Zn^{II} Complex with a Phenanthroline-Containing Macrocycle as Receptor for Amino Acids and Dipeptides − Hydrolysis of an Activated Peptide Bond

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The interaction between several L-amino acids and dipeptides and the Zn^{II} complex with ligand L, a macrocycle containing a triamine chain linking the 2,9 positions of a phenanthroline moiety, has been studied by means of potentiometric and ^1H NMR spectroscopic measurements in aqueous solutions. In the $[ZnL]^{2+}$ complex, the metal ion displays an unsaturated coordination environment and the metal can act as a binding site for these substrates. Amino acids and dipeptides in their neutral (zwitterionic) form bind to Zn^{II} through the carboxylate function and, when in their anionic form, through the amine group. In this case the carbonyl of the amide function is probably also involved in metal coordination. Amino acids containing aromatic pendants form the most stable complexes, due to hydrophobic and/or π-stacking interactions between the aromatic subunits of the substrates and the phenanthroline moiety of the metal receptor. The hydrolytic properties of the Zn^{II} complex with L, involving the cleavage of the peptide bond, have been tested by using L-leucine-*p*-nitroanilide (LNA), which contains an activated peptide bond. This substrate forms stable $[ZnL(LNA)]^{2+}$ and $[ZnL(OH)(LNA)]^{+}$ complexes. The formation of the $[ZnL(OH)(LNA)]^{+}$ complex is followed by LNA hydrolysis, through a nucleophilic attack of the Zn–OH function on the peptide bond.

Introduction

Over the years a great deal of interest has focused on the role of metal ions in the active centers of hydrolytic metalloenzymes (e.g. phosphatase, amino- and carboxypeptidase, carbonic anhydrase, P1-nuclease).[1−15] Hydrolytic metalloenzymes generally contain one or more Zn^{II} ions in their active site. The catalytic role of zinc is generally ascribed to two main functions: i) binding and activation of the substrates, and ii) deprotonation of Zn^{II}-coordinated water molecules to give Zn−OH functions, which can act as nucleophiles in the hydrolytic mechanism. The catalytic site is often lodged in a hydrophobic pocket, which may contribute to substrate binding and can also assist in the formation of the nucleophilic Zn^{II}−OH functions.

Aminopeptidases are one of the Zn^{II} enzymes and contain either one or two zinc ions per active site and specifically hydrolyze the *N*-terminal residue of polypeptide chains, through a catalytic mechanism which involves coordination of the peptide function to zinc, followed by a nucleophilic attack of a Zn−OH group on the carbonyl carbon and cleavage of the peptide bond.[4,5,7,12−15]

As one of the approaches to the study of Zn^{II} enzymes, a number of synthetic Zn^{II} complexes have recently been used as simple model systems for hydrolytic enzymes.[16−50] Examples of functional models for aminopeptidases are less common, due to the marked hydrolytic inertness of the peptide bond to hydrolysis. Examples of Cu^{II},[17,18,51−53] Pt^{II},[54] Pd^{II},[55−57] Co^{III} [18,58] and Ce^{IV} [59] complexes able to cleave the peptide bond in aqueous solutions are known; peptide bond cleavage induced by Zn^{II} complexes has rarely been observed,[40] and occurs only at high temperature in aqueous solution, due to the low tendency of Zn^{II} to hydrolyze the amide bonds. As a matter of fact, binding of free Zn^{II} to peptides may even inhibit the cleavage of the peptide bond.[37]

We have recently reported on the synthesis of a series of phenanthroline-containing polyamine macrocycles,[60,61] such as L (Figure 1), able to form stable Zn^{II} complexes in aqueous solutions.[62]

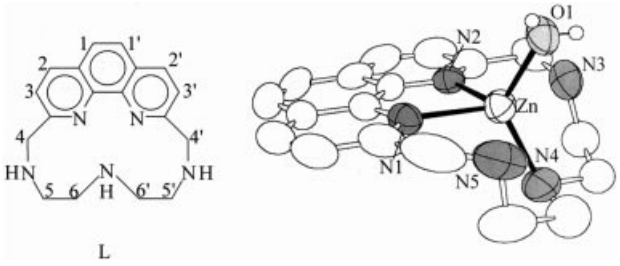

Figure 1. L drawing (with atom labelling used in NMR spectroscopic experiments) and crystal structure of the $[ZnL(H_2O)]^{2+}$ cation.

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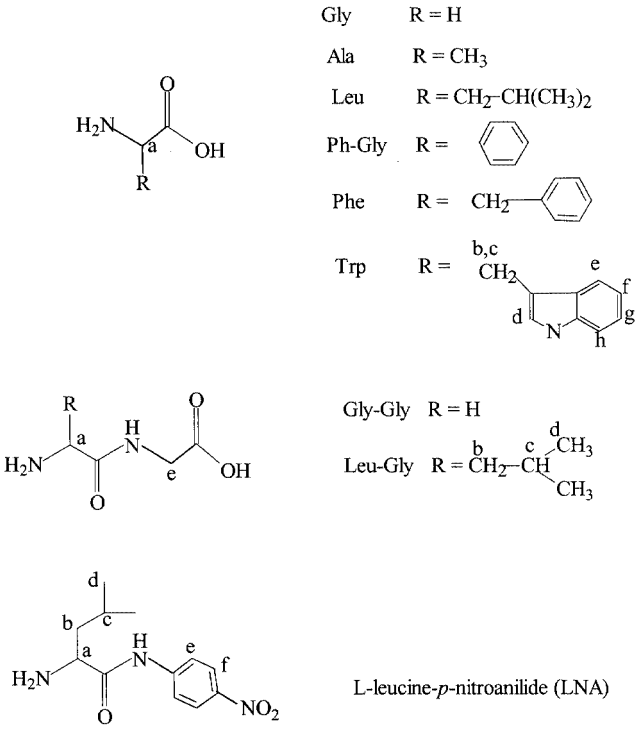
The rather high stability of the complexes is mainly due to the heteroaromatic nitrogens, which can offer an optimal binding site for the metal ion. At the same time, the rigidity of the phenanthroline unit does not allow for the involvement of all the aliphatic amine groups in the metal binding. Actually, the crystal structure of the $[ZnL(H_2O)]^{2+}$ cation (Figure 1) shows a Zn^{II} coordination environment not saturated by the ligand donors, since the two benzylic nitrogens are not involved in metal binding.[62] A water molecule is coordinated to Zn^{II}, to give an almost tetrahedral coordination environment for the metal. The presence of "free" binding sites at the Zn^{II} ion makes this complex a promising metallo-receptor for substrate molecules.

A solution study showed that the coordinated water molecule deprotonates at a slightly alkaline pH to give a $Zn^{II}-OH$ function. A dihydroxo complex $[ZnL(OH)_2]$ is also formed at an alkaline pH. Finally, phenanthroline can generate a rather hydrophobic environment for the metal. These characteristics make this complex a potential candidate for a model system of hydrolytic enzymes.

With this in mind, we decided to investigate the effective binding properties of this complex for amino acids and dipeptides as well as its hydrolysis of a peptide bond. The substrates involved in this study, along with the atom labeling used in the NMR spectroscopy experiments, are shown in Scheme 1.



Scheme 1

Results and Discussion

Crystal Structure of $[ZnL(HGly)]_2(ClO_4)_4 \cdot 1.5H_2O$

The crystal structure consists of $[ZnL(HGly)]^{2+}$ cations, perchlorate anions and water solvent molecules. The $[ZnL(HGly)]^{2+}$ cation contains the amino acid in its neutral form, herein indicated by HGly. The asymmetric unit contains two $[ZnL(HGly)]^{2+}$ independent cations. The ORTEP[63] drawings of the two $[ZnL(HGly)]^{2+}$ cations (herein indicated A and B) with atom labelling are shown in Figure 2 and Table 1 lists selected bond angles and distances for the coordination sphere of Zn^{II}. The coordination environment for the Zn^{II} ion is almost equal in both the A and B cations and can be described as a distorted tetrahedron. In complex A, the Zn1 ion is coordinated by the N1 and N2 heteroaromatic nitrogens, the central N4 amine of the aliphatic chain and the O1g oxygen of the carboxylate group of the amino acid.

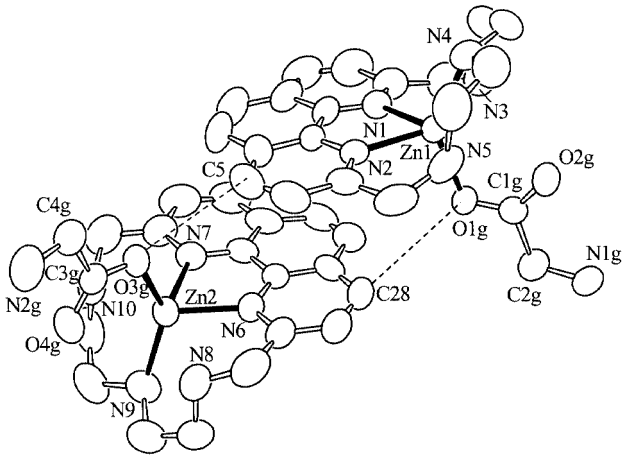


Figure 2. ORTEP drawing of the $[ZnL(HGly)]^{2+}$ complex, representing the A and B independent units coupled by π-stacking and C–H···O hydrogen bond interactions

Table 1. Bond lengths [Å] and angles [°] for the metal coordination environment in the A and B cations of $[ZnL(HGly)]_2(ClO_4)_4 \cdot 1.5H_2O$

A Unit			
Zn1–O1g	1.989(4)	Zn1–N1	2.165(5)
Zn1–N2	2.171(5)	Zn1–N4	2.094(6)
O1g–Zn1–N1	103.79(19)	N1–Zn1–N2	74.64(19)
O1g–Zn1–N2	104.79(17)	N4–Zn1–N1	110.5(2)
O1g–Zn1–N4	135.5(2)	N4–Zn1–N2	110.9(2)
B unit			
Zn2–O3g	1.990(4)	Zn2–N7	2.167(5)
Zn2–N6	2.150(5)	Zn2–N9	2.095(6)
O3g–Zn2–N6	104.83(18)	N6–Zn2–N7	75.0(2)
O3g–Zn2–N7	104.42(19)	N9–Zn2–N6	110.7(2)
O3g–Zn2–N9	136.1(2)	N9–Zn2–N7	109.0(2)

The Zn–N bond lengths are in the range 1.99–2.17 Å. Their values are typical for Zn^{II} complexes with polyamine compounds. The benzylic nitrogens N3 and N5 are not coordinated, lying far apart from the metal ion [Zn1···N3 2.440(6) Å, Zn1···N5 2.448(7) Å].

In complex B, the Zn2 ion is coordinated by three donor atoms of the ligand molecule (N6, N7 and N9) and the

O3g oxygen atom of glycine, while the N8 and N10 benzylic nitrogen atoms are not coordinated (Table 1).

It is worth noting that, in both $[ZnL(HGly)]^{2+}$ independent cations, the amino acid is in its zwitterionic form. In both A and B complexes, the C−O bond lengths of the carboxylic group of HGly are equal within their e.s.d. [C1g−O1g 1.234(8) Å, C1g−O2g 1.238(8) Å, C3g−O3g 1.259(7) Å, C3g−O4g 1.226(8) Å], indicating that this group is in its anionic form. The unbound amine group is therefore protonated. Such a monodentate binding mode of Gly is rather common for amino acids in their zwitterionic form.[24−26,35−37,64]

In both A and B, the coordination geometry of the metal ion is very similar to that found in the crystal structure of $[ZnL(H_2O)](ClO_4)_2$. Actually, in the present structure an oxygen donor of the carboxylate unit simply replaces the Zn^{II}-coordinated water molecule of $[ZnL(H_2O)](ClO_4)_2$. The overall conformation of the ligand is also similar in the two complexes. As already found in $[ZnL(H_2O)]^{2+}$, the ligand is folded along the line connecting the benzylic nitrogen atoms, giving rise to dihedral angles of 82.4(3)° (A complex) and 84.5(3)° (B complex) between the mean planes defined respectively by the benzylic nitrogen atoms and the aromatic unit (max deviations 0.15(1) Å for C8 in the A complex and 0.154(8) Å for C25 in the B complex) and by the three secondary nitrogen atoms of the aliphatic chains.

Inspection of the crystal packing reveals the presence of π-stacking interactions between the phenanthroline moiety of the two independent cations. As shown in Figure 2, the two heteroaromatic groups are almost parallel [the dihedral angle between the planes containing the aromatic groups is 7.1(2)°] and lie 3.55 Å apart from each other. In addition, two H-bonding interactions between a C−H aromatic carbon atom of each complex and a carboxylate oxygen atom belonging to the other [C5···O3g 3.34(1) Å and C28···O1g 3.412(9) Å] contribute to the molecular pairing.[65]

Binding of L-Amino Acids and L-Dipeptides to the Zn^{II} Complex Containing L

The binding of the amino acids glycine (HGly), L-phenylglycine (Hph-Gly), L-alanine (HAla), L-phenylalanine (HPhe), L-leucine (HLeu) and L-tryptophan (HTrp) to the $[ZnL]^{2+}$ complex was studied by means of potentiometric and ^1H NMR spectroscopic measurements in aqueous solutions; the results of the potentiometric investigation are reported in Table 2.

Table 2. Equilibrium constants for the addition of L-amino acids to the Zn^{II} complex with the ligand L (NMe_4NO_3 0.1 M, 298 K).

Reaction	log *K*					
	Gly	*ph-Gly*	*Ala*	*Phe*	*Leu*	*Trp*
$[ZnL]^{2+} + A^- = [ZnL(A)]^+$	2.96(4)	3.61(3)	2.95(4)	3.83(4)	2.11(4)	5.12(6)
$[ZnL]^{2+} + AH = [ZnL(AH)]^{2+}$	2.58(4)	2.71(5)	2.43(2)	2.79(3)	−	3.71(3)
$[ZnL(OH)]^+ + A^- = [ZnL(OH)(A)]$						5.02(6)

All of the amino acids under investigation, with the exception of leucine, interact with the $[ZnL]^{2+}$ complex, both in their neutral and deprotonated forms, herein indicated as HA and A^-, respectively, to give the $[ZnL(HA)]^{2+}$ and $[ZnL(A)]^+$ species (Table 2). Among the six amino acids, leucine gives the weakest interaction, and only the complex with its deprotonated form is formed in aqueous solutions. On the contrary, tryptophan gives the strongest interaction with $[ZnL]^{2+}$. Figure 3 shows the distribution diagram of complexes formed by tryptophan in the presence of our Zn^{II} complex. The formation of the complex with the zwitterionic form of the amino acid $[ZnL(HTrp)]^{2+}$ occurs at an acidic pH and is followed by deprotonation of the amino acid at neutral pH to give the [ZnL(Trp)] complex. In this case a third species, $[ZnL(Trp)OH]^+$, is found at an alkaline pH.

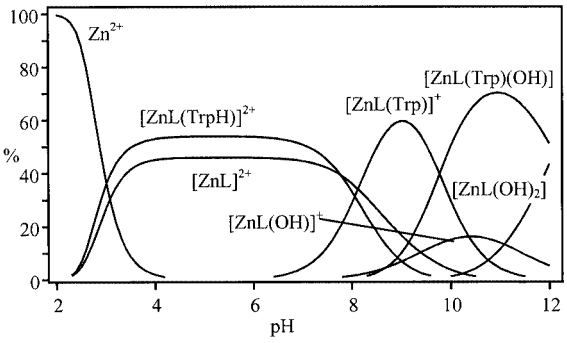


Figure 3. Species distribution diagram for the system $[ZnL]^{2+}$/Trp in 1:1 molar ratio (NMe_4NO_3 0.1 M, 298 K).

The data in Table 2 clearly outline that the stability of the complexes of our metallo-receptor with the neutral form of amino acids (HA) is lower than that found with their anionic form (A^-). A similar behavior was found for the addition of amino acids to the "free" Zn^{II} ion; it is generally accepted that coordination to Zn^{II} involves the anionic carboxylate function in the $[Zn(HA)]^{2+}$ complex and both the carboxylate and the amine groups, with the formation of a six-membered chelate ring, in the deprotonated $[Zn(A)]^+$ complex.[35−37,47,64] To shed further light on the binding mode of amino acids to our Zn^{II} complex we recorded ^1H NMR spectra at different pH values. Figure 4 (a) shows the pH dependence of the Trp signals in the presence and in the absence of our Zn^{II} complex, while Figure 4 (b) shows the ^1H NMR spectrum of a D_2O solution containing $[ZnL](ClO_4)_2 \cdot H_2O$ and tryptophan in a 1:1 molar ratio at pH 9.3, where the $[ZnL(Trp)]^+$ is the prevalent species in solution, compared with those of deprotonated tryptophan (Trp^-) and $[ZnL]^{2+}$.

As shown in Figure 4(a), the formation of the $[ZnL(HTrp)]^{2+}$ complex in the pH range 3−7 does not lead to a significant variation in the chemical shifts of the ^1H NMR signals of the protons in the α-position to the carboxylate and the amine function (*a* in Scheme 1) with respect to the same signals of the amino acid HA (prevalent in solution in the pH range 3−8), where the acidic proton is located on the amine group. This would indicate that in

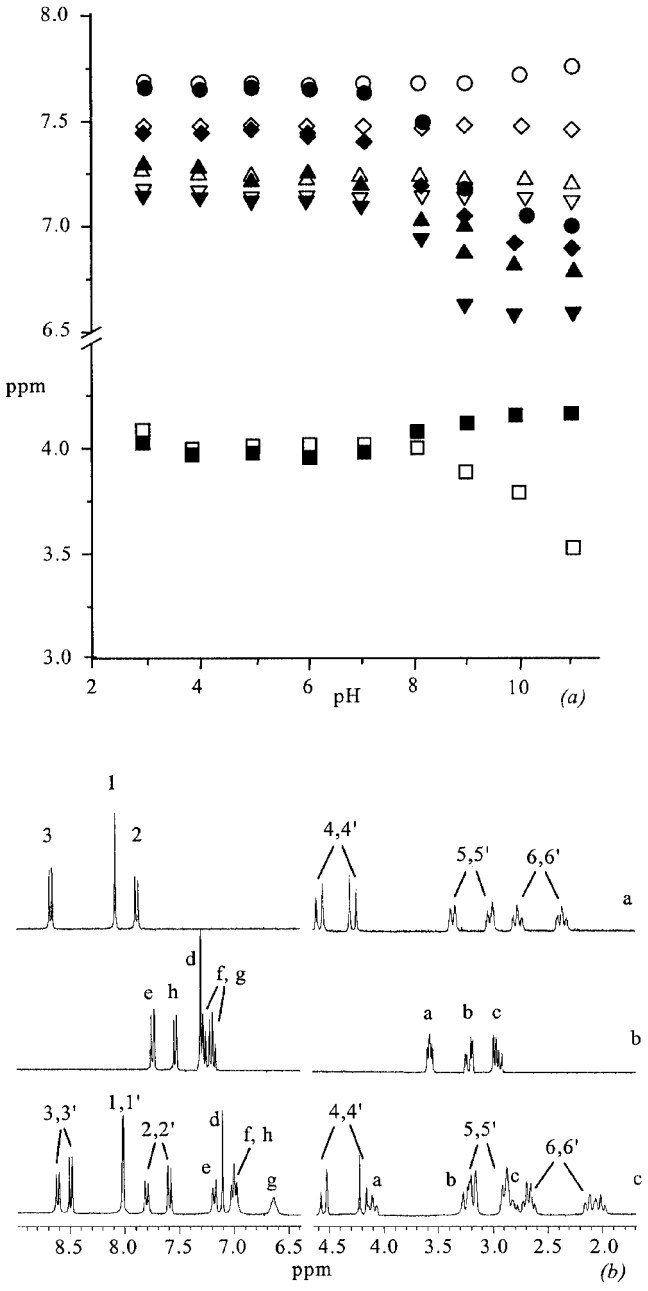


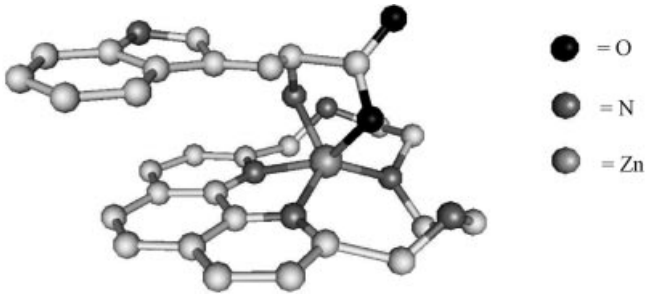
Figure 4. (*a*): pH dependence of the ^1H NMR chemical shifts of selected protons of Trp in the absence (□: a proton; ▽: g; △: f; ◇: h; ○: e). and in the presence of the Zn^{II} complex with L (■: *a* proton; ▼: *g*; ▲: *f*; ◆: *h*; ●: *e*). The signals of the protons *b*, *c* and *d* are omitted for clarity. (*b*): ^1H NMR spectra of $[ZnL]^{2+}$ (a), Trp^- (b) and $[ZnL(Trp)]^+$ (pH, 9.3) (c). See Scheme 1 and Figure 1 for the labelling used.

$[ZnL(HA)]^{2+}$ the acidic proton localization is not changed by HA binding to zinc and, therefore, HA coordination takes place through the carboxylate group, while the amine remains protonated. This hypothesis is corroborated by the crystal structure of the $[ZnL(HGly)]^{2+}$ cation, which shows the metal coordinated by one oxygen atom of the carboxylate function, while the amine group is protonated.

At an alkaline pH, the *a* signal of tryptophan alone shifts upfield, due to deprotonation of the amine group. On the contrary, the formation of the $[ZnL(Trp)]^+$ complex above pH 7 leads to a minor, but significant, downfield shift of the *a* resonance [Figure 4 (a)]. Similar downfield shifts for the signal of the *a* protons are also found for all the amino acids under investigation [up to 0.4 ppm in the case of Trp, see Figure 4 (b)]. This indicates the involvement of the NH_2 group in Zn^{II} binding. However, a bidentate coordination mode of the deprotonated amino acids, involving both the amine and the carboxylate functions, has generally been found in binary and ternary Zn^{II} complexes with these substrates,[24−26,35−37,47,64] and can be presumed to a reasonable extent in the present case.

The most interesting finding in Table 2 is the higher stability of the adducts with amino acids containing an aromatic side arm. In particular, tryptophan, which contains the largest aromatic subunit, indole, shows addition constants to the $[ZnL]^{2+}$ complex that are two orders of magnitude higher than Gly, Ala or Leu. This is rather surprising, since the addition constants of Gly or Ala to the "free" Zn^{II} ion are somewhat higher than those reported for ph-Gly, Phe and Trp.[66] Figure 4 (a) also shows that the formation of the $[ZnL(Trp)]^+$ complex remarkably affects the spectrum of the indole units, with a marked upfield shift of the signals of the aromatic protons with respect to the corresponding signals of "free" Trp^-. At the same time, the phenanthroline subspectrum displays a dramatic loss of symmetry. Besides the downfield shift of the *a* signal of the $[ZnL(Trp)]^+$ complex, discussed above, Figure 4 (b) displays a splitting into two doublets of each signal of the protons 2 and 3. The singlet attributed to 1 is also split into a doublet. Similarly to the indole resonances, the signals of the phenanthroline protons show a marked upfield shift with respect to the corresponding signals in $[ZnL]^{2+}$.

These spectral characteristics account for the presence of π-stacking interactions between phenanthroline and the aromatic pendant arm of Trp (Scheme 2), which can contribute to the stability of the complex.



Scheme 2

In the case of Phe and ph-Gly, substrate complexation by $[ZnL]^{2+}$ is also accompanied by upfield shifts of the resonances of the aromatic protons of the substrates and phenanthroline. The shifts, however, are generally smaller (ca. 0.1−0.2 ppm) than those observed for Trp and no loss of symmetry was found for the phenanthroline unit. This accounts for a reduced π-stacking pairing between the phenyl units and phenanthroline. Accordingly, the stability of the Phe and ph-Gly complexes is lower than that of the Trp complex.

Interestingly, no significant shift of the signals of the aromatic protons are observed at acidic pH, where the complexes with the protonated forms (HA) of the amino acids are present in solution [Figure 4(a)]. This experimental observation points out that the pairing between the two aromatic moieties is disrupted by coordination to Zn^{II} through the carboxylate function, due to protonation of the amine group in the $[ZnL(HA)]^{2+}$ complex.

Similar π-stacking interactions have already been observed by Sigel et al. in the case of ternary metal complexes[67,68] involving Trp and dipyridine or ATP. In the latter case the indole unit of Trp and the adenine moiety of the nucleotide are paired *via* π-stacking.[67] Yamauchi et al. also found intramolecular stacking interactions in Cu^{II} ternary complexes with Trp and dipyridine or phenanthroline, both in solution and in the solid state.[47][69−72]

In conclusion, our Zn^{II} complex may behave as a multifunctional receptor for amino acids, presenting two distinct binding sites, i.e., the metal center, which forms coordination bonds with amino acids, and the heteroaromatic unit, which can give stabilizing hydrophobic and/or π-stacking interactions with the aromatic portion of the substrates. These interactions lead to an unusual selectivity pattern in amino acid binding by our Zn^{II} complex, since the amino acids with aromatic pendant arms are strongly bound with respect to the aliphatic amino acids Gly or Ala.

In order to evaluate the effective binding properties of our complex towards the peptide amide group, we decided to extend our analysis to dipeptides. Unfortunately, most of the dipeptides tested, such as tryptophan-containing dipeptides, form low soluble complexes with $[ZnL]^{2+}$. Our study was therefore limited to L-glycylglycine (HGly-Gly) and L-leucylglycine (HLeu-Gly). Table 3 reports the equilibrium constants for the addition of these dipeptides to the $[ZnL]^{2+}$ complex in aqueous solution.

Table 3. Equilibrium constants for the addition of Gly-Gly and Leu-Gly to the Zn^{II} complex with the ligand L (NMe_4NO_3 0.1 M, 298 K).

Reaction	log *K*	
	Gly-Gly	*Leu-Gly*
$[ZnL]^{2+} + A^- = [ZnL(A)]^+$	2.84(9)	3.91(4)
$[ZnL]^{2+} + HA = [ZnL(AH)]^{2+}$	1.9(1)	3.06(2)
	pK_a	
$[ZnL(A)]^+ + H_2O = [ZnL(A)(OH)] + H^+$	8.77(6)	8.95(3)

The formation of the complex with the neutral form of the dipeptide ($[ZnL(HLeu\text{-}Gly)]^{2+}$) at acidic pH is followed by deprotonation of the substrate to give $[ZnL(Leu\text{-}Gly)]^+$ at a slightly alkaline pH. A further deprotonation step is monitored above pH 9. This process could be attributed, in principle, to deprotonation of the amide function to give the $[ZnL(Leu\text{-}GlyH_{-1})]^+$ complex or to deprotonation of a coordinated water to give a hydroxylated ternary complex. Gly-Gly shows similar binding features towards $[ZnL]^{2+}$ (Table 3). As already found in the case of binding amino acids, the addition constants of the deprotonated dipeptides to the $[ZnL]^{2+}$ complex are about one order of magnitude higher than those of the dipeptides in their neutral (zwitterionic) form. To clarify the binding mode of these dipeptides to Zn^{II}, we decided to record ^1H NMR spectra on solutions containing the dipeptides in the presence of $[ZnL]^{2+}$ at different pH values. The formation of the $[ZnL(HLeu\text{-}Gly)]^{2+}$ complex at pH 5 does not significantly change the spectrum of the dipeptide in its neutral form, which contains a protonated amine and a carboxylate group in its anionic form. Only a minor shift (ca. 0.05 ppm) of the CH_2 protons in the α-position to the carboxylate group is observed. As in the case of amino acids, this may suggest that in $[ZnL(HLeu\text{-}Gly)]^{2+}$ the dipeptide is coordinated to Zn^{II} through the carboxylate group, while the amine group still remains protonated. On the contrary, the comparison of the ^1H NMR spectra of the anionic form of the Leu-Gly dipeptide in the absence and in the presence of our $[ZnL]^{2+}$ complex at pH 8.5 shows that the formation of the $[ZnL(Leu\text{-}Gly)]^+$ species leads to a marked upfield shift of the signal of the proton in the α-position (*a* in Scheme 1) to the carbonyl carbon atom and the deprotonated NH_2 group (Figure 5).

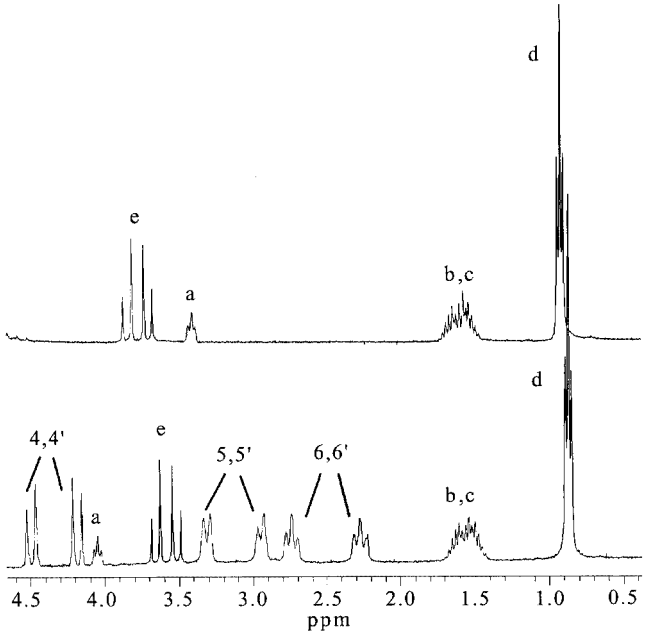


Figure 5. ^1H NMR spectra of Leu-Gly$^-$ (a) and $[ZnL(Leu\text{-}Gly)]^+$ (pH, 8.5) (b). (See Scheme 1 and Figure 1 for the labelling used).

A similar behavior is also found in the case of Gly-Gly. As already proposed in the case of amino acids, the potentiometric and ^1H NMR spectroscopic data suggest that Leu-Gly, in its anionic form, binds to Zn^{II} by using the amine group. However, the coordination of the amide carbonyl function in metal binding can also be supposed, in agreement with the binding mode generally found for dipeptides to Zn^{II} alone as well as in ternary Zn^{II} complexes.[35−37] The deprotonation process observed in the pH range 9−12 does not significantly alter the ^1H NMR spectrum of coordinated Leu-Gly$^-$, suggesting that the de-

protonation process involves a coordinated water molecule to give a [ZnL(Leu-Gly)(OH)] complex.

The latter complex contains both a Zn^{II}-coordinated dipeptide and a Zn−OH function, as a potential nucleophile in a hydrolytic process. On the other hand, dipeptide hydrolysis in the presence of our model complex is too slow to be confidently followed at a physiological temperature. This observation prompted us to test the effective hydrolytic ability of our Zn^{II} complex by using L-leucine-*p*-nitroanilide (LNA), a substrate containing an activated peptide bond.

L-Leucine-*p*-nitroanilide (LNA) Binding and Hydrolysis by the Zn^{II} Complex Containing L

LNA, a substrate generally used to test the hydrolytic ability of leucine-aminopeptidases involving the *N*-terminal peptide bond,[73] behaves as a weak base (log K = 7.37(3) for the equilibrium LNA + H^+ = $LNAH^+$ at 308.1 K) and can also cause deprotonation at a strongly alkaline pH to give a $[LNA(H_{-1})]^-$ species (pK_a = 10.6).

LNA binding by $[ZnL]^{2+}$ was studied by means of potentiometric and ^1H NMR spectroscopic experiments at 308.1 K.[74] The $[ZnL]^{2+}$ complex forms rather stable adducts with this substrate, with a log K value of 5.55 for the equilibrium $[ZnL]^{2+}$ + LNA = $[ZnL(LNA)]^{2+}$. A small interaction is also detected with the protonated form of the substrate $HLNA^+$ {log K = 2.1 for the equilibrium $[ZnL]^{2+}$ + $HLNA^+$ = $[ZnL(HLNA)]^{3+}$}. Finally, the potentiometric measurements show that the $[ZnL(LNA)]^{2+}$ complex releases a single proton at pH > 8 to give a deprotonated species.[74] Once again, this process could be ascribed to deprotonation of the amide group of LNA, giving a complex, or a metal-bound water molecule, yielding, respectively, a $[ZnL(LNAH_{-1})]^+$ or a $[ZnL(LNA)(OH)]^+$ species. The distribution diagram, derived from the potentiometric measurements (Figure 6) shows that the formation of the $[ZnL(LNA)]^{2+}$ complex, in the pH range 4−7, is followed by complex deprotonation in the pH range 8−10.

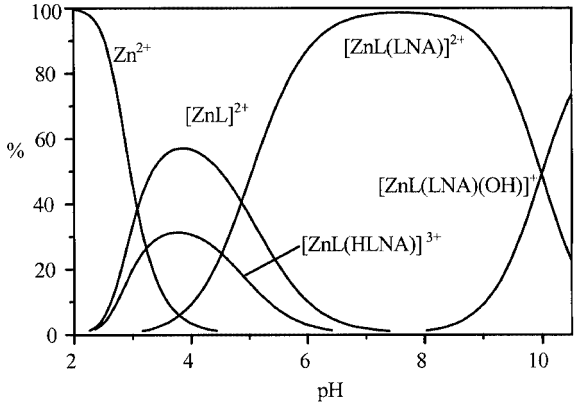


Figure 6. Species distribution diagram for the system $[ZnL]^{2+}$/LNA in 1:1 molar ratio (NMe_4NO_3 0.1 M, 308.1 K).

Figure 7 (a) displays the ^1H chemical shifts of selected protons of LNA in the presence and in the absence of the Zn^{II} complex with L at different pHs, while Figure 7 (b) shows the ^1H NMR spectroscopic chemical shift of the $[ZnL(LNA)]^{2+}$ complex, compared with those of LNA and $[ZnL]^{2+}$.

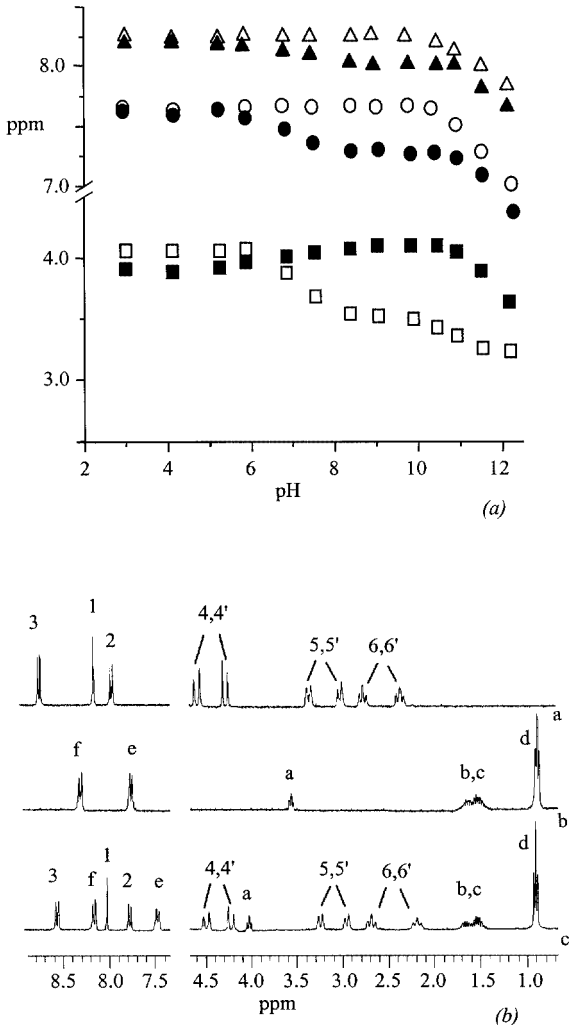


Figure 7. (*a*) pH dependence of the ^1H NMR chemical shifts of selected protons of LNA in the absence (□: *a* proton; △: f; ○: e). and in the presence of the $[ZnL]^{2+}$ complex (■: *a* proton; ▲: *f*; ●: *e*). The signals of protons *b*, *c* and *d* are omitted for clarity. Their chemical shift does not significantly change in the pH range investigated. (*b*) ^1H NMR spectra of $[ZnL]^{2+}$ (a), LNA (b) and $[ZnL(LNA)]^{2+}$ (pH, 7.5) (c). (see Scheme 1 and Figure 1 for the labelling used).

The signal of the *a* proton of "free" LNA displays an upfield shift in the pH range 6−8.5, resulting from the deprotonation of the amine group of $HLNA^+$ to give the neutral LNA form. The ^1H signals of the aromatic protons, on the other hand, do not significantly shift, up to pH 10. LNA deprotonation above pH 10 yields the $[LNA(H_{-1})]^-$ anion, which is accompanied by a marked upfield shift of the aromatic protons *e* and *f*. [Figure 7 (a)]. In the presence of Zn^{II}, the formation of the $[ZnL(LNA)]^{2+}$ complex in the pH range 4−7 (see Figure 6) gives rise to a slight, but significant, downfield shift of the *a* resonance. Actually, the *a* signal in $[ZnL(LNA)]^{2+}$ is ca 0.5 ppm downfield shifted with respect to that of unbound LNA [Figure 7 (b)].

These observations are indicative of the involvement of the amine group in metal coordination. Although these

NMR spectroscopic data do not allow conclusions to be inferred as to the role of the peptide bond in metal binding, the rather high value of the equilibrium constant for the addition of LNA to the Zn^{II} complex and the analogies of the LNA structure with amino acids and dipeptides, leads to the supposition that the carbonyl oxygen of the amide function is also involved, or partially involved, in Zn^{II} binding, with the formation a stabilizing six-membered chelate ring. Figure 7 (a and b) also shows that both the phenanthroline and phenyl protons are upfield shifted above pH 5, where the $[ZnL(LNA)]^{2+}$complex occurs, due to π-stacking and hydrophobic interactions between the aromatic units of metallo-receptor and substrate. Therefore, as already observed in the binding of the amino acids, our Zn^{II} complex behaves as a multifunctional binding site for LNA, since both coordination bonds and supramolecular interactions contribute to the complex stability.

According to Figure 6, the $[ZnL(LNA)]^{2+}$ complex deprotonates in the pH range 8−10; the ^1H NMR signals of coordinated LNA do not shift appreciably in this pH region [Figure 7(a)], indicating that deprotonation occurs on a coordinated water molecule, with the formation of the $[ZnL(LNA)(OH)]^{+}$ hydroxo complex.

Although the potentiometric measurements do not reveal the formation of other complexes between LNA and $[ZnL]^{2+}$in the pH range investigated (2.5−10.5), the analysis of the ^1H NMR spectra of LNA recorded at a strongly alkaline pH (pH >10.5) in the presence of $[ZnL]^{2+}$ reveals a marked upfield shift of the signals of the *a*, *e* and *f* protons [Figure 7 (a)]. A similar upfield shift is also observed at a strongly alkaline pH for the corresponding signals of "free" LNA, due to deprotonation of the amide function to give the *N*-deprotonated $[LNA(H_{-1})]^{-}$ species. This comparison suggests that the $[ZnL(LNA)(OH)]^{+}$ complex undergoes deprotonation in strongly alkaline solutions to give a neutral $[ZnL(LNAH_{-1})(OH)]$ complex. A similar complex, containing a *N*-deprotonated LNA unit coordinated to a Zn^{II} complex has recently been characterized by Vahrenkamp et al.[26] Our attention, however, was focused on the $[ZnL(LNA)(OH)]^{+}$ complex, which contains a Zn^{II}-coordinated neutral LNA molecule and a Zn−OH function, in close proximity, as a potential nucleophile for cleavage of the peptide bond.

Indeed, the $[ZnL(LNA)(OH)]^{+}$ complex promotes LNA hydrolysis in aqueous solution at 308.1 K to give L-leucine and *p*-nitroanilide. The hydrolysis products were identified by means of ^1H NMR spectroscopic measurements. Pseudo-first order rate constants k_{LNA} have been determined at different pH values for the complex. In Figure 8 k_{LNA} values for the Zn-L complexes are reported as a function of pH, together with the distribution curve of the $[ZnL(LNA)(OH)]^{+}$ species at 308.1 K. A good fit between the k_{LNA} values and the distribution curve of the $[ZnL(LNA)(OH)]^{+}$ was found. No promoted hydrolysis was observed below pH 8, where the $[ZnL(LNA)(OH)]^{+}$ is absent from the solution. Accordingly, it can be concluded that the $[ZnL(LNA)(OH)]^{+}$ is the kinetically active species. We can propose a two-step hydrolytic mechanism where LNA is bound to the Zn^{II} complex, probably in a bidentate binding mode, which involves the peptide bond. Hydrophobic interactions also contribute to complex stabilization. Coordination of the peptide bond to the electrophilic Zn^{II} ion would activate the amide carbon atom leading to the subsequent intramolecular nucleophilic attack of the Zn-bound hydroxide or to intermolecular attack of hydroxide from the medium. On the other hand, the nucleophilic character of a Zn−OH function is only slightly lower than that of hydroxide.[75] At the same time, in $[ZnL(LNA)(OH)]^{+}$ the metal-bound hydroxide is close to the substrate. This suggests that the peptide bond cleavage occurs via an intramolecular pathway, as sketched in Scheme 3.

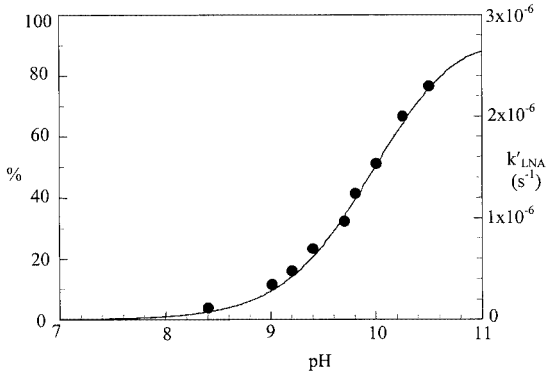
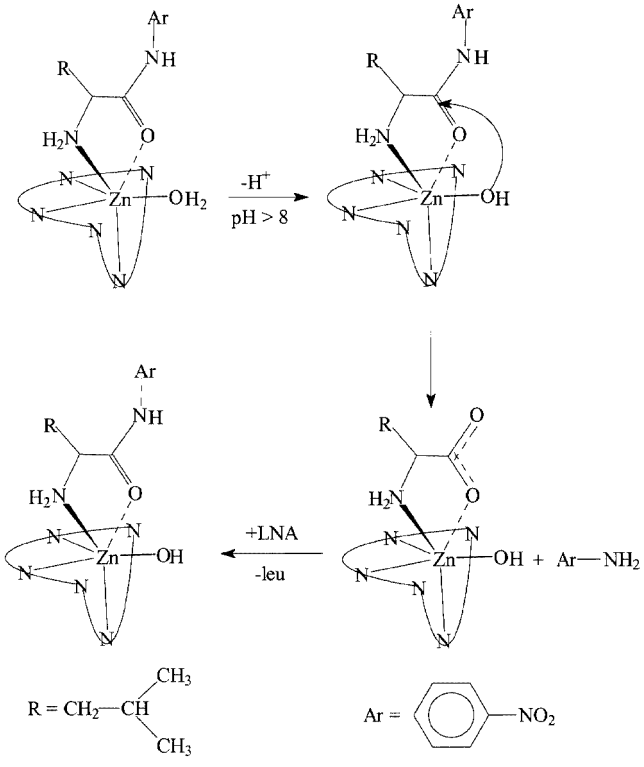


Figure 8. Plot of the distribution curve of $[ZnL(LNA)(OH)]^{+}$ (solid line, right *y* axis) and k_{LNA} values (●, left *y* axis) as a function of pH (0.1 M NMe_4NO_3, 308.1 K).



Scheme 3

The $[ZnL(LNA)(OH)]^+$ complex is formed in a yield of 70% percentage, at most, in the pH range used in the kinetic measurements (Figure 8). As a consequence, the first order rate constants, k'_{LNA}, have been determined from the maximum k_{LNA} value by using the following equation:

$$v = k_{LNA}[\text{total } Zn^{II} \text{ complex}] = k'_{LNA}\,[ZnL(LNA)(OH)^+]$$

By using this expression, a k'_{LNA} value of $3.0 \cdot 10^{-6}$ s^{-1} can be calculated. The hydrolysis rate is much lower than those reported for aminopeptidases,[73] but, at the same time, almost two orders of magnitude higher than the rate constant for spontaneous hydrolysis of LNA.

The addition constant of LNA to the $[ZnL]^{2+}$ is ca 3100 times higher than the corresponding constant of Leu; this consideration implies that LNA hydrolysis promoted by our Zn^{II} is free, for the major part, of inhibiting agents generated by its own hydrolysis, due to the replacement of Leu by the strongly bound LNA substrate in the coordination sphere of $[ZnL]^{2+}$.

The hydrolytic process is too slow at 308.1 K to determine the reaction turnover. However, an experiment carried out at 343.1 K and pH 9.7, with an excess of substrate showed that 1 equiv. of the complex can completely cleave as many as 11 equiv. of LNA.

Conclusion

The particular molecular topology of the phenanthroline-containing macrocycle L leads to an unsaturated coordination sphere of the metal in the $[ZnL]^{2+}$ complex. Therefore, the metal ion can behave as a binding site for substrate molecules and anions. Actually, the $[ZnL]^{2+}$ forms stable complexes with amino acids and dipeptides in their zwitterionic or anionic forms. The phenanthroline moiety can act as a further binding site for substrates containing aromatic pendants, through stacking and/or hydrophobic interactions, giving a peculiar selectivity pattern in amino acid binding. In some cases, a [ZnL(A)OH] species is also formed in aqueous solution. This species contains, in close proximity, both a coordinated substrate and a $Zn^{II}-OH$ unit, a potential nucleophile towards cleavage of the peptide bond. Our complex is indeed able to hydrolyze the peptide bond of the activated substrate L-leucine-*p*-nitroanilide (LNA). The cleavage process occurs in two successive steps i) substrate binding, through coordination bonds to Zn^{II} and stacking interactions and ii) nucleophilic attack of a Zn−OH function, formed at alkaline pH values. Although hydrolysis only occurs at alkaline pH values, this mechanism resembles that proposed for peptide hydrolysis by aminopeptidases.

In particular, selective recognition of amino acids, due to stabilizing π-stacking interactions, is a striking result; it could be used, in principle, for anchoring the complex onto a specific peptide bond of polypeptide chains. This effect, coupled with the hydrolytic properties of the Zn−OH function, encourage the development of other metal complexes with phenanthroline-containing ligands, with the purpose to generate more strongly nucleophilic M−OH functions in aqueous solutions.

Experimental Section

General Remarks: Ligand L and its Zn^{II} complex $[ZnL(H_2O)](ClO_4)_2$ were prepared as already described.[61][62] 300.07 MHz ^1H spectra in D_2O solutions at different pH values were recorded at 298.1 K in a Varian Unity 300 MHz spectrometer. Peak positions are reported relative to HOD at δ = 4.79 ppm. ^1H-^1H and ^1H-^{13}C 2D correlation experiments were performed to assign the signals. Small amounts of 0.01 mol dm^{-3} NaOD or DCl solutions were added to solutions containing the substrates and $[ZnL(H_2O)](ClO_4)_2$ to adjust the pD. The pH was calculated from the measured pD values using the following relationship:[76] pH = pD − 0.40

UV/Vis spectra were recorded on a Shimadzu UV-2101PC spectrophotometer.

Potentiometric Measurements: All the pH metric measurements (pH = −log $[H^+]$) were carried out in degassed 0.1 mol·dm^{-3} NMe_4NO_3 solutions, at 298.1 or 308.1 K, by using the equipment and procedure that have already been described.[60] The combined Ingold 405 S7/120 electrode was calibrated as a hydrogen concentration probe by titrating known amounts of HCl with CO_2-free NMe_4OH solutions and determining the equivalent point by the Gran's method[77][78] which allows for the determination of the standard potential E^o, and the ionic product of water (pK_w = 13.83(1) at 298.1 K and 13.40(1) at 308.1 K in 0.1 mol·dm^{-3} NMe_4NO_3). All equilibria involved in the studied systems were determined, or redetermined,[79] under the present experimental conditions in order to obtain a consistent set of data. The concentration of $[ZnL(H_2O)](ClO_4)_2$ was 1×10^{-3} mol·dm^{-3} in all experiments, while the concentrations of the substrates were varied in the range $1 \times 10^{-3}-4 \times 10^{-3}$ mol·dm^{-3}. At least three measurements (about 150 data points for each) were performed for each system in the pH range 2.5−10.5 and the relevant e.m.f. data were treated by means of the computer program HYPERQUAD[79] that furnished the relevant equilibrium constants reported in Table 2 and 3.

Kinetics of Leucine-*p*-Nitroanilide (LNA) Hydrolysis: The hydrolysis rate of LNA in the presence of the Zn^{II} complexes with L to give Leu and *p*-nitroaniline was measured by an initial slope method following the increase in the 405 nm absorption of the released *p*-nitroaniline at 308.1 K. The molar absorbance of *p*-nitroaniline was determined at the pH of each measurement. The ionic strength was adjusted to 0.1 M with NMe_4NO_3. TAPS (pH, 7.8−8.9), CHES (pH, 8.9−9.7) and CAPS (9.7−10.5) buffers were used (50 mM). In a typical experiment, with LNA (2−5 mM) and $[ZnL(H_2O)](ClO_4)_2$ (0.5 mM) in aqueous solutions at the appropriate pH (the reference experiment does not contain the Zn^{II} complex) were mixed, the UV absorption decay was recorded immediately and was followed until 5% of *p*-nitroaniline had been formed.

At a given pH value, a plot of the hydrolysis rates vs. $[ZnL(LNA)(OH)^+]$ concentration, calculated at the different LNA concentrations, gave a straight line, allowing for the determination of the pseudo-first order rate constants k_{LNA} from the slope of the line. Errors on k_{LNA} values were about 5%.

X-ray Crystallographic Study: Crystals of $[ZnL(HGly)]_2(ClO_4)_4 \cdot 1.5H_2O$ were obtained by slow evaporation of an aqueous solution containing $[ZnL(H_2O)](ClO_4)_2$ and HGly in a 1:1 molar

ratio at pH 5. Crystal data: $C_{40}H_{55}Cl_4N_{12}O_{21.5}Zn_2$, MW = 1320.50; a = 18.520(1) Å, b = 15.171(1) Å, c = 19.884(2) Å, β = 106.107(7)°; Z = 4, calculated density 1.634 Mg/m^3, monoclinic, $P2_1/a$. Data Collection: P4 SIEMENS X-ray diffractometer, λ = 1.54178 Å (Cu-$K\alpha$), graphite monochromated, 0.3 × 0.2 × 0.15 mm, θ−2θ up to θ = 60° at room temperature, 7885 reflections collected, 6637 symmetry-independent reflections, $I > 2\sigma(I)$, μ = 3.710 mm^{-1}, Psi-Scan method, transmission factors 0.44−0.57. Structural analysis and refinement: direct method of the SIR97 program;[81] full-matrix least-squares method of SHELXL-97;[82] All the non-hydrogen atoms were anisotropically refined. The hydrogen atoms were introduced in calculated positions and their coordinates and thermal factors refined in agreement with those of the linked atoms. Parameter/F_o ratio = 0.11, final agreement factors $R1$ = 0.0701, $wR2$ = 0.1908 [$I > 2\sigma(I)$]; $R1$ = 0.0790, $wR2$ = 0.2013(all data).

CCDC-198421 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; Fax: (internat.) +44−1223/336−033; E-mail: deposit@ccdc.cam.ac.uk].

Acknowledgments

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