HYDROLYSIS AND DEUTERATION OF GLYCYLGLYCINE CATALYZED BY
DINUCLEAR [BMXDCu₂]⁴⁺ COMPLEX

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ABSTRACT

Kinetic studies of hydrolysis and deuteration of glycylglycine by dinuclear [BMXDCu₂]⁴⁺ complexes were following by NMR¹H. Two parallel reactions were observed for the ternary system BMXD-Cu₂-Glycylglycine: peptide bond hydrolysis and NCH₂ deuteration reactions. The reaction rates show a first order behavior to the concentration of the ternary [BMXDCu₂Glycylglycine] complex. The specific rate constants for the hydrolysis reaction are: $k_{LCu_2HGG^{4+}}$ ($L = BMXD$ and $GG = glycylglycine$) = $1,8 \times 10^{-6} s^{-1}$; $k_{LCu_2GG^{3+}} = 2,3 \times 10^{-6} s^{-1}$; $k_{LCu_2H_{-1}GG^{2+}}$, $k_{LCu_2(OH)H_{-1}GG^{+}}$ and $k_{LCu_2(OH)_2H_{-1}GG} = 0$, and the specific deuteration rate constants for individual species are: $k_{LCu_2HGG^{4+}} = 3,9 \times 10^{-6} s^{-1}$; $k_{LCu_2GG^{3+}} = 4,3 \times 10^{-6} s^{-1}$; $k_{LCu_2H_{-1}GG^{2+}}$, $k_{LCu_2(OH)H_{-1}GG^{+}}$ and $k_{LCu_2(OH)_2H_{-1}GG} = 0$. The results show that the most active species toward hydrolysis and deuteration reactions are the protonated and non-protonated species, the former being the most reactive species. Semi-empirical calculations for energy minimization showed that the binuclear [BMXD-Cu₂]⁴⁺ complexes adopt the boat-type conformation, in order to accommodate the dipeptide glycylglycine.

KEYWORDS: hydrolysis; deuteration; dinuclear [BMXD-Cu₂]⁴⁺ complexes, glycylglycine.

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RESUMO

Estudos cinéticos da hidrólise e deuteração da glicilglicina pelo complexo binuclear $[BMXDCu_2]^{4+}$ foram acompanhados por NMR¹H. Duas reações paralelas foram detectadas para o sistema ternário BMXD-Cu₂-GG: reação de hidrólise da ligação peptídica e a deuteração do NCH₂. A velocidade de reação demonstra um comportamento de primeira ordem para o complexo ternário $[BMXDCu_2GG]$. As constantes de velocidade específicas para as reações de hidrólise são: $k_{LCu_2HGG^{4+}}$ (L = BMXD e GG = glicilglicina) = $1,8 \times 10^{-6} s^{-1}$; $k_{LCu_2GG^{3+}} = 2,3 \times 10^{-6} s^{-1}$; $k_{LCu_2H_{-1}GG^{2+}}$, $k_{LCu_2(OH)H_{-1}GG^+}$ e $k_{LCu_2(OH)_2H_{-1}GG} = 0$, e as constantes de velocidade específicas para a reação de deuteração das espécies são: $k_{LCu_2HGG^{4+}} = 3,9 \times 10^{-6} s^{-1}$; $k_{LCu_2GG^{3+}} = 4,3 \times 10^{-6} s^{-1}$; $k_{LCu_2H_{-1}GG^{2+}}$, $k_{LCu_2(OH)H_{-1}GG^+}$ e $k_{LCu_2(OH)_2H_{-1}GG} = 0$. Os resultados demonstram que as espécies ativas para as reações de hidrólise e deuteração são a protonada e a não-protonada. Cálculos semi-empíricos para minimização de energia mostram que o complexo binuclear $[BMXDCu_2]^{4+}$ adota a conformação do tipo “cuia” para acomodar o dipeptídeo glicilglicina.

1. INTRODUCTION

Macrocyclic ligands containing two chelating subunits linked by two bridges may form both mononuclear and dinuclear complexes with metal cations.¹ Of special interest among this growing body of complexing agents are the macrocyclic ligands because they can impose a high degree of preorganization on metal complex formation.² The hexamine 3,6,9,17,23-hexaazatricyclo [23.3.1.1] triaconta 1(29), 11(30), 12, 14, 25, 27-hexaene (L) (Figure 1), is a ligand with considerable flexibility, that forms a dinuclear arrangement by inclusion of two cations in its molecular cavity, leaving additional coordination sites in the metals available.³ The structure of dinuclear copper(II) complex of BMXD was determined by X-ray methods, showing that BMXD adopts preferred spatial configurations dependent on the substrate coordinated in its cavity.³ Basallote and co-workers published kinetic studies of formation of mono- and dinuclear copper(II) complexes with ligand BMXD under slightly acidic solutions⁴ and extremely basic solutions.⁵

BMXD

Glycylglycine

Figure 1. Formulas of ligand BMXD and dipeptide Glycylglycine

Many biological processes involve the hydrolysis of proteins and peptides⁶ and it has been shown that metal complexes of dinuclear macrocyclic ligands may also serve as models for certain metalloproteins that require the presence of two metal centers for activity.⁷ Copper(II) ion catalysis of the hydrolysis of glycylglycine has been reported.⁸ Hydrolysis of glycylglycine (Figure 1) by a mononuclear macrocyclic copper(II) complex, Cu[9]aneN₃Cl₂, has been reported using a catalytic process.⁹ In agreement with Bordignon-Luiz¹⁰, the dinuclear macrocyclic complex [OBISDIENCu₂]⁴⁺ was proposed as a catalyst to the glycylglycine hydrolysis; the reaction was studied at various pD values (pD = -log[D⁺]) and the glycine was the observed product.

In this paper, kinetic studies of ternary system BMXD-Cu₂-GG are reported and a mechanism is proposed for hydrolysis and deuteration of glycylglycine catalyzed by dinuclear [BMXDCu₂]⁴⁺ complexes. A spatial conformation of BMXD-Cu₂-GG system is presented, with the aid of the calculations based on minimization of potential energy.

2. EXPERIMENTAL

BMXD was synthesized by a method described in the literature.¹¹ Glycylglycine (Aldrich) was reagent grade material and was used without further purification. A stock solution of Cu(II) chloride dihydrate (CuCl₂·2H₂O – Vetec) was standardized by titration with EDTA (ethylenediamine-tetraacetic acid – Vetec) with murexide as the indicator.¹² Deuterated water (D₂O 99% - Acros Organics) and a solution of deuterated sodium

hydroxide, NaOD 30t% wt in D₂O (Aldrich), were used to prepare the experimental solutions utilized in the H-NMR experiments.

2.1. NMR Measurements

The NMR spectra of the compounds in 99% D₂O were recorded with a 200MHz Bruker AC 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 pD values were prepared by adding a NaOD/D₂O solution that was prepared from a 30% wt/wt solution in 99% D₂O. The concentration of the system was ca. 0.01 mol L⁻¹ BMXD, 0.02 mol L⁻¹ Cu(II) and 0.01- 0.03 mol L⁻¹ glycylglycine. The pD values of the solutions were measured by using a Micronal B-474 pH meter equipped with a combination electrode (Ingold). The instrument was standardized with diluted HCl solutions and the readings were converted to pD by the relation $pD = pH_{meas.} + 0.40$ ¹³, and the pD of the reaction was maintained constant with additions of small amounts of concentrated NaOD with a Gilmon burette. The solutions were incubated in a 70.0 ± 0.1°C constant temperature bath (Etica Equipments Cientifics S.A.). The kinetics reactions were carried out at 70.0°C because they are very slow at 25.0°C.

The values of k_{obs} were calculated by following the disappearance of the NCH₂ (CH₂ adjacent to the amino group, C5, Figure 1), and the CCH₂ resonance (CH₂ adjacent to the carboxyl group, C2) when possible. Since the reaction was slow, the integrations of the resonances were normalized with respect to the CH₂ resonances of the macrocycle.

2.2. Characterization of Hydrolysis Product

The hydrolysis product was characterized by H-NMR. The experimental solutions were allowed to react and after an interval of time copper(II) ion were precipitated with H₂S generated from the reaction of iron(II) sulfide and concentrated HCl. The suspension was then centrifuged (Centrifuge Excelsa Baby II – Model 206-R, Fanem).

2.3. Calculation Method

Using semi-empiric models, where only the valence electrons of the atoms are considered, lower energy conformations were obtained for the complexes [BMXDCu₂]⁴⁺

and $[\text{BMXDCu}_2]^{4+}$ + Glycylglycine. The calculation of energy minimization was carried out with the program CAChe¹⁴ (Computer-Aided Chemistry) version 5.0 (Fujitsu), using the AM1-d¹⁵ (Austin Model 1) as parametrization method, and with the EF¹⁶ (Eigenvector Following) as the optimization model.

3. RESULTS AND DISCUSSION

3.1. Kinetic Measurements

Equilibrium studies of BMXD-Cu₂-GG system had been previously carried out, and the stability constants¹⁷ was used with a different concentrations of reagents to generate a species distribution plot¹⁸ (Figure 2), which was used to select the appropriate conditions to perform kinetic runs. Two parallel reactions were observed: hydrolysis of peptide bond and deuteration of NCH₂ (CH₂ adjacent to the amino group).

Figure 2. Species distribution curves of BMXD-Cu₂-GG system as a function of pH. $[\text{BMXD}] = 1.00 \times 10^{-2} \text{ molL}^{-1}$; $[\text{Cu(II)}] = 2.00 \times 10^{-2} \text{ molL}^{-1}$; $[\text{GG}] = 1.00 \times 10^{-2} \text{ molL}^{-1}$; $\mu = 0,100 \text{ molL}^{-1}$ (KCl).

The proton NMR spectra of the BMXD-Cu₂-GG system at pD 6.1 (Figure 3) shows a resonance at 3.80 ppm due to the CH₂ adjacent to the amino group, and a resonance at

3.89 ppm due to the CH₂ adjacent to the carboxylate group of glycylglycine. The deuteration reactions were studied following the sequence I, II, III, shown in Figure. 3. Deuteration occurs at the NCH₂ of the peptide, while the CCH₂ does not deuterate under these conditions. The product appears at 3.62 ppm as a singlet. Deuteration of some peptides has been reported previously and was found to occur at the NCH₂ group.^{10,19}

Figure 3. Selected 200 MHz proton NMR spectra of the 1:2:1 BMXD-Cu₂-GG system for a solution initially containing $1.00 \times 10^{-2} \text{ molL}^{-1}$ BMXD, $2.00 \times 10^{-2} \text{ molL}^{-1}$ Cu(II) and $1.00 \times 10^{-2} \text{ molL}^{-1}$ GG at pD 6.1, showing the decrease in the signal and resonance due to glycylglycine CH₂ protons; (I) = initial; (II) = after 50 h and (III) = after 72 h in D₂O; T = $70.0 \pm 0.1^\circ\text{C}$ and $\mu = 0.100 \text{ molL}^{-1}$ (KCl).

The kinetic results are interpreted with Equations (1) and (2).

where k_h is the hydrolysis constant and k_d is the deuteration constant. The observed rate constant is the sum of these two constants, Equation (3).

$$k_{\text{obs}} = k_h + k_d \quad (3)$$

The disappearance of the NCH_2 resonance with time was measured to calculate the values of k_{obs} . The observed rate constants were calculated from the straight-line slope of $\ln[\text{NCH}_2]$ versus time (Figure 4). The hydrolysis constants were calculated with the aid of Equation (4)²⁰ and the values of the deuterated constants, k_d , were determined with the aid of Equation (3) and are listed in Table 1.

$$[\text{GG}] = \frac{k_h [\text{GG}]_0 (1 - e^{-k_{\text{obs}}t})}{k_{\text{obs}}} \quad (4)$$

Figure 4. Plot of $\ln[\text{NCH}_2]$ vs. time for the deuteration and hydrolysis of CH_2 adjacent to the amino group of the dipeptide glycylglycine in D_2O at pD 6.1, $t = 70.0 \pm 0.1^\circ\text{C}$ and $\mu = 0.100 \text{ molL}^{-1}$ (KCl).

Table 1. Observed deuteration and hydrolysis constants for glycylglycine ($T = 70.0 \pm 0.1^\circ\text{C}$ and $\mu = 0.100 \text{ molL}^{-1} \text{ KCl}$)

pD	$k_{\text{obs}} (10^{-6} \text{ s}^{-1})$	$k_{\text{h}} (10^{-6} \text{ s}^{-1})$	$k_{\text{d}} (10^{-6} \text{ s}^{-1})$
4,5	3.3(2)	0.9(2)	2.4(2)
6,1	5.0(1)	1.6(1)	3.4(1)
7,1	1.5(2)	0.5(2)	1.0(3)
8,0	0	0	0
10,4	0	0	0

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

The hydrolysis constants were calculated independently of deuteration by following the disappearance of the CCH_2 resonance in the proton NMR spectrum, illustrated in Figure 5. In this way the hydrolysis is independent of deuteration, since both species, deuterated and non deuterated glycylglycine, undergo hydrolysis. The CCH_2 group is the CH_2 adjacent to the carboxylic group of glycylglycine.

Figure 5. Plot of $\ln[\text{CCH}_2]$ vs. time for the hydrolysis of the dipeptide glycylglycine in D_2O at pD 6.1, $T = 70.0 \pm 0.1^\circ\text{C}$ and $\mu = 0.100 \text{ molL}^{-1} \text{ (KCl)}$.

The specific rate constants for the hydrolysis reaction for each active species present in the system were calculated with Equation (5), and the concentrations of each species were obtained from Figure 2. The species distribution curves at 25.0°C were used in this study, because it was not possible to carry out the equilibrium studies at 70 °C because the reactions occur at this temperature. The equilibrium constants are not expected to change substantially with a 45.0°C temperature variation.

$$k_h[\text{LCu}_2\text{GG}]_T = k_1[\text{LCu}_2\text{HGG}^{4+}] + k_2[\text{LCu}_2\text{GG}^{3+}] + k_3[\text{LCu}_2\text{H}_1\text{GG}^{2+}] + k_4[\text{LCu}_2(\text{OH})\text{H}_1\text{GG}^+] + k_5[\text{LCu}_2(\text{OH})_2\text{H}_1\text{GG}] \quad (5)$$

$[\text{LCu}_2\text{GG}]_T$ is the total concentration of species in the BMXD-Cu(II)-GG system, and k_1 , k_2 , k_3 , k_4 and k_5 are the specific hydrolysis constants of each species: monoprotonated, non-protonated, amide deprotonated, monohydroxide and dihydroxide species, respectively. The values of specific rate constants determined for these species are given in Table 2.

Table 2. Specific hydrolysis and deuteration rate constants of N-terminal CH₂ residue of glycylglycine (T = 70.0 ± 0.1°C and μ = 0.100 mol L⁻¹ KCl)

Species	k_h (10 ⁻⁶ s ⁻¹) (hydrolysis)	k_d (10 ⁻⁶ s ⁻¹) (deuteration)
LCu ₂ HGG ⁴⁺	1.8(6)	3.9(4)
LCu ₂ GG ³⁺	2.3(1)	4.3(1)
LCu ₂ H ₁ GG ²⁺	0	0
LCu ₂ (OH)H ₁ GG ⁺	0	0
LCu ₂ (OH) ₂ H ₁ GG	0	0

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

The most active species with regard to the hydrolysis reaction is the non-protonated species. Deprotonation of amide bond stabilizes the peptide bond in relation to the hydrolysis reaction, and the specific rate constants for the amide deprotonated and the two hydroxo species (which are also amide deprotonated) indicate zero activity or too low to be measured with the method employed.

The same method of calculation was carried out for the deuteration reaction and the specific deuteration rate constants were calculated with the aid of Equation 6, which is similar to Equation 5. The most active species is also the non protonated species, which is to be expected since both reactions have the same intermediate, a ternary dinuclear BMXD-Cu(II) glycylglycine complex.

$$k_d[\text{LCu}_2\text{GG}]_T = k_1[\text{LCu}_2\text{HGG}^{4+}] + k_2[\text{LCu}_2\text{GG}^{3+}] + k_3[\text{LCu}_2\text{H}_1\text{GG}^{2+}] + k_4[\text{LCu}_2(\text{OH})\text{H}_1\text{GG}^+] + k_5[\text{LCu}_2(\text{OH})_2\text{H}_1\text{GG}] \quad (6)$$

3.2.Semi-Empirical Calculation

Theoretical results showed that the macrocycle structure loses degrees of freedom in its conformation when the copper II take its place in the structure, as can be seen in the Figure 6 (top view). Although the binuclear macrocycle complex $[\text{BMXDCu}_2]^{4+}$ has a more rigid structure, being more symmetrical, the complex appears to be able to be adapted, depending on the ligand that is complexed. Calculations involving one and two hydroxyl groups, as well as water molecules, were used to address these considerations. Thus, depending on the substrate coordinated in the macrocycle cavity, variations in the distance of the copper (II) atoms are observed.

Figure 6. Conformations of low energy of the complex $[\text{BMXDCu}_2]^{4+}$.

The molecule presents certain symmetry, where the aromatic rings are moved to the bottom, and the two diethylenetriamine subunits are turned to the same side, characterizing

a “boat” type conformation. The inter-atomic distance of the two copper (II) centers is 6.9 Å.

The glycylglycine must approach the complex at the top, since underneath there is a large steric hindrance, due to the nuclei of the atoms involved, and also due to an electronic steric hindrance, as this area is already occupied by many electrons (aromatic rings).

As previously mentioned, even though the minimization calculations reproduce the complex $[\text{BMXDCu}_2\text{GG}]^{2+}$ structure, showing that the macrocycle adopts the “boat” type conformation, this is not necessarily the structure that the complex would adopt in solution, since the calculations did not consider the relationship of these structures with molecules or potentials that would characterize a solvent interaction.

4. Conclusion

The proposed mechanism for dinuclear $[\text{BMXDCu}_2]^{4+}$ catalyzed hydrolysis and deuteration reactions of glycylglycine can be seen in Scheme 1. Coordination of glycylglycine in the cavity of the “host” complex BMXD-dicopper(II) yields the ternary complex 7 (Scheme 1). In this complex, the amino group is coordinated to the Cu(II) ion on one side, and the amide carbonyl is labilized by the metal center on the other side of the dinuclear $[\text{BMXDCu}_2]^{4+}$ complex, allowing a nucleophilic attack by the carboxylate group of the dipeptide. The reactions are first-order in the ternary complex concentration, since they depend on the intramolecular attack of the terminal carboxylate group on the amide carbonyl, yielding a cyclic intermediate (8), as required for the two parallel reactions: hydrolysis of the peptide bond and deuteration of NCH_2 group. The hydrolysis reaction follows the path “a” and the breaking of the C-N bond yields the intermediate anhydride. The anhydride intermediate undergoes a fast nucleophilic attack, yielding (9) and two glycine molecules. The coordination of a new glycylglycine molecule closes the catalytic cycle.

The deuteration reaction follows path “b”. Deuteration of intermediate (10) yields a monodeuterated molecule of glycylglycine (11), which may be substituted by a new molecule of the dipeptide, closing the catalytic cycle. The two cycles occur in parallel producing deuterated and non deuterated glycine molecules.

5. REFERENCES

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