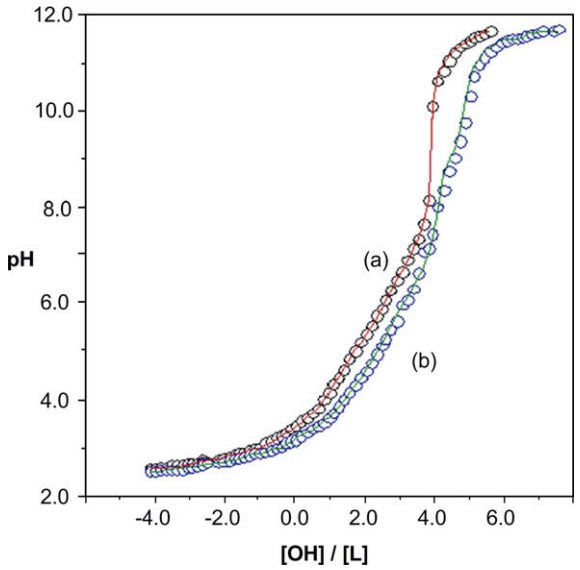


Figure 1. pH titration curves for 5.0×10^{-4} M of **L** in the presence of 2.76×10^{-3} M HNO_3 in aqueous MeOH (50%, v/v) at I = 0.1 M $NaNO_3$ and 25 °C: (a) free ligand and (b) in the presence of an equimolar amount of Zn^{2+}.

Table 1
Protonation constants, pK_a of the ligand L

Chemical species	pK_a
$H_6L^{4+} = H_5L^{3+} + H^+$	<2.00
$H_5L^{3+} = H_4L^{2+} + H^+$	<2.00
$H_4L^{2+} = H_3L^+ + H^+$	3.00
$H_3L^+ = H_2L + H^+$	4.69
$H_2L = HL^- + H^+$	5.93
$HL^- = L^{2-} + H^+$	6.63

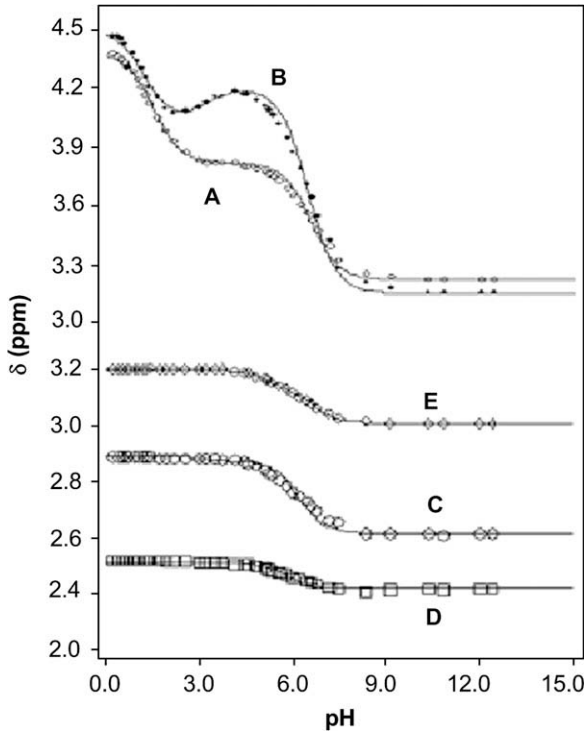
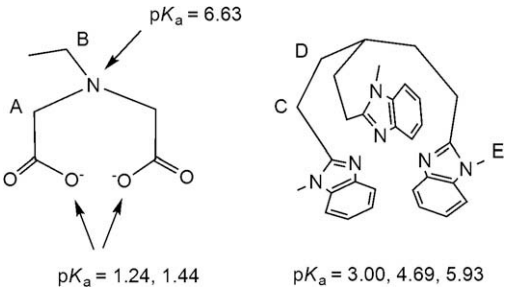


Figure 2. Chemical shifts of protons (A–E) versus pH curves for a 5×10^{-4} M solution of **L** in aqueous MeOH (50%, v/v).



Scheme 4. The protonation constants determined from the ^{1}H NMR spectra of the ligand system as a function of pH.

affected, (ii) if protonation occurs at the tertiary nitrogen site, the A and B protons would be expected to be affected to approximately the same extent, and (iii) if the deprotonation occurs at the benzimidazole nitrogens, protons C, D, H4, H5,6, and H7 would be affected. To illustrate, we consider the protonation of certain model compounds. An example of a compound involving a pure nitrogen interaction is trimethylamine (Me_3N) for which a strong pH dependence has been noted for the chemical shifts of the CH_3 protons.[21] A simple compound capable of both nitrogen and carboxylate interactions is methyliminodiacetic acid.[22]

The acid–base behavior of the ligand in methanolic (wt. 33%) solution was also investigated by means of ^{1}H NMR studies, which showed that the pK_a values of the tertiary amine nitrogen and the three benzimidazole nitrogens, were determined to be 6.63, 5.93, 4.69, and 3.00, respectively. These values were in good agreement with those determined by pH titration, whereas the two carboxylate protons are expected to deprotonate at pH values lower than 2. By increasing the pH value to 2, the methylene protons A were shifted upfield compared to the protonated form. In Figure 2, the chemical shifts of the methylene protons A show that the two carboxylate protons are deprotonated and overlapped as one signal, whereas the methylene protons B remained constant without shifting. By increasing the pH values above 6, both the A and B protons were shifted upfield compared to the protonated form. In Figure 2, the methylene protons B show that the two carboxylates and the tertiary amine nitrogen are deprotonated and showed two inflections for A and one inflection for B. Evidence for the rapid exchange of these two types of protons is provided by the absence of additional resonances in the spectrum. The average pK_a value of the benzimidazole nitrogens (4.5) is lower than that of the free benzimidazole (5.6) as supported by the investigation of Buhler et al.,[23] who determined the pK_a values of a series of bis(pyridin-2-yl)alkanes with an increasing number of carbon atoms between the pyridine rings and found that longer aliphatic chains had less effect on the protonation processes, where the interaction of the aromatic rings decreases the basicity of the nitrogen donors.

The potentiometric pH titration curve for ligand **L** in the presence of equimolar Zn^{2+} revealed complex formation until [OH]/[**L**] = 4, suggesting deprotonation and coordination of the four nitrogen donor atoms (tertiary and benzimidazole nitrogens). After [OH]/[**L**] = 4, the zinc(II) complex reacted with more OH^- than was needed to fully deprotonate the ligands. This excess base consumption was assigned to proton loss from the coordinated water molecule forming a **L**Zn(OH) species. The obtained stability constants logK_{st} and deprotonation constants pK_a (H_2O) of the zinc(II) complex are listed in Table 1.

The pH titration curve was best fitted by taking into account eight different species in the pH range 2–12, differing from each other only in their protonation states: Zn^{2+}, $ZnH_3\mathbf{L}^{3+}$, $ZnH_2\mathbf{L}^{2+}$, $ZnH\mathbf{L}^{+}$, Zn**L**, $Zn_2\mathbf{L}_2$, Zn**L**OH^-, and $Zn_2\mathbf{L}_2OH^-$. The stability constants of these complexes are lower compared to those found in zinc complexes with benzimidazole ligands as investigated by Brown et al.,[24] who determined the stability constants for N–CH_3 imidazole derivatives and compared them to their N–H imidazole analogs. In every case, N-methylation reduced the metal binding ability of the ligand by 3–4 pK units. While there is no obvious reason for the stronger binding ability of N–H versus N–CH_3 imidazoles, the phenomenon may be related to better solvation of the former when bound to the metal. A typical diagram of species distribution as a function of pH is displayed in Figure 3. The most significant finding is the presence of mono- and dinuclear zinc complexes (Zn**L**, $Zn_2\mathbf{L}_2$) as well as deprotonation of the coordinated water molecule at a pK_a value of 8.85. This was confirmed by an ESI-mass spectrum for **L**/Zn^{2+} (1:1 ratio) in aqueous methanol (Fig. 4), which showed the presence of Zn**L** (m/z = 741.3) and $Zn_2\mathbf{L}_2$ (m/z = 1485.5).

A ^{1}H NMR study was carried out at different metal-to-ligand ratios at pH 9 to obtain a better insight into the complexes formed in the **L**/Zn^{2+} system, and also to distinguish between the several models to describe the pH-titration curves. Figure 5 shows the benzimidazole regions (H4, H7, and H5,6) and the methylene regions (C and D) as well as the methylene regions (A and B).

The important line broadening of the –CH_2– signals (A, B, C, and D) as well as the benzimidazole protons (H4, H7, and H5,6) upon the incremental addition of Zn^{2+} ions up to R = 2 suggest subse-

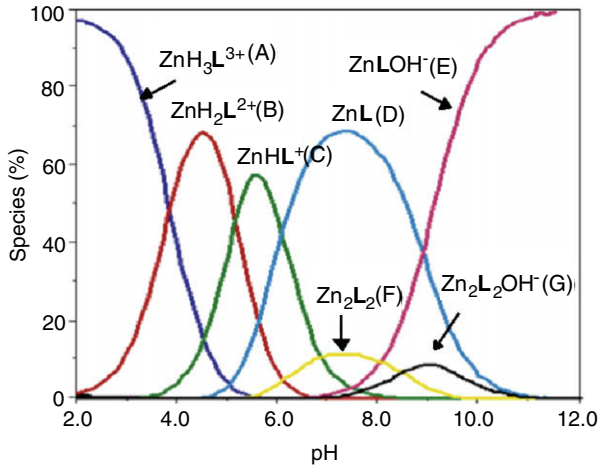


Figure 3. Species distribution curves for 5.0×10^{-4} M solutions of **L** in the presence of 2.76×10^{-3} M HNO_3 in aqueous MeOH (50%, v/v) at I = 0.1 M $NaNO_3$ and 25 °C: (A) logk_{st} = 5.27, (B) logk_{st} = 6.12, (C) logk_{st} = 6.90, (D) logk_{st} = 7.48, (E) pk_a = 8.85, (F) logk_{st} = 16.92, and (G) pk_a = 8.45.

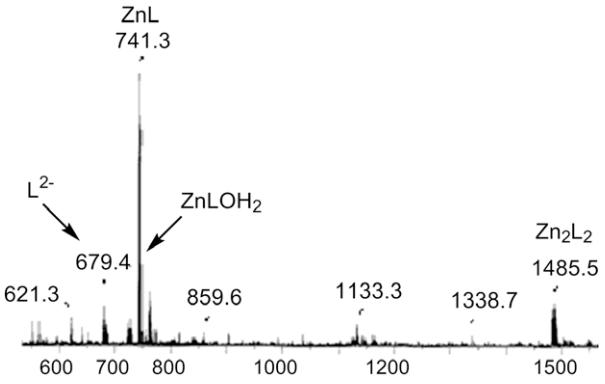


Figure 4. ESI-MS spectrum of the Zn^{2+}:**L** species in aqueous MeOH (50%, v/v) in a ratio of 1:1.

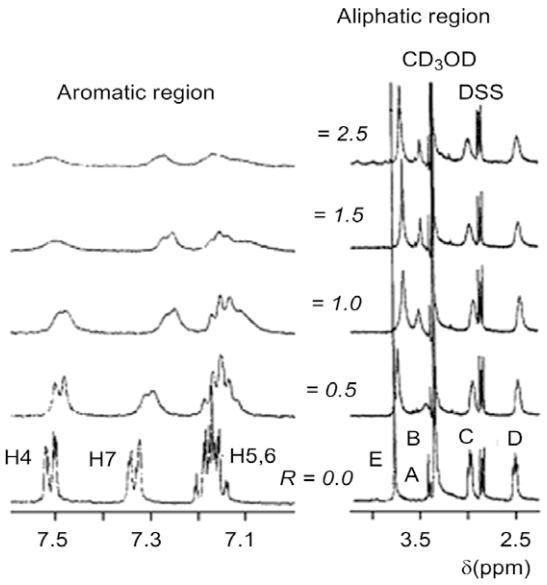


Figure. 5. Zinc(II) titration of a 5×10^{-4} M solution of **L** as a function of R = $[Zn^{2+}]$/[**L**] at 0.1 M $NaNO_3$ (50%, v/v) at pH 9.0 and 25 °C.

quent coordination of the ionizable groups in the species formed. It is shown from the ^{1}H NMR curves that up to R = 1, the chemical shifts of all the protons close to the ionizable groups were affected and shifted upfield compared to those found in the free ligand, suggesting the formation of a weakly coordinated mononuclear complex, which showed lower stability. After R = 1, the chemical shifts of these protons were shifted downfield compared to those found when R = 0.2–1.0, suggesting the formation of a new species in solution, that is, a dinuclear zinc complex.

A preliminary investigation of CO_2 fixation using the model zinc(II) complex was undertaken. CO_2 gas was bubbled for 6 h into an aqueous methanol solution (50%, v/v) containing $\mathbf{L}^{2-}$ (5.0×10^{-3} M) and $Zn(ClO_4)_2{\cdot}6H_2O$ at 1:1 molar ratio in the presence of an equimolar amount of $Ca(ClO_4)_2{\cdot}4H_2O$ at pH 9. The solution became a suspension, which was filtered to give a white precipitate and clear solution. ESI-MS spectrometry (Fig. 4) of the resultant white precipitate showed Zn**L** (m/z = 741.3) as a species in solution. The white precipitate formed after adjusting the pH value of the clear solution to 10–11 showed IR absorption bands at 870 and 1410 cm^{-1}, which were assigned to out-of-plane deformations and asymmetric stretching of CO_3^{2-} of calcite.[25] Since we encountered problems with the solubility of the zinc(II) complex, we could not study the formation of $CaCO_3$ using ^{13}C NMR spectroscopy.

Acknowledgments

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18. Casella, L.; Monzani, E.; Gullotti, M.; Gliubich, F.; De Gioia, L. *J. Chem. Soc., Dalton Trans.* **1994**, 3203.
19. *Compound* **II**: Calcd for $C_{33}H_{38}N_8O{\cdot}3H_2O$ (570.77 + 54.05): C, 63.43; H, 7.09; N, 17.94. Found: C, 63.32; H, 6.74; N, 17.57. δ_H (DMSO-d_6, 400 MHz) 7.72–7.62 and 7.39–7.29 (m, 12H, benzimidazole protons), 3.91 (s, 9H, CH_3–N protons), 3.74 (s, 2H, CH_2–NH_2), 3.23 (t, ^3J = 5.58 Hz, 6H, benzimidazole–CH_2), and 2.44 ppm (t, 6H, ^3J = 5.32 Hz, CH_2–N). δ_C (DMSO-d_6, 100 MHz) 155.1, 142.1, 135.9, 121.9, 121.0, 118.1, and 110.5 (benzimidazole carbons), 57.0 (C–NH_2), 45.0 (C–NH–), 31.5 (–CH_2–N), 29.4 (benzimidazole–CH_2), 21.1 (CH_3–N), and 173 ppm (–CO–NH–).
20. Ligand **L**: Calcd for $C_{37}H_{40}N_8O_5Na_2{\cdot}5.5H_2O$ (722.76 + 99.08): C, 54.07; H, 6.21; N, 13.64. Found: C, 54.29; H, 6.34; N, 13.61. δ_H (D_2O, 400 MHz) 7.34–7.33, 7.14–7.13 and 7.01–6.99 (m, 12H, benzimidazole protons), 3.49 (s, 9H, CH_3–N protons), 3.11 (s, 4H, –CH_2COONa), 3.05 (s, 2H, CH_2–NH–), 2.70 (t, ^3J = 5.51 Hz, 6H, benzimidazole–CH_2), and 2.31 ppm (t, ^3J = 5.38 Hz, 6H, CH_2–N). δ_C (D_2O, 100 MHz) 155.9, 141.0, 135.8, 122.9, 122.7, 117.8, and 110.6 (benzimidazole carbons), 59.3 (CH_2–N), 54.6 (C–NH–), 31.1 (–CH_2–N), 30.1 (benzimidazole–CH_2) and 21.2 ppm (CH_3–N), 174.2 (–CO–NH–), and 179.3 ppm (COONa). FAB-MS, M^+ 678.
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