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Inorganica Chimica Acta 287 (1999) 134–141

Inorganica Chimica Acta

Molecular recognition of dipeptides. Catalysis of deuteration and hydrolysis of glycylglycine by dinuclear OBISDIEN Zn(II) complexes

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Received 14 May 1998; accepted 25 November 1998

Abstract

Potentiometric equilibrium studies of the system: glycylglycine–Zn(II)–OBISDIEN are described. Kinetic studies of hydrolysis and deuteration of glycylglycine catalyzed by dinuclear Zn(II)–OBISDIEN complexes have been carried out. The hydroxyl groups coordinated to the metal ion in the complex play an important role in the hydrolysis and deuteration reactions. The specific deuteration rate constants for individual species are $k_{LZn_2HP^{4+}}$ (P = glycylglycine) and $k_{LZn_2P^{3+}} = 0$; $k_{LZn_2(OH)P^{2+}} = 7.0 \times 10^{-7}$ s^{-1}; $k_{LZn_2(OH)_2P^{+}} = 42.5 \times 10^{-7}$ s^{-1} and $k_{LZn_2(OH)_3P^{+}} = 42.5 \times 10^{-7}$ s^{-1} also. The specific rate constants for the hydrolysis reaction are $k_{LZn_2HP^{4+}}$ and $k_{LZn_2P^{3+}} \cong 1.0 \times 10^{-7}$ s^{-1}; $k_{LZn_2(OH)P^{2+}} = 4.6 \times 10^{-7}$ s^{-1}; $k_{LZn_2(OH)_2P^{+}} = 4.8 \times 10^{-7}$ s^{-1} and $k_{LZn_2(OH)_3P^{+}} = 4.9 \times 10^{-7}$ s^{-1}. The results show that the most active species toward hydrolysis are the hydroxo species. The hydroxide groups are bound to the metal centers in the cavity of OBISDIEN, and glycylglycine probably has its carboxylate group coordinated to the Zn^{2+} on one side and the amide carbonyl coordinated to the other Zn^{2+} at the other side of the macrocycle. The role of the hydroxide ion coordinated to the metal ion at the active center of hydrolytic enzymes seems to be important, and this model shows that the hydroxide ion coordinated to the metal center increases the rate constant of hydrolysis by an average of 4.8 times, and the hydroxo complexes are the only active species for the deuteration reactions.

Keywords: Molecular recognition; Hydrolysis; Zinc complexes; Hexamine complexes; Dipeptide complexes; Dinuclear complexes

1. Introduction

Nature has developed many metalloenzymes which are involved in the hydrolysis of important biomolecules, such as proteins, phospholipids and DNA [1]. Although the amide bond is unreactive [2,3] (the half life for hydrolysis in neutral aqueous solution is approximately 7 years [4]) hydrolysis of peptides catalyzed by zinc-containing enzymes is an important biochemical process.

A large body of dinucleating macrocyclic ligands capable of binding two metal ions in close proximity has appeared in the literature [5,6] as the result of their relevance to various aspects of coordination chemistry as well as to biochemical processes. The hexamine OBISDIEN (1,4,7,13,16,19-hexaaza-10,22-dioxacyclotetracosane) is a ligand with considerable flexibility. It has been shown to coordinate two metal ions, and secondary anionic guests bound to the metal centers [7–14]. Metal complexes of dinucleating macrocyclic ligands may also serve as models for certain metallo-

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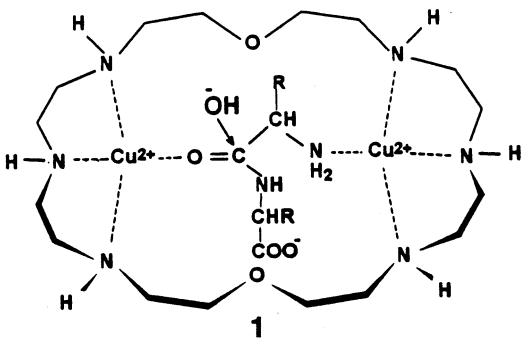
[1] Abstracted in part from a dissertation submitted by M.T.B. Luiz to the faculty of Universidade Federal de Santa Catarina in partial fulfillment of the requirements for the degree of Doctor of Chemistry.

0020-1693/99/$ - see front matter
PII: S0020-1693(98)00416-2

proteins that require the presence of two metal centers for activity [15]. The dinuclear metal complexes are now recognized [16] as efficient intermediates in biochemical relevant hydrolysis reactions.

Copper(II) ion catalysis of the hydrolysis of glycylglycine has been reported [17]. In that study reaction kinetics were performed at pH values of 3.6–6.0 and a maximum rate was observed at pH 4.2, probably due to the fact that at higher pH copper(II) deprotonates the amide bond, to some extent stabilizing this bond [17]. Hydrolysis of the dipeptide glycylglycine by a macrocyclic copper(II) mononuclear complex, Cu[9]aneN_3Cl_2 was reported [18]; the reaction was studied at pH 8.1 and the peptide was observed to hydrolyze slowly near to physiological pH [18]. The interaction and recognition of dipeptides by dinuclear copper(II) OBISDIEN complexes were determined and dinuclear macrocyclic metal complexes were proposed as promoting hydrolysis of peptides. Formula **1** shows the suggested arrangement of the dipeptide in the dinuclear cavity of the receptor complex [19], and how it is activated toward hydrolysis by coordination of Cu(II).



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In the present work equilibrium studies of Zn(II)–OBISDIEN complexes of the dipeptide glycylglycine were performed, and a Zn(II)–OBISDIEN–glycylglycine complex is proposed in order to explain the intramolecular catalysis whereby the peptide carbonyl undergoes nucleophilic attack by the coordinated hydroxide ion.

2. Experimental

The OBISDIEN employed was synthesized by the method described in the literature [20,21]. The glycylglycine (Aldrich) was reagent grade material and was used without further purification. The stock solution of Zn(II) nitrate hexahydrate ($Zn(NO_3)_2 6H_2O$) was standardized by titration with EDTA (ethylenediaminetetraacetic acid) with Erio T as indicator [22]. Carbonate-free solutions of 0.100 M KOH were prepared from Baker Dilut-It ampoules and were standardized by titration against standard potassium acid phthalate purchased from Sigma Chemical Co.

2.1. Potentiometric equilibrium measurements

Potentiometric studies of OBISDIEN in the presence and absence of Zn(II), and the dipeptide (glycylglycine) was carried out with a Micronal-B 375 pH meter fitted with blue-glass and calomel reference electrodes calibrated to directly read $-\log [H^+]$, designated as p[H]. The ionic strength of the solution was maintained at 0.100 M by addition of KCl. The temperature was maintained at 25.00 ± 0.05°C and the experimental solutions, adjusted to 0.100 M ionic strength by addition of KCl, were titrated with 0.100 M standard CO_2-free KOH. Depending on the stoichiometry, 0.1–0.2 or 0.05–1.0 mmol quantities of reagents were used in each system. The p[H] measurements were made as KOH was added to the system in small increments and the value of $K_w = [H^+][OH]$ used in the computations was $10^{-13.78}$ [23]. Each titration was performed in such a manner as to collect 10–11 equally spaced points per equivalent of base added, with up to 20 increments added beyond that required. Although in all cases preliminary titrations were performed, these were done only for comparison but never used in the calculations. Beyond the preliminary titration only a single final titration was used in the calculations. Thus the glycylglycine titration provided 25 points; 1:1 Zn(II):glycylglycine 34 points; OBISDIEN alone provided 65 points: 2:1 Zn(II):OBISDIEN system provided 88 points and the ternary system 1:2:1 provided 101 points. OBISDIEN alone provided 61 points; 2:1 Zn(II)–OBISDIEN system provided 87 points and the ternary system 1:2:1 provided 100 points.

2.2. Calculations

Equilibrium constants were calculated with the program BEST [23]. Species distribution diagrams were calculated with the aid of the SPE program [23] and plotted on a Laser Jet with SPEPLOT [23].

2.3. NMR measurements

The NMR spectra of the compounds in 99.9% D_2O were recorded with a 200 MHZ Bruker AM 200 spectrometer at 25.0°C. Internal reference was sodium 3-(trimethylsilylpropanesulfonate (DSS), and the water peak was also used as a reference. Sample solutions with various p[D] values were prepared by adding a KOD/D_2O solution that was prepared from a 40% wt/wt solution in 98% D_2O (Aldrich). The concentration of the system was ca. 0.01 M OBISDIEN, 0.02 M Zn(II) and 0.01–0.03 M glycylglycine. The p[H] values of the solutions were measured by using a Digimed DMPH-3 pH meter equipped with a combination electrode (Ingold). The instrument was standardized with diluted standard HCl solutions and the readings were

converted to p[D] by the relation $p[D] = p[H]_{means} + 0.44$ [24]. The solutions were incubated in a 70.0 ± 0.1°C constant temperature bath (Etica Equip. Cient SA).

The values of k_{obs} were calculated by following the disappearance of the NCH_2 resonance (CH_2 adjacent to the amino group), and the CCH_2 resonance (CH_2 adjacent to the carboxyl group) when possible. Since the reaction was slow, the integrations of the resonances were normalized with respect to the CH_2 resonances of the macrocycle.

3. Results and discussion

3.1. Equilibrium studies

After testing various combinations of likely species, the formation of ternary species defined by Eqs. (1)–(5) were considered present in these systems. They were derived from potentiometric data under the present experimental conditions. For the dipeptide HP, we may write the following expressions:

$$LZn_2^{4+} + P^- \rightleftharpoons LZn_2P^{3+}$$

$$K = \frac{[LZn_2P^{3+}]}{[LZn_2^{4+}][P]} \quad (1)$$

$$LZn_2P^{3+} + H^+ \rightleftharpoons LZn_2HP^{4+}$$

$$K_1^H = \frac{[LZn_2HP^{4+}]}{[LZn_2P^{3+}][H^+]} \quad (2)$$

$$LZn_2(OH)P^{2+} + H^+ \rightleftharpoons LZn_2P^{3+}$$

$$K_1^{OH} = \frac{[LZn_2P^{3+}]}{[LZn_2(OH)P^{2+}][H^+]} \quad (3)$$

$$LZn_2(OH)_2P^+ + H^+ \rightleftharpoons LZn_2(OH)P^{2+}$$

$$K_2^{OH} = \frac{[LZn_2(OH)P^{2+}]}{[LZn_2(OH)_2P^+][H^+]} \quad (4)$$

$$LZn_2(OH)_3P + H^+ \rightleftharpoons LZn_2(OH)_2P^+$$

$$K_3^{OH} = \frac{[LZn_2(OH)_2P^+]}{[LZn_2(OH)_3P][H^+]} \quad (5)$$

where L is the macrocyclic ligand OBISDIEN.

3.1.1. Species formed in the glycylglycine-bridged dinuclear Zn(II)–OBISDIEN system

The potentiometric curve for a mixture of Zn(II), OBISDIEN and glycylglycine in a 2:1:1 molar ratio is shown in Fig. 1 (curve E), together with curves of glycylglycine (A), glycylglycine–Zn(II)(II) (B), OBISDIEN (C), and OBISDIEN–Zn(II) (D).

The curves were analyzed by considering the formation of several mixed complexes, and the ones with stoichiometry LZn_2P^{3+}, LZn_2HP^{4+}, and $LZn_2(OH)_xP^{+3-x}$, $x = 1, 2, 3$, were found, where LZn_2P is the (μ-peptide)dizinc(II) OBISDIEN complex, LZn_2HP^{4+} is the protonated species, and $LZn_2(OH)_xP^{+3-x}$ is the hydroxo species. The resulting equilibrium system is shown in Scheme 1 and the species are related by protonation steps. The values of the equilibrium constants appear in Table 1. The protonation step leading to LZn_2HP^{4+} probably occurs on the amino group of the dipeptide, which is close to the macrocyclic cavity. Protonation of the carboxylate group is not possible in

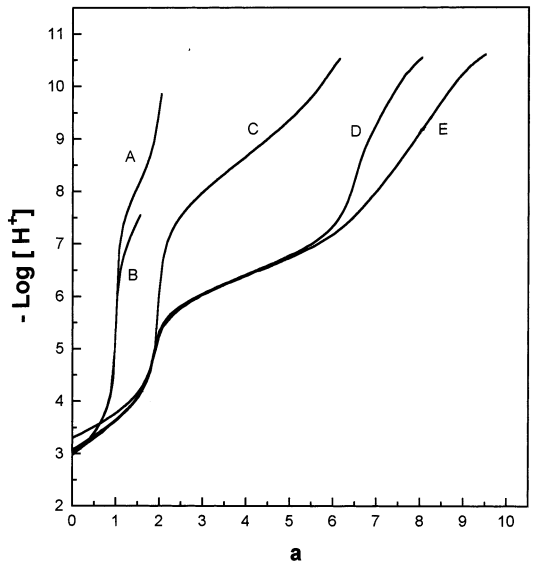
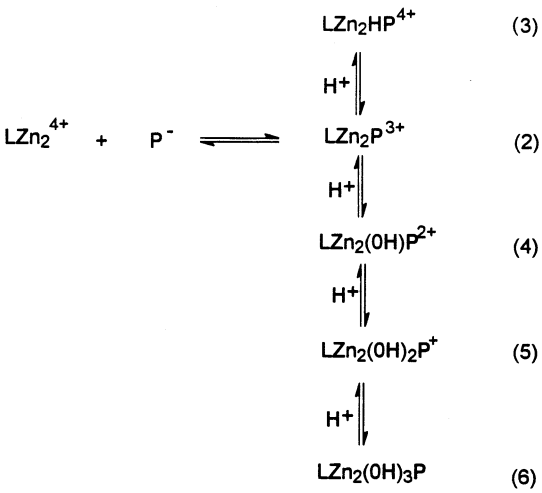


Fig. 1. Potentiometric equilibrium curves for the glycylglycine–Zn(II)–OBISDIEN system at 25.00 ± 0.50°C and $\mu = 0.100$ M (KCl). (A) 0.11231 mmol of glycylglycine in 50.00 ml of solution; (B) 0.1242 mmol of glycylglycine and 0.0979 mmol of Zn(II) in 50.00 ml of solution; (C) 0.1056 mmol of OBISDIEN in 50.00 ml of solution; (D) 0.1056 mmol of OBISDIEN and 0.1795 mmol of Zn(II) in 66.10 ml of solution; (E) 0.1056 mmol of OBISDIEN, 0.1795 mmol of Zn(II) and 0.1064 mmol of glycylglycine dissolved in 77.76 ml of solution (a = moles of KOH added per mole of ligand).



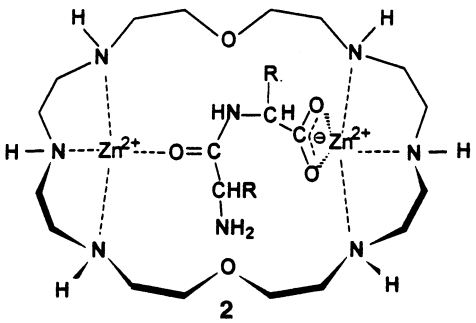
Scheme 1.

Table 1
Log values of stability constants and protonation constants for ternary species formed by reactions of Zn(II) complexes of OBISDIEN with glycylglycine (GG) at 25.00 ± 0.05°C, $\mu = 0.100$ M (KCl)[a]

$[LZn_2P^{3+}]/[LZn_2^{4+}][P^{-1}]$	4.87
$[LZn_2HP^{4+}]/[LZn_2P^{3+}][H^+]$	6.65
$[LZn_2P^{3+}]/[LZn_2(OH)P^{2+}][H^+]$	7.65
$[LZn_2(OH)P^{2+}]/[LZn_2(OH)_2P^+][H^+]$	9.27
$[LZn_2(OH)_2P^+]/[LZn_2(OH)_3P][H^+]$	10.50

[a] Ref. [25] 25°C, $\mu = 0.1$, $\sigma_{fit} = 0.014$ log K $[ZnGG^+]/[Zn^{2+}][GG^-] = 3.52$ (3.44); log K $[ZnGG_2][ZnGG^+][GG^-] = 6.20$ (6.31); log K $[ZnHGG^{2+}]/[ZnGG^+][H^+] = 6.27$ (5.60); log K $[ZnGG^+]/[ZnOHGG][H^+] = 8.51$ (8.24).

these complexes since it is coordinated to one of the two metal centers as shown in Formula **2**.



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In order to determine whether protonation occurs at the terminal amino group of the dipeptide in the complex or at one of the macrocyclic amino groups, a plot of the chemical shifts of the CH_2 resonances of the protons adjacent to the amino group of glycylglycine, NCH_2, alone and in the complex, were performed (Fig. 2). Log K values found from this curve were 8.7 and 7.3, for free and complexed glycylglycine, respectively, in D_2O. The log K values in H_2O are 8.1 and 6.7. Protonation constants determined in D_2O and H_2O for most ligands differ by about 0.5–0.7 log units, a quantity that has been shown to increase with pK_a according to the relationship in Eq. (6) [26]. Thus the results indicate that protonation occurs at the terminal amino group of the dipeptide, and not at one of the macrocyclic amino groups.

$$pK(D_2O) - pK(H_2O) = 0.41 + 0.020pK(H_2O) \quad (6)$$

The species distribution curves of glycylglycine–Zn(II)–OBISDIEN system as a function of p[H] (Fig. 3) show that formation of ternary species begins to occur at p[H] 5.5, with the protonated species reaching a maximum at p[H] 6.6 where only 18.9% has formed, 42.3% of the non-protonated species has formed at p[H] 7.3. Above p[H] 7.0 the hydroxo species predominates; the monohydroxo being 63.5% at p[H] 8.5, the dihydroxo being 58.9% formed at p[H] 9.9 and the trihydroxo is 85.5% at p[H] 12.0.

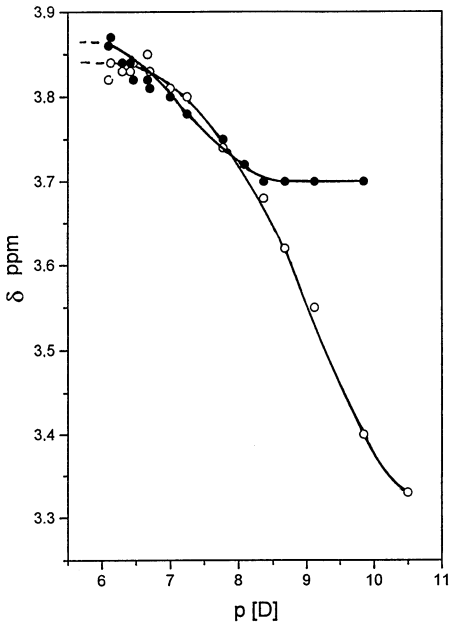


Fig. 2. Variation in pH of chemical shifts of NCH_2 protons of glycylglycine–Zn(II)–OBISDIEN (●) and of glycylglycine alone (○) at 25.0°C and $\mu = 0.100$ M (KCl).

3.2. Kinetic measurements

Two parallel reactions were observed to occur in the glycylglycine–Zn(II)–OBISDIEN system: deuteration of NCH_2 (CH_2 adjacent to the amino group) and hydrolysis of peptide bond.

The proton NMR spectra of the glycylglycine–Zn(II)–OBISDIEN system at p[D] 8.37 (Fig. 4) shows

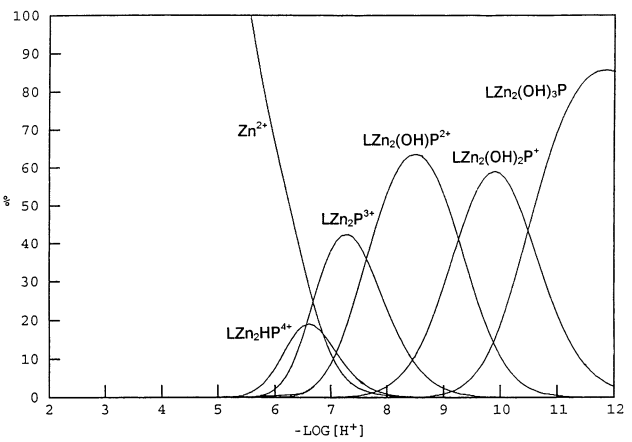


Fig. 3. Species distribution curves of 1:2:1 OBISDIEN–Zn(II)–glycylglycine as a function of $-\log [H^+]$ for a solution initially containing 1.00×10^{-2} M OBISDIEN, 2.00×10^{-2} M Zn(II), and 1.00×10^{-2} M glycylglycine, where LZn_2P^3, $LHZn_2P^{4+}$ and $LZn_2(OH)P^{+2-x}$ are the non-protonated, monoprotonated and hydroxo species of the glycylglycine dinuclear Zn(II)–OBISDIEN complex. The curve labeled Zn^{2+} represents the free aquo Zn(II) ion, $t = 25.00 \pm 0.05$°C and $\mu = 0.100$ M (KCl), % = per cent concentration with Σ [OBISDIEN] = 100%. Only the peptide–Zn(II)–OBISDIEN species are shown.

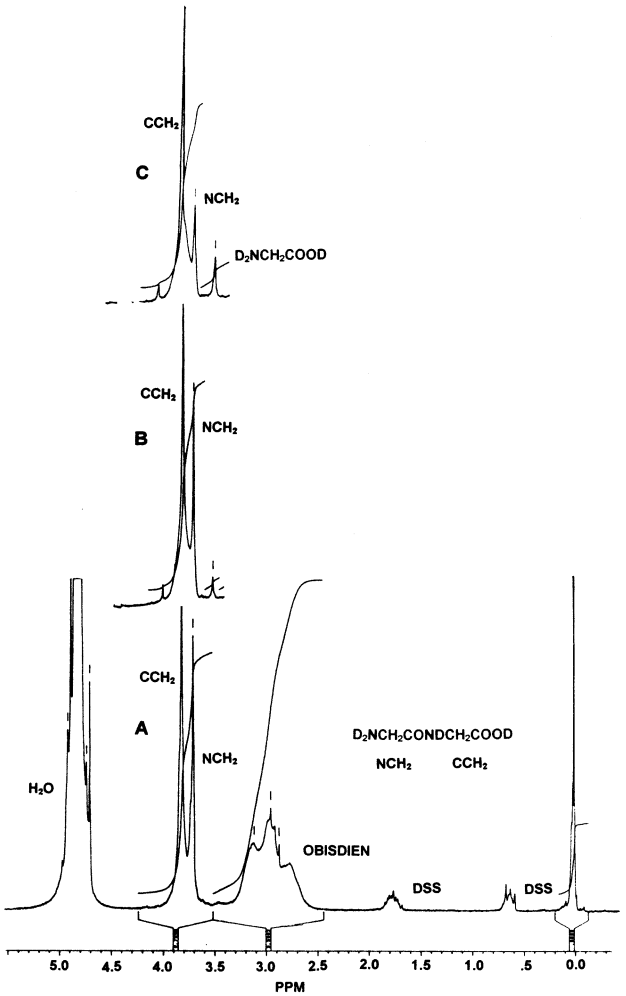


Fig. 4. The 200 MHz spectra of 1:2:1 OBISDIEN–Zn(II)–glycylglycine system for a solution initially containing 1.00×10^{-2} M OBISDIEN, 0.100×10^{-2} M Zn(II) and 3.50×10^{-2} M glycylglycine at p[D] 8.37, showing the decrease in the NCH_2 signal and resonance due to glycine CH_2 protons; (A) = zero; (B) after 91 h 30 min; and (C) after 210 h 30 min in D_2O, $t = 70.0 \pm 0.5$°C and $\mu = 0.100$ M (KCl).

a resonance at 3.75 ppm due to the CH_2 adjacent to the amino group (NCH_2), and a resonance at 3.85 ppm due to the CH_2 adjacent to the carboxylate group (CCH_2) of glycylglycine. The deuteration reactions were studied following the sequence A, B, C, shown in Fig. 4. Deuteration occurs at the NCH_2 of the dipeptide while the CCH_2 does not deuterate under these conditions. The product appears at 3.47 ppm as a singlet. Deuteration of several dipeptides has been reported previously and was found to occur at the NCH_2 group [27].

The kinetic results are interpreted with Eqs. (7) and (8).

$$\text{glycylglycine} \xrightarrow{k_h} 2 \text{ glycine} \quad (7)$$

$$\text{glycylglycine} \xrightarrow{k_d} \text{deuterated glycylglycine} \quad (8)$$

where k_h is the hydrolysis constant and k_d is the deuteration constant. The observed constant is the sum of

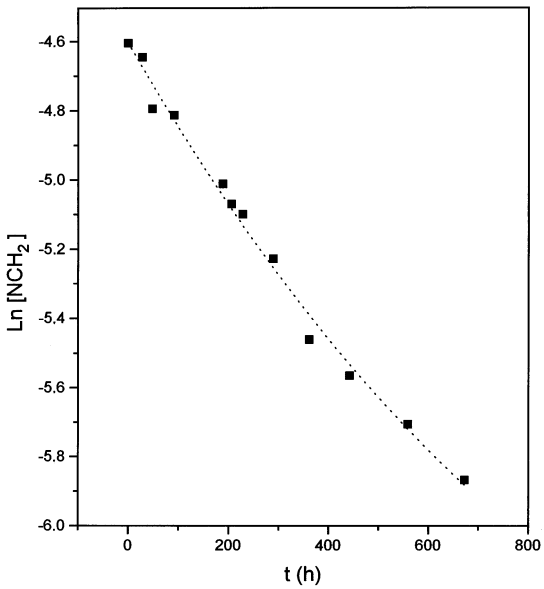


Fig. 5. Log of ln[n-CH_2] vs. time for the deuteration of the N-terminal amino acid of the dipeptide glycylglycine in D_2O at p[D] 3.37, $t = 70.0 \pm 0.2$°C; $\mu = 0.100$ M (KCl).

these two constants (Eq. (9)).

$$k_{obs} = k_h + k_d \quad (9)$$

The values of k_{obs} were calculated from the disappearance of the NCH_2 resonance. The constants were calculated from the slope of the tangent to the curve Ln[NCH_2] versus time, in zero time (Fig. 5). The hydrolysis constants were calculated with the aid of Eq. (10) [28].

$$[\text{glycylglycine}] = \frac{k_h[\text{glycylglycine}]_0[1 - e^{-k_{obs}t}]}{k_{obs}} \quad (10)$$

The values of the deuteration constant, k_d, were determined with Eq. (9) and they are listed in Table 2.

The hydrolysis constants were also calculated independently of deuteration by following the disappearance of the CCH_2 proton NMR resonance illustrated in Fig. 6. The CCH_2 group is the CH_2 group adjacent to the carboxylate group of glycylglycine. This was done

Table 2
Observed deuteration rate constants and hydrolysis constants for the dipeptide glycylglycine ($t = 70.0 \pm 0.2$°C, $\mu = 0.100$ M KCl)[a]

p[D]	$k_{obs} \times 10^7$ s^{-1}	$k_h \times 10^7$ s^{-1}	$k_d \times 10^7$ s^{-1}
7.01	0.5 (3)	0.4 (3)	0.1 (3)
8.37	6.3 (3)	2.6 (2)	3.7 (2)
8.72	14.0 (3)	3.5 (2)	11.5 (3)
9.8	27.0 (5)	3.8 (6)	23.2 (6)
10.6	40.0 (6)	4.3 (6)	35.7 (6)

[a] The numbers in parentheses are the estimated errors in the last significant figure.

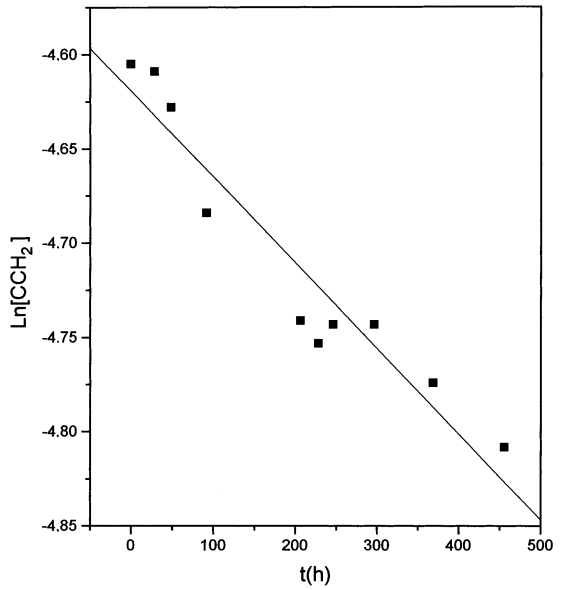


Fig. 6. Plot of $\ln[[AA]^{\infty} - [AA]_t]$ vs. time for the hydrolysis of the dipeptide glycylglycine in D_2O, p[D] 8.37, $t = 70.0 \pm 0.2$°C, $\mu = 0.100$ M (KCl).

for p[D] 8.37 and 8.72 and k_h values obtained were 2.6×10^{-7} s^{-1} and 3.5×10^{-7} s^{-1}, respectively. These values are in agreement with the constants calculated with the aid of Eq. (9).

The specific rate constants for each active species present in the system were calculated with Eq. (11). The concentration of each species was taken from Fig. 3. We assumed the activity of protonated species, LZn_2HP^{4+}, to be the same as the non-protonated species, LZn_2P^{3+}, since the formulas are very similar.

$$k_h[LZnP]_T = k_1[LZn_2HP^{4+}] + k_2[LZn_2P^{3+}] + k_3[LZn_2(OH)P^{2+}] + k_4[LZn_2(OH)_2P^{+}] + k_5[LZn_2(OH)_3P] \quad (11)$$

$[LZn_2P]_T$ is the total concentration of species in the peptide–Zn(II)–OBISDIEN system, and k_1, k_2, k_3, k_4 and k_5 are the specific hydrolysis constants of each species, the monoprotonated, non-protonated, monohydroxide, dihydroxide and trihydroxide, respectively.

Table 3
Specific hydrolysis and deuteration rate constants of N-terminal CH_2 residue of glycylglycine ($t = 70.0 \pm 0.2$°C, $\mu = 0.100$ M KCl)[a]

Species	$k \times 10^7$ s^{-1} (hydrolysis)	$k' \times 10^7$ s^{-1} (deuteration)
LZn_2HP^{4+}, LZn_2P^{3+}	1.0 (2)	0.0
$LZn_2(OH)P^{2+}$	4.6 (3)	7.0 (3)
$LZn_2(OH)_2P^{+}$	4.8 (4)	42.5 (6)
$LZn_2(OH)_3P$	4.9 (4)	42.5 (6)

[a] The numbers in parentheses are the estimated errors in the last significant figure.

The values of specific rate constants determined for these species are given in Table 3. The activities of the hydroxide species are about the same, with the trihydroxide species slightly higher. It is five times the activity of the non-hydroxide species. Intramolecular catalysis is evident in these systems. The nucleophilic hydroxide ion bonded to the metal center is in the cavity of the complex, close to the peptide carbonyl of the glycylglycine which is activated by coordination to the other metal center.

The same methods of calculation were carried out for the deuteration reactions and the specific deuteration rate constants were calculated with the aid of Eq. (12).

$$k_d[LZnP]_T = k_{1'}[LZn_2HP^{4+}] + k_{2'}[LZn_2P^{3+}] + k_{3'}[LZn_2(OH)P^{2+}] + k_{4'}[LZn_2(OH)_2P^{+}] + k_5[LZn_{2'}(OH)_3P] \quad (12)$$

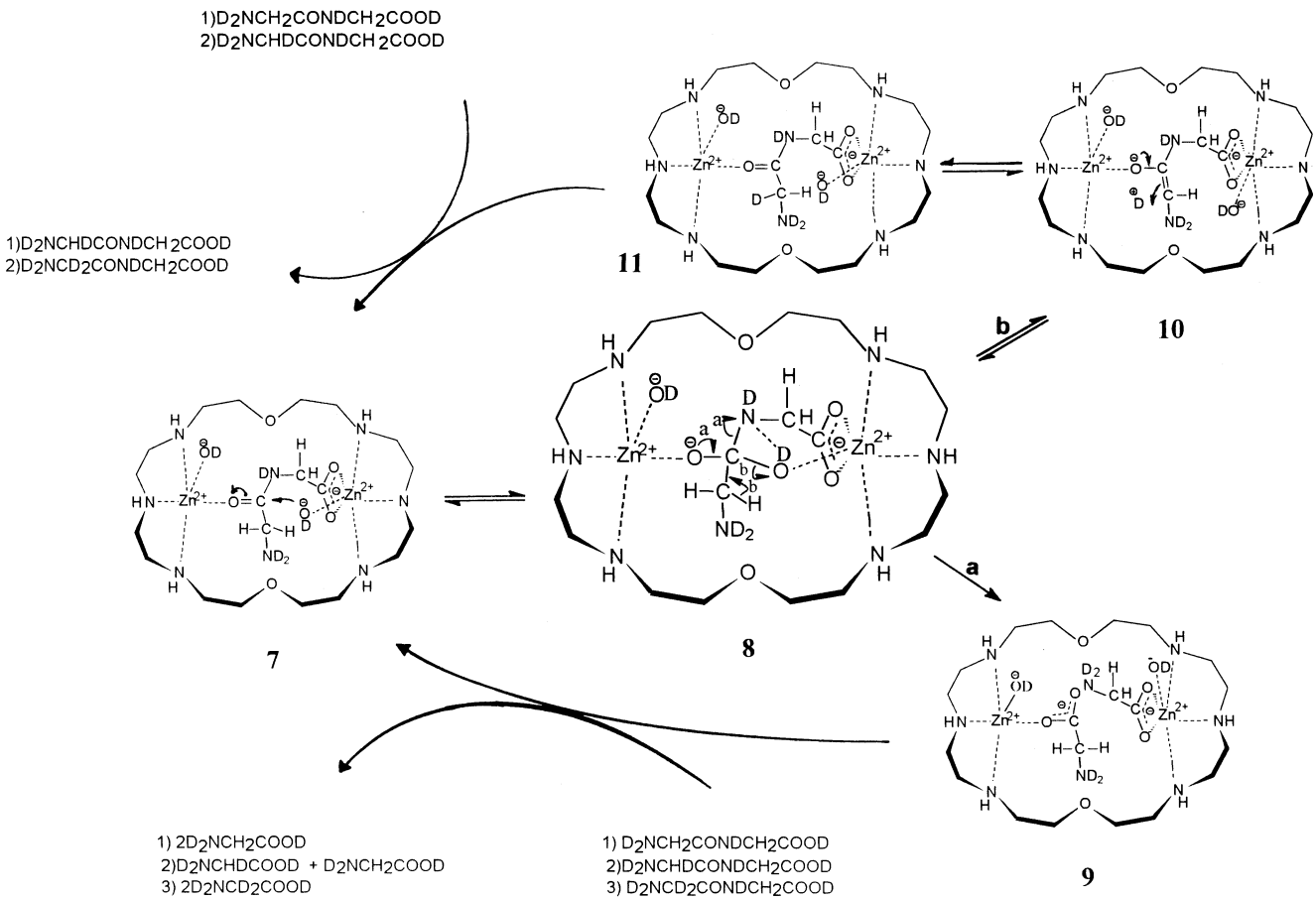
The hydroxo species are the most active species for the deuteration reaction, and the non-hydroxo is inactive. The results listed in Table 3 show that the di- and trihydroxo species are the most active, they are six times faster when compared to the monohydroxo species.

3.3. Reaction mechanism

The proposed mechanisms for dinuclear Zn(II)–OBISDIEN catalyzed hydrolysis and deuteration reactions of glycylglycine appear in Scheme 2.

Coordination of glycylglycine in the cavity of the 'host' complex (dihydroxo) dizinc(II)–OBISDIEN yields the ternary complex **7** (Scheme 2). In this complex, the carboxylate group is coordinated to the Zn(II) ion on one side, and the amide carbonyl is labilized by the metal center at the other side of the dinuclear Zn(II)–OBISDIEN complex as in **8**, allowing a nucleophilic attack by the OH^- coordinated to the Zn(II) ion. This is one of the most active species for hydrolysis and deuteration reactions. It is suggested that the intermediate **8** accounts for the two parallel reactions, hydrolysis and NCH_2 deuteration. Following path a, hydrolysis of glycylglycine is completed and two molecules of glycine are formed in **9**. Coordination of a new molecule of the peptide completes the catalytic cycle. Following b, deuteration of the intermediate **10** produces a NCH_2-deuterated molecule. Substitution of this molecule into **11** by a new peptide molecule completes the cycle. Both cycles can go forward, deuteration of the NCH_2 group of the peptide, and hydrolysis of the amide bond yielding a deuterated glycine and a non-deuterated glycine.

The hydroxo species are on average about five times more active in the hydrolysis reaction, compared with the non-hydroxo species. The non-hydroxo species have low activity toward hydrolysis and are inactive toward



Scheme 2.

deuteration. However, deuteration was observed for the hydroxo species. The dihydroxo species with a hydroxyl group bonded to each metal center, has a higher probability for nucleophilic attack on the coordinated dipeptide because the OH^- group coordinated to a Zn^{2+} ion is in a favorable position for such an attack (as in **7**, Scheme 2). This species is six times more active than the monohydroxo form. Addition of another OH^- group coordinated to one of the metal ions, as in the trihydroxo species, has no effect or it is very small.

The results show that intramolecular catalysis was observed for both reactions, and the coordinated OH^- ions play an important role in this process. The hydrolysis of glycylglycine alone and in the presence of Zn(II) was too slow to be measured following the same procedure used for the glycylglycine–Zn(II)–OBISDIEN system.

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