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ApA Cleavage Promoted by Oxa-aza Macrocycles and Their Zn(II) Complexes. The Role of pH and Metal Coordination in the Hydrolytic Mechanism*

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The thermodynamic parameters for protonation and Zn(II) complex formation with ligand 1,4,7,16,19,22-hexaza-10,13,25,28-tetraoxacyclotriacontane (L1) have been determined. L1 forms stable dizinc complexes from neutral to alkaline pH. The hydrolytic ability toward adenylyl(3′-5′)adenosine (ApA) of L1 and its dizinc(II) complexes have been analyzed by means of HPLC chromatography. Only partially protonated species of L promote ApA hydrolysis suggesting that the cleavage process is cooperatively promoted by a general base catalysis by neutral amine groups and a general acid catalysis by protonated ammonium functions. Concerning the Zn(II) complexes, the hydrolysis rates increase in the presence of the hydroxo complexes $[Zn_2L1(OH)]^{3+}$ and $[Zn_2L1(OH)_2]^{2+}$. This indicates that Zn-OH functions play a crucial role in the hydrolytic process, assisting the deprotonation of the 2′-OH group of ApA, which may act as nucleophile in the cleavage process. Both binuclear L1 complexes are better catalysts than the mononuclear $[ZnL2(OH)]^+$ complex (L2 = 1,4-Dioxa-7,10,13-triazacyclopentadecane), indicating a cooperative role of the two Zn(II) ions in ApA cleavage by $[Zn_2L1(OH)]^{3+}$ and $[Zn_2L1(OH)_2]^{2+}$, probably due to a bridging coordination of the phosphate moiety of ApA to the two metal centers.

Keywords: Macrocycles; Zinc; Zn(II) complexes; RNA cleavage

INTRODUCTION

In the last few years a great deal of interest has been devoted to the study of the mechanism of DNA and RNA hydrolysis, mainly because it is essential for further developments in biotecnology, molecular biology and therapy [1].

As one of the approaches, several simple organic molecules [2–4] or metal complexes [5–8] have been tested as models for hydrolytic enzymes. RNA cleavage by simple organic compounds, such as imidazole, has been deeply investigated by Breslow *et al.* [2] in order to clarify the role in the hydrolytic mechanism played by this heterocycle, contained in the side chain of the amino acid histidine in many hydrolytic enzymes, such as ribonuclease A. Oligomeric

*Dedicated to Professor Piero Paoletti in the occasion of his 70th birthday.

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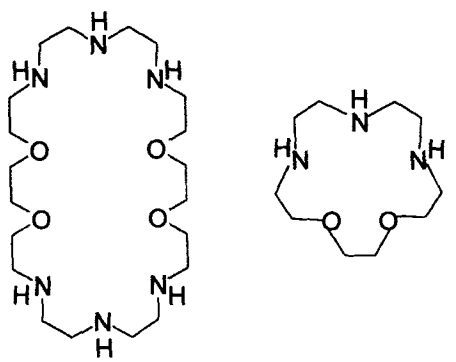
amines (ethylendiamine, triethylentetramine and pentaethylenhexamines) have also been used as efficient catalyst for RNA cleavage at neutral pH by Komiyama and coworkers [3]. In this case the presence of both protonated and unprotonated amine groups play a key role in a cooperative general acid-base hydrolytic mechanism.

Synthetic metal complexes has been also investigated as potential simple models for hydrolytic enzymes [5–8]; many enzymes, including those that hydrolyze phosphate esters, contains one or more metal ions in their active site. Particular attention has been focused on Zn(II) complexes, since in hydrolytic metallo-enzymes the metal ions are often Zn(II). Recent work by Breslow [8], Komiyama [6] and Kimura [5] has shown that RNA and diribonucleotide hydrolysis is efficiently promoted by dinuclear Zn(II) complexes, with a bifunctional catalytic mechanism involving coordination of the substrate to the dimetal core and Zn(II)-assisted deprotonation of the 2′-OH function, which can give a nucleophilic attack at the phosphorous.

Polyamine macrocycles are potential candidates as catalysts for RNA cleavage. They can form, in fact, polyprotonated species at neutral or slightly acidic pH's, and, at the same time, they can give stable metal complexes. Recently, we reported on the synthesis, protonation and Zn(II) coordination by the macrocyclic ligand **L1** [9a].



L1 **L2**

LIGAND DRAWINGS

This ligand gives a stable dizinc complex from neutral to alkaline pH. In this dinuclear complex each metal is bound by a triamine unit and presents an unsaturated coordination sphere. This complex, therefore, may be a promising receptor for bridging substrates. Actually, it is able to cleave activated phosphate esters, such bis(*p*-nitrophenyl)phosphate, through a hydrolytic mechanism which involves bridging coordination of the phosphate ester and simultaneous attack of a Zn-OH function at the phosphorous [9b]. At the same time, this polyamine ligand, in the absence of metal cations, gives highly protonated species at slightly acidic pH.

In this paper we have analyzed the hydrolytic properties of **L1** toward the diribonucleotide adenylyl(3′–5′)adenosine (ApA), both in the presence and in the absence of Zn(II), in a wide pH range. We hoped that ApA hydrolysis could takes place both at slight acidic pH's, due to the protonated species of **L1**, and at neutral or alkaline pH values, where the dizinc complex is largely prevalent in aqueous solutions, thus achieving a *switch* of the cleavage mechanism, determined by both pH and metal coordination. Furthermore, we have also analyzed ApA hydrolysis promoted by the Zn(II) complex with ligand **L2**. This latter can be considered as "half" **L1** and gives a stable mononuclear complex in aqueous solution. The comparison of the cleavage activity of the dinuclear **L1** complex and the mononuclear **L2** ones can get further insight into the role played by the presence of two coordinated Zn(II) ions at close distance within the same macrocyclic framework.

RESULTS AND DISCUSSION

Ligand Protonation and Zn(II) Binding at 323 K

The determination of ligand protonation constants and Zn(II) complexation with **L1** at the same temperature used for kinetic

measurements (323 K) are necessary in order to determine the effective active specie in ApA hydrolysis by ligand **L1** and its Zn(II) complexes. Reliable values for the protonation and stability constants at this temperature, however, are of difficult achievement with commonly used potentiometric systems. Therefore, we determined the enthalpy changes for ligand protonation and Zn(II) complexation by means of direct calorimetric measurements at 298.1 K. Since ligand protonation constants and Zn(II) complexation constants at 298.1 K are known [9], the ΔS° values at this temperature can be also calculated. In the temperature range 298–323 K, the ΔH° and ΔS° values can be approximately considered constant, allowing to calculate the protonation and stability constants at 323 K by using the Gibbs equation. The enthalpy and entropy changes for **L1** protonation and for the formation of the binuclear Zn(II) complexes at 298.1 K are listed in Tables I and II, respectively, together with the corresponding constants calculated at 323 K. Figure 1 reports a distribution diagram for the Zn(II) complexes with **L1**, calculated with a 1:2 ligand to metal molar ratio at 323 K.

Considering the data in Table II and Figure 1, some main considerations can be outlined. With a 1:2 ligand to metal molar ratio, only binuclear Zn(II) complexes are formed in solution. The formation of the dinuclear $[Zn_2\mathbf{L1}]^{4+}$ complex at pH > 6 is followed at neutral pH by deprotonation of a Zn(II)-coordinated water to give the monohydroxo complexes $[Zn_2\mathbf{L1}(OH)]^{3+}$, denoting facile deprotonation of the coordinated water with pK_{a1} values (Tab. I) of 7.08 and 7.7 at 298.1 K and 323 K, respectively. These rather low pK_a values are often attributed to a bridging coordination of hydroxide to the two metal centers. Actually, the crystal structure of $[Zn_2(\mu\text{-OH})\mathbf{L1}]^{3+}$ showed that each metal is coordinated to a polyamine moiety and the bridging OH^- [9]. The formation of a dihydroxo species is observed at alkaline pH's, with pK_{a2} value of 8.64 and 8.5 at 298.1 and 323 K, respectively. These pK_a values are higher than those usually found for bridging hydroxide

TABLE I Thermodynamic parameters for protonation of ligand **L1** (298.1 K, I = 0.1 M) and calculated protonation constants at 323 K

	Log K[a] (298.1 K)	$-\Delta H^\circ$ kcal mol^{-1}	$T\Delta S^\circ$ kcal mol^{-1}	Log K[b] (323 K)
$\mathbf{L1} + H^+ = \mathbf{L1}H^+$	9.46(6)	8.9(2)	4.0(2)	8.7(2)
$\mathbf{L1}H^+ + H^+ = \mathbf{L1}H_2^{2+}$	8.88(5)	9.4(2)	2.7(2)	8.2(2)
$\mathbf{L1}H_2^{2+} + H^+ = \mathbf{L1}H_3^{3+}$	8.05(7)	11.1(2)	−0.1(2)	7.4(2)
$\mathbf{L1}H_3^{3+} + H^+ = \mathbf{L1}H_4^{4+}$	7.43(6)	10.2(2)	−0.1(2)	6.8(2)
$\mathbf{L1}H_4^{4+} + H^+ = \mathbf{L1}H_5^{5+}$	4.12(9)	8.0(2)	−2.4(2)	3.8(2)
$\mathbf{L1}H_5^{5+} + H^+ = \mathbf{L1}H_6^{6+}$	3.21(8)	8.4(2)	−4.0(2)	3.0(2)

[a] Values taken from Ref. [9].
[b] Calculated by means of the Gibbs equation.

TABLE II Thermodynamic parameters for the formation of the binuclear Zn(II) complexes with **L** (298.1 K, I = 0.1 M) and calculated formation constants at 323 K

Reaction	Log K[a] (298.1 K)	$-\Delta H^\circ$ (kcal mol^{-1})	$T\Delta S^\circ$ (kcal mol^{-1})	Log K[b] (323 K)
$2Zn^{2+} + \mathbf{L1} = Zn_2\mathbf{L1}^{4+}$	13.36(7)	6.1(1)	12.1(1)	12.3(2)
$Zn_2\mathbf{L1}^{4+} + OH^- = Zn_2\mathbf{L1}(OH)^{3+}$	6.74(4)	4.0(1)	5.2(1)	6.2(2)
$Zn_2\mathbf{L1}(OH)^{3+} + OH^- = Zn_2\mathbf{L1}(OH)_2^{2+}$	4.94(6)	5.1(2)	1.6(2)	4.5(2)

[a] Values taken from Ref. [9].
[b] Calculated by means of the Gibbs equation.

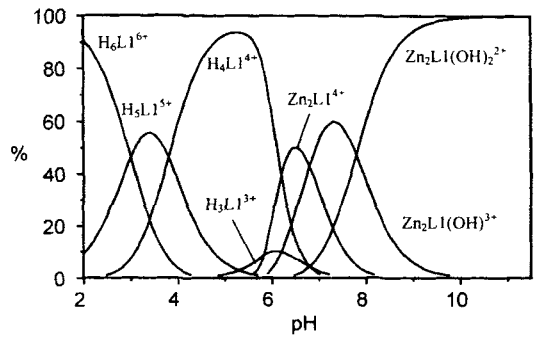
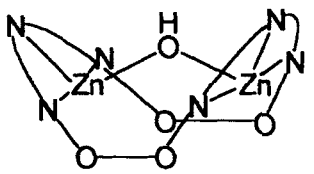


FIGURE 1 Distribution diagram for the system Zn(II)/**L1** (2:1 molar ratio) calculated at 323 K and $I = 0.1$ M ([**L1**] = $1 \cdot 10^{-3}$ M, [Zn(II)] = $2 \cdot 10^{-3}$ M).

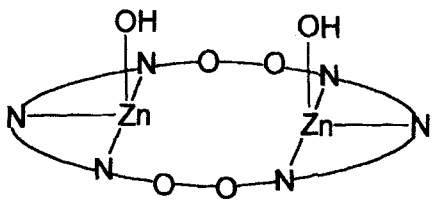
groups, suggesting that the second hydroxide anion binds to a single metal, as sketched in Scheme 1.

ApA Hydrolysis Promoted by Ligand L1 and its Zn(II) Complexes

ApA hydrolysis at 323 K in the presence of 2 equiv. of Zn(II) and 1 eq. of **L1** at different pH values was followed by means of reversed-phase HPLC and a typical HPLC pattern for



$[Zn_2\mathbf{L1}(OH)]^{3+}$



$[Zn_2\mathbf{L1}(OH)_2]^{2+}$

SCHEME 1

ApA cleavage (pH 8) is shown in Figure 2. The products were adenosine (A) and its 2′- and 3′-phosphate derivatives (A2′P and A3′P in Fig. 2, respectively), as usually observed for promoted hydrolysis of ApA. Formation of small but definite amount of adenosine 2′,3′-cyclic phosphate (A2′3′P) as the hydrolysis intermediate was also detectable. This pattern is in good accord with the two-steps mechanism generally proposed for dinucleotide hydrolysis promoted by polyamines or metal complexes [2–8]. In the rate-determining step of the cleavage reaction, ApA is firstly hydrolyzed to A2′3′P, with consequent release of A. The A2′P and A3′P, subsequently formed from the A2′3′P intermediate, are finally hydrolyzed to A. Pseudo-first-order kinetics were satisfactorily followed at any pH values. No significant hydrolytic effect is given by Zn(II) at any pH values in the absence of the ligand.

Figure 3 reports the pseudo-first-order rate constants of ApA hydrolysis in the presence of **L1** and Zn(II) (1:2 molar ratio) as a function of pH. The most interesting finding is the observation of two distinct stages of rate constant variation. In fact, Figure 3 clearly shows a well defined bell-shaped pH *vs* rate profile at acidic pH values, with a maximum at pH 5.4, followed by a marked increase of the rate constants from neutral to alkaline pH.

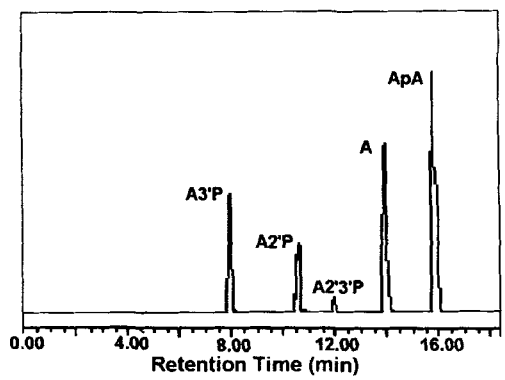


FIGURE 2 Typical HPLC pattern for ApA cleavage (pH 8) in presence of **L1** and Zn(II) in 1:2 molar ratio.

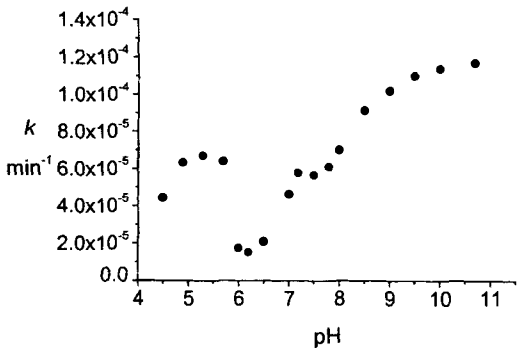


FIGURE 3 Pseudo-first-order rate constant for ApA hydrolysis at different pH values in the presence of Zn(II) and **L1** in 2:1 molar ratio.

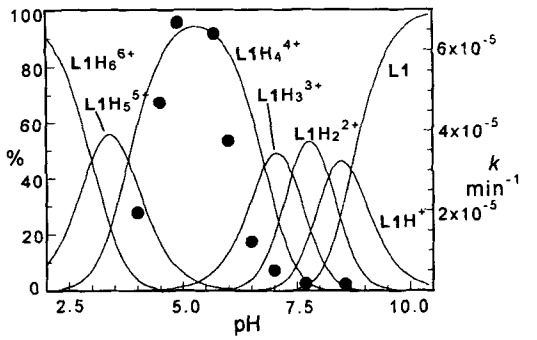


FIGURE 4 Pseudo-first-order rate constant for ApA hydrolysis in the presence of **L1** (•, right y axis) and distribution curves for the protonated species of **L1** in 2:1 molar ratio (solid line, left y axis).

On the other hand, the distribution diagram for the system **L1**/Zn(II) in 1:2 molar ratio (Fig. 1) shows that Zn(II) complexation takes place mainly from neutral to alkaline pH values, with the formation of the dinuclear Zn(II) complexes, while protonated species of the ligand are largely predominant in the acidic pH region. This consideration strongly suggests that ApA hydrolysis takes place *via* different mechanisms, involving two different active species, *i.e.*, protonated forms of the ligand at acidic pH and dinuclear metal complexes at alkaline pH.

ApA Cleavage by the Protonated Receptor L1

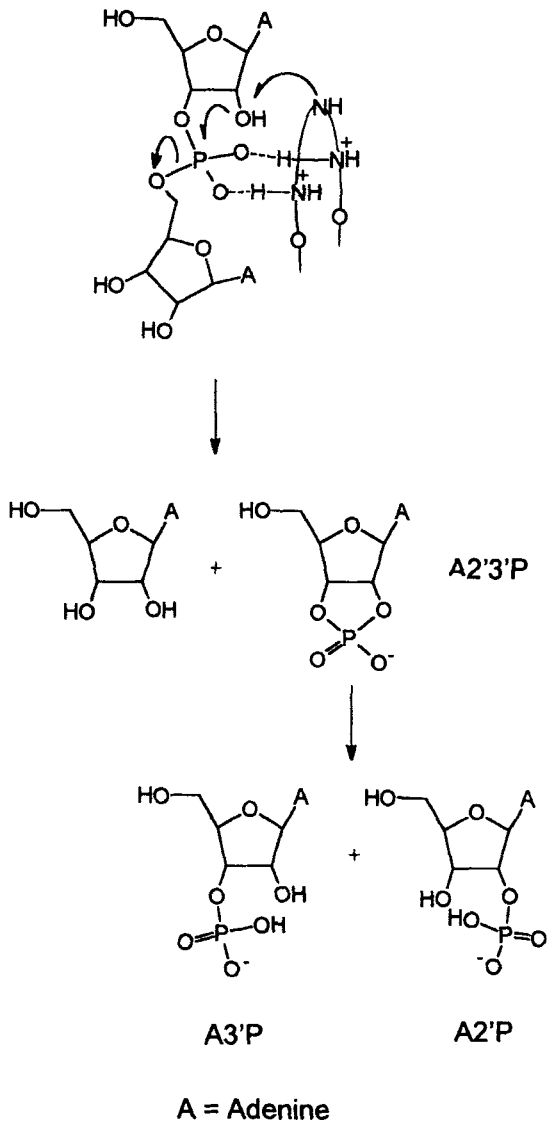
In order to confirm the above hypothesis, we analyzed ApA hydrolysis by ligand **L1** in the absence of Zn(II) and the results are reported in Figure 4, where the pseudo-first-order rate constants for ApA cleavage at different pH values are superimposed to the distribution diagram of the **L1** protonated species. At acidic pH, both the pH *vs* rate profile and the values of the rate constants are almost equal to those found in the presence of Zn(II). On the contrary, no promoted hydrolysis is observed at alkaline pH.

According to Figure 4, ApA hydrolysis is exclusively promoted by the tetraprotonated species of **L1**, which is formed at slightly acidic pH values. This result indicates that the presence of a protonated receptor, containing at the same time charged ammonium functions and unprotonated amine groups, such as the tetraprotonated $\mathbf{L1H_4^{4+}}$ form, is necessary to achieve ApA hydrolysis.

These data account for a hydrolytic mechanism cooperatively promoted by a general base catalysis by neutral amine groups and a general acid catalysis by protonated ammonium functions. This mechanism would involve prior or simultaneous activation of the phosphodiester by the ammonium groups, trough proton transfer or partial charge-transfer *via* hydrogen bond from the protonated amine to the anionic phosphate, and nucleophilic attack of the 2′-OH toward the phosphorous, assisted by the unprotonated amine group (Scheme 2).

Such a bifunctional general acid/general base hydrolytic mechanism is in good accord with those proposed by Komiyama in the case of RNA hydrolysis catalyzed by partially protonated open-chain amines [3] and by Breslow for dinucleotides cleavage in imidazole buffer [2]. The formation of a monoanionic intermediate, containing a trigonal-bipyramidal phosphorous has been also suggested [2, 3].

In our case, however, hydrolysis seems to take place only in the presence of an highly charged form of the receptor, the $\mathbf{L1H_4^{4+}}$ species. Less charged forms of the receptor do not promote hydrolysis. This observation may suggest that



SCHEME 2

the formation of a receptor-substrate adduct, held by charge-charge interactions and/or hydrogen bonding, is also necessary to achieve ApA hydrolysis. Less protonated species of **L1** would not be able to give stable complexes with the substrate, not promoting the cleavage process. From this point of view, protonation of ApA to give a neutral form of the dinucleotide, could also contribute to reduce the interaction with the protonated receptor, leading to decrease of the hydrolysis rate at acidic pH (pH < 4.5, Fig. 4).

ApA Cleavage by the Dizinc Complexes with L1

As observed above, Figure 3 shows that the hydrolysis rate of ApA bears a remarkable increase above pH 6, where binuclear Zn(II) are formed in aqueous solutions. In order to confirm the effective activity of these species, pseudo-first order rate constants k_{obs} were determined by using solutions containing ApA and the preformed complex $[Zn_2\mathbf{L1}](ClO_4)_4 \cdot 2H_2O$ [9]. The rate constants determined in these conditions are almost equal to those found in the previous experiment. In Figure 5 the observed rate constants (k_{obs}) are compared with the distribution curves of the binuclear Zn(II) complexes. As displayed by this figure, the hydrolysis rate increases significantly with the formation of the $[Zn_2\mathbf{L1}(OH)]^{3+}$ and $[Zn_2\mathbf{L1}(OH)_2]^{2+}$ complexes, indicating that these two species promote ApA hydrolysis, while the $[Zn_2\mathbf{L1}]^{4+}$ complex is almost ineffective. At each pH value, therefore, the hydrolysis rate is given by the following equation:

$$v = k_{obs}[\text{total Zn(II)complex}]$$
$$= k_1[Zn_2\mathbf{L1}(OH)^{3+}] + k_2[Zn_2\mathbf{L1}(OH)_2^{2+}]$$

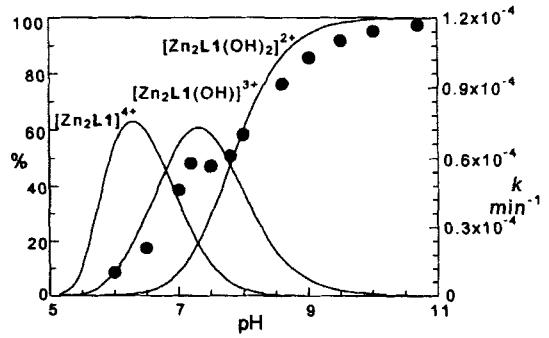


FIGURE 5 Pseudo-first-order rate constant for ApA hydrolysis in the presence of the $[Zn_2\mathbf{L1}](ClO_4)_4 \cdot 2H_2O$ (•, right y axis) and distribution curves of the binuclear Zn(II) complexes with **L1** (solid line, left y axis).

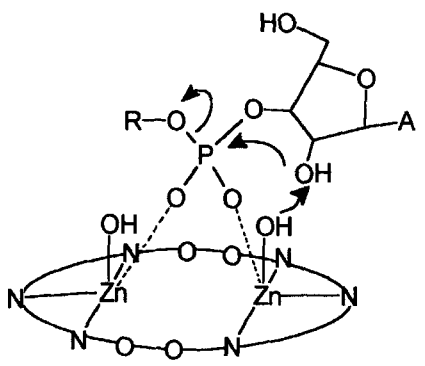
The contributions, k_1 and k_2, of the two active species, to the measured rates constants were then determined by using a least-square fitting method.

By using this method, rate constants of $(7.1 \pm 0.7) \cdot 10^{-5}\,min^{-1}$ and $(1.2 \pm 0.1) \cdot 10^{-4}$ min^{-1} can be calculated for the $[Zn_2\mathbf{L1}(OH)]^{3+}$ and $[Zn_2\mathbf{L1}(OH)_2]^{2+}$ complexes, respectively. The rate obtained for the $[Zn_2\mathbf{L1}(OH)_2]^{2+}$ species is equal, within the experimental error, to that measured at pH 10.8, where this complex is the unique species present in solution.

These values are lower than those previously found with other synthetic dizinc complexes. Two main points, however, should be noted.

Firstly, the formation of Zn-OH functions plays a key role in the hydrolytic process. This is in accord with a mechanism involving deprotonation of the 2′-OH group, assisted by the Zn(II)-hydroxide group, followed by the nucleophilic attack of the 2′-OH to the phosphorous to generate 2′,3′-cyclic phosphate (Scheme 3). The observation of this product in HPLC chromatogramms supports this hypothesis.

Secondly, the monohydroxo $[Zn_2\mathbf{L1}(OH)]^{3+}$ complex exhibits a lower rate constant than the corresponding dihydroxo $[Zn_2\mathbf{L1}(OH)_2]^{2+}$ one. As discussed above, in the $[Zn_2\mathbf{L1}(OH)]^{3+}$ complex the OH^- bridges the two electrophilic metal centers. This structural feature reduces the pK_a of the coordinated water molecule, and, therefore, the basicity of the dizinc-bound hydroxide. Actually, the crystal structure of the $[Zn_2\mathbf{L1}(\mu\text{-OH})](ClO_4)_3$ salt [9] showed that the $[Zn_2(\mu\text{-OH})]$ unit is tightly encapsulated within the macrocyclic framework, making the dizinc hydroxo function low available for assisting the deprotonation of the 2′-OH group for the hydrolytic process. Addition of a second hydroxide anion to a Zn(II) ion would lead to detachment, or partial detachment, of the bridging OH^- from one of the metal, giving rise to a more "opened" conformation of the macrocycle and to less hindered Zn-OH functions, as sketched in Scheme 1. This determines the higher pK_{a2} values found for deprotonation of a Zn(II)-bound water molecule to give the $[Zn_2\mathbf{L1}(OH)_2]^{2+}$ complexes and increases the basicity of the resulting Zn-OH functions.



A = Adenine
R = Adenilyl-5′

SCHEME 3

A Comparison Between Mono and Binuclear Zn(II) Complexes as Catalysts for ApA Hydrolysis

The mononuclear Zn(II) complex with **L2** also promotes ApA hydrolysis. Ligand **L2** forms a stable Zn(II) complex, $[Zn\mathbf{L2}]^{2+}$, at neutral pH's and gives a monohydroxo species, $[Zn\mathbf{L2}(OH)]^{+}$, at alkaline pH values [10]. Figure 6 reports the pseudo-first-order rate constants as a function of pH, superimposed to the distribution curve of the $[Zn\mathbf{L2}(OH)]^{+}$ complex. No hydrolytic effect is observed below pH 7, where the $[Zn\mathbf{L2}]^{2+}$ is largely predominant in solution. Figure 6 clearly shows that the hydroxo complex $[Zn\mathbf{L2}(OH)]^{+}$ is the kinetically active species, in accord with the results found for the binuclear **L1** complex. The $[Zn\mathbf{L2}(OH)]^{+}$ complex, however, shows a much lower ability in ApA hydrolysis than both **L1** hydroxo complexes, with a k_3 constant of $(2.6 \pm 0.3) \cdot 10^{-5}$ min^{-1} at pH 11. The higher activity of the **L1** complexes may be ascribed to a cooperative role played by the two Zn(II) metals in the hydrolytic

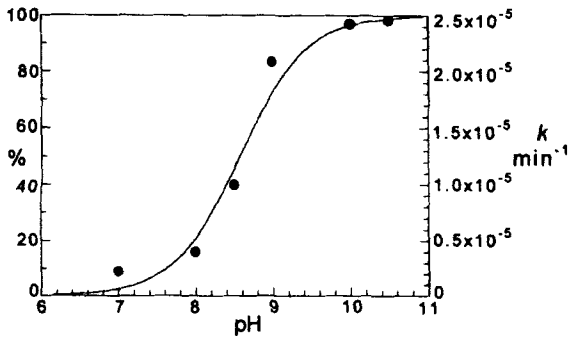


FIGURE 6 Pseudo-first-order rate constant for ApA hydrolysis in the presence of the $[ZnL2](ClO_4)_2$ complex (•, right y axis) and distribution curve of the $ZnL2(OH)^+$ complex (solid line, left y axis).

mechanism, through an "associative" mechanism, in which the substrate approaches the $[Zn_2L1(OH)_2]^{2+}$ complex and two oxygens of ApA associate with the two electrophilic metal Zn(II) ions, through a bridging coordination (Scheme 3). A similar bridging coordination of the phosphate oxygens have been recently found in the $[Zn_2L1(PP)_2]^{2+}$ complex, where diphenylphosphate (PP^-) bridges the two Zn^{2+} ions [9b]. Simultaneously a zinc bound hydroxide promotes deprotonation of the 2'-OH function, which may give a nucleophilic attack at the phosphorous. It is possible, however, that the synergic role of the two Zn(II) ions also resides in promotion of 2'-OH deprotonation, as well as in stabilization of a five-coordinated phosphorous intermediate.

EXPERIMENTALS

Materials

Ligand **L1** ad its dizinc complex, $[Zn_2L1](ClO_4)_4 \cdot 2H_2O$, were prepared as already described [9a]. Adenylyl(3'–5')adenosine, A2'P, A3'P, A2'3'P and A were purchased from Pharmacia.

Microalorimetric Measurements

The enthalpies of ligand protonation and Zn(II) complexation were determined in 0.1 M NMe_4NO_3 aqueous solutions at 298.1 K by means of the apparatus which has been already described [11]. Protonation enthalpies were determined by addition of NMe_4OH (0.1 M, addition volumes 0.015 cm^3) to acidic solution of the ligand (5×10^{-3} M, 1.2 cm^3). The complexation enthalpies were determined by means of titrations with NMe_4OH (0.1 M, addition volumes 0.015 cm^3) of acidic solutions, containing ligand and Zn(II) (with a Zn(II) concentration of 5×10^{-3} mol dm^{-3} and a metal to ligand molar ratio varying from 0.4:1 to 0.8:1). The ionic medium was NMe_4NO_3(0.1 M). Under the reaction conditions and employing the protonation and/or stability constants determined at 298.1 K, the concentrations of the species present in solution before and after addition were calculated and the corresponding enthalpies of reaction were determined from the calorimetric data by means of the AAAL program [12]. At least three titrations were performed for each system. The titration curves for each system were treated either as a single set or as separated entities without significant variation in the values of the enthalpy changes.

Kinetics of ApA Hydrolysis

ApA cleavage at different pH values was monitored by a reversed-phase HPLC. In typical kinetic experiment, a solution of ApA (0.1 mM), **L1** or the Zn(II) complexes with **L1** or **L2** ($[Zn_2L1](ClO_4)_4 \cdot 2H_2O$ and $[ZnL2](ClO_4)_2$, both of them 5 mM) and the appropriate buffer (50 mM) were mixed together and the pH was adjusted with small amounts of 0.01 M NMe_4OH or HNO_3. The ionic strength was adjusted to 0.1 M with NMe_4NO_3 and the solution was allowed to react at 323 K in a thermostatized air-bath. At an appropriate interval, a 20 µl portion of the reaction was sampled, injected onto a C-18 reversed-phase column (Symmetry 5 µm, 4.6 × 250 mm) and eluted for 3 min with a 1% aqueous solution of CH_3CN, followed by a 1–7% linear gradient of CH_3CN in aqueous solution over 7 min and then by a 7% aqueous

solution of CH_3CN for 5 min with a flow rate of 1 mL/min. At each pH values, measurements were also carried out in absence of the catalyst (**L1** or its dizinc complex), in order to determine the contribution of spontaneous hydrolysis of ApA. This contribution was then subtracted from the rate constants measured in the presence of the catalysts. The reaction was followed generally until 10–20% hydrolysis of ApA. All reaction products were identified by means of separated HPLC measurements carried out on commercially available compounds (ApA, A2′P, A3′P, A2′3′P and A) in the same conditions used for ApA hydrolysis. Potassium hydrogen phtalate (pH 3–5.5), MES (pH 5.5–6.7), MOPS (pH 6.7–7.8), TAPS (pH 7.8–9.1), CHES (pH 8.6–9.8) and CAPS (pH 9.8–11) buffers were used (50 mM). Errors on the rate constants were about 5%.

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