Metal-ion-promoted hydrolysis of uridylyl(3′,5′)uridine: internal *vs.* external general base catalysis

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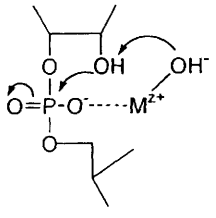
The hydrolysis of uridylyl(3′,5′)uridine promoted by Mg^{2+}, Zn^{2+} and Zn^{2+}[12]aneN_3 ([12]aneN_3 = 1,5,9-triazacyclododecane) has been studied in imidazole, HEPES and triethanolamine† buffers and the origin of marked electrolyte effects observed with Zn^{2+}[12]aneN_3 has been examined. The results obtained suggest that the basic buffer constituent does not serve as an external general base, but the catalytic activity of the metal ion species is influenced by the coordination to the buffer base and other Lewis bases present in the solution. The data lend additional support to a bifunctional mechanism, which consist of coordination of the metal ion to the anionic phosphodiester and intracomplex general base (or nucleophilic) catalysis by its hydroxo ligand.

Introduction

Numerous reports on the metal-ion-promoted hydrolysis of nucleoside phosphoesters have been published since the early 1970s.[1] The mechanisms of these reactions have been considered to be of interest for three main reasons: (*i*) metal-ion-promoted hydrolysis of phosphoesters constitutes a simple model system for phosphate transfer by metallo enzymes,[2] (*ii*) the action of catalytic RNAs, ribozymes, depends on metal ions[3] and (*iii*) metal ion chelates tethered to oligonucleotides may be exploited as artificial nucleases that hydrolyse RNA in a sequence-selective manner.[4–7]

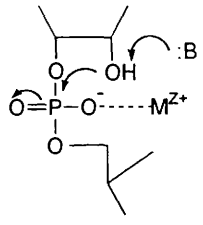
Several lines of evidence suggest a bifunctional mechanism for the metal-ion-promoted hydrolysis of the 3′-phosphodiesters of ribonucleosides, including dinucleoside 3′,5′-monophosphates.[8–11] According to this mechanism, the metal ion acts as an electrophile by coordinating to the phosphate function and its hydroxo ligand serves as an intracomplex general base deprotonating the attacking 2′-hydroxy function (Scheme 1). Consistent with the suggested mechanism, the rate-



Scheme 1

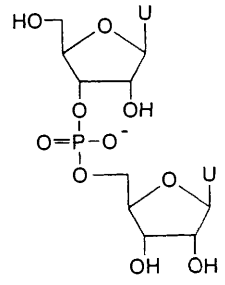
accelerating effect of various metal ions and metal ion chelates on the hydrolysis of dinucleoside monophosphates and their simple models correlates with the acidity of their aquo ion.[8–12] The reaction is first-order in hydroxide ion concentration at pH < pK_a of the metal aquo ion and levels to a constant rate under conditions where the metal ion species is converted to its hydroxo complex.[8,9,11–13] Metal ions, such as Mg^{2+} and Ca^{2+}, having a pK_a value greater than 12, exhibit a barely noticable catalytic activity.[10,11,14]

The bifunctional mechanism described above is not, however, unanimously accepted, but the action of the metal ion as a mere electrophile has also been suggested.[14,15] Accordingly, the role of the metal ion would only be to lower the electron density at phosphorus by binding to the anionic phosphoryl oxygen, while the general base catalyst needed to deprotonate the attacking nucleophile would be an external one, in the simplest case the basic constituent of the buffer (Scheme 2). This mechanism is largely based on the observation



Scheme 2

according to which the hydrolysis rate of uridylyl(3′,5′)uridine (UpU; **1**) passes at a fixed Zn^{2+} concentration (1×10^{-3} mol



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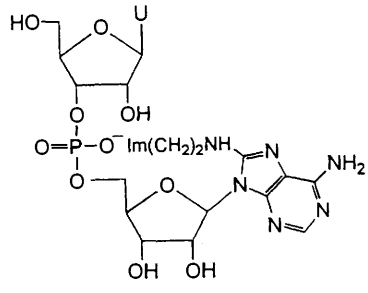

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dm^{-3}) through a maximum when the concentration of imidazole (Im) buffer is increased.[14] The rate acceleration occurring at [Im] < 1.5×10^{-2} mol dm^{-3} has been attributed to catalysis by external imidazole base, and the rate deceleration observed at higher imidazole concentrations to competition of imidazole for Zn^{2+}. An analogous mechanism has more recently been suggested for the hydrolysis of a derivative of uridylyl(3′,5′)adenosine bearing a histamino group at C8 of the adenine base (**2**): Zn^{2+} is suggested to act as a mere electrophile and the imidazole moiety of the histamino group is assumed to deprotonate the attacking 2′-hydroxy function.[16,17]

In striking contrast to the rate-acceleration by imidazole buffer, the Zn^{2+}-promoted hydrolysis of UpU has been shown to undergo modest rate-retardation with the increasing

† HEPES = *N*-(2-Hydroxyethyl)piperazine-*N*′-(ethane-2-sulfonic-acid). Triethanolamine = 2,2′,2″-nitrilotriethanol.

concentration of HEPES buffer,[11] in spite of the fact that HEPES is approximately as strong a base as imidazole.[18,19] The possible cooperative action of Zn^{2+} and imidazole is of special interest, because an imidazole tethered oligodeoxyribonucleotide has been shown to exhibit sequence-selective RNase activity in the presence of Zn^{2+}.[7] To study this subject in more detail, comparative kinetic measurements with the following metal/buffer systems have been carried out, paying special attention to electrolyte effects: (*i*) Zn^{2+}/Im, (*ii*) Zn^{2+}/HEPES, (*iii*) Mg^{2+}/Im, (*iv*) Zn^{2+}[12]aneN$_3$/Im, (*v*) Zn^{2+}[12]aneN$_3$/HEPES and (*vi*) Zn^{2+}[12]aneN$_3$/triethanolamine. If the catalysis involves participation of two mutually independent species, as suggested by the mechanism depicted in Scheme 2, replacement of one of the catalysts with another related species should not affect the catalytic behaviour of the other catalyst. Although in principle the nature of the metal ion coordinated to the phosphate group could affect the acidity of the 2′-hydroxy function and hence the susceptibility to general base catalysis, this is in all likelihood of minor importance, owing to the fact that the inductive effect is transmitted through several σ bonds. A marked influence is expected only if the metal ions interact with the hydroxy function, either *via* direct coordination or *via* an aquo/hydroxo ligand. In other words, any metal ion of comparable charge, size and affinity to phosphate, could be expected to replace Zn^{2+} in the sense that a buffer catalysis is still observed and any buffer of comparable basicity ought to be able to replace imidazole. The results obtained argue against the mechanism in Scheme 2.

Results and discussion

If the metal ions promote the hydrolysis of UpU by simply lowering the electron density at phosphorus, as suggested by the mechanism depicted in Scheme 2, then all metal ions having similar charge, size and affinity to the phosphodiester bond could be expected to exert comparable effects on the hydrolysis rate. For example, Mg^{2+} and Zn^{2+} ions are in these respects rather similar; the ionic radius of Mg^{2+} is slightly smaller than that of Zn^{2+} (0.066 nm and 0.074 nm respectively[20]), and the stability constants of phosphate complexes of *e.g.*, Mg^{2+} are about one quarter of those of the corresponding Zn^{2+} complexes.[21] For example, the log β values of the complexes of Mg^{2+} and Zn^{2+} with the monoanionic PO(OH)$_2$(O$^-$) ligand are 0.7 and 1.2 (I = 0.15 mol dm^{-3}; T = 310 K), respectively.[22] Furthermore, the effect of these ions on the ^{31}P shifts of UpU suggests that the Mg^{2+} and Zn^{2+} exhibit comparable affinity to the monoanionic phosphodiester bond.[23] Accordingly, the rate-promoting effect of Mg^{2+} on the hydrolysis of UpU could be expected to be similar to that of Zn^{2+}. However, our previous results[11] show that under conditions where Zn^{2+} promotes the hydrolysis of UpU by a factor of 80 ([M^{2+}] = 5 × 10^{-3} mol dm^{-3}; pH 5.6; T = 363.2 K), Mg^{2+} increases the rate only two-fold. This finding as such argues against the participation of the metal ion as a mere electrophile (Scheme 2). A more conclusive piece of evidence against this mechanistic alternative is, however, that the Mg^{2+}-promoted reaction is not catalysed by imidazole buffer (Fig. 1), in striking contrast to the Zn^{2+}-enhanced hydrolysis. Since Mg^{2+} is known to complex, as a hard Lewis acid, with heteroaromatic nitrogen bases much less firmly than Zn^{2+},[24] one may argue that the rate acceleration observed with the Zn^{2+}/Im system does not result from general base catalysis by imidazole, but from formation of a Zn^{2+}Im complex that is catalytically more active than Zn^{2+} aquo ion.

Studies on Zn^{2+}[12]aneN$_3$-promoted hydrolysis of UpU lend additional support to the argument presented above. This chelate is an even more efficient catalyst than Zn^{2+} aquo ion, but as seen from Fig. 1, its rate-accelerating effect is only slightly

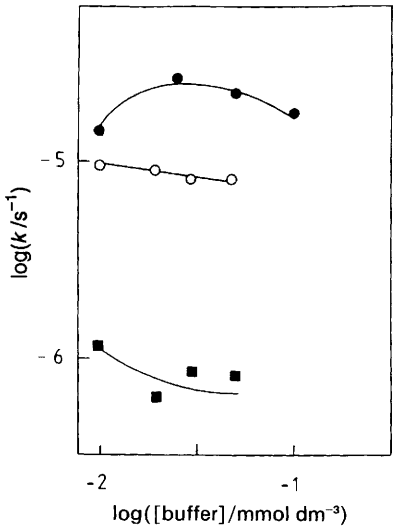

Fig. 1 Logarithmic first-order rate constants for the metal-ion-promoted hydrolysis of UpU in imidazole–imidazolium perchlorate buffers at 363.2 K (I = 0.1 mol dm^{-3} with NaClO$_4$). (●) [Zn^{2+}] = 1 × 10^{-2} mol dm^{-3}, [Im]:[ImH$^+$] = 3:7, pH = 6.3; (○) [Zn^{2+}(12)aneN$_3$] = 3 × 10^{-3} mol dm^{-3}, [Im]:[ImH$^+$] = 3:7, pH 6.6; (■) [Mg^{2+}] = 1 × 10^{-2} mol dm^{-3}, [Im]:[ImH$^+$] = 3:7, pH = 6.7. [Im] = the total concentration of free imidazole base and its metal ion complexes. The pH values refer to 298.2 K.

dependent on imidazole concentration, being continuously reduced upon addition of imidazole. These data strongly suggest that complexing of the buffer base with the metal ion cannot be ignored on interpreting the rate-promoting effect of Zn^{2+}. Evidently binding of imidazole to Zn^{2+}[12]aneN$_3$, having already a tridentate aza ligand, converts the chelate to a catalytically less active tetraaza species, while binding of imidazole to Zn^{2+} aquo ion enhances the catalytic efficiency of the metal ion. One should bear in mind that according to the bifunctional mechanism depicted in Scheme 1, Zn^{2+} requires two ligand sites for the catalytic action, one occupied by the phosphoryl oxygen and the other by a hydroxo ligand. While the Zn^{2+}Im complex (and possibly Zn^{2+}Im$_2$ and Zn^{2+}Im$_3$) may well fulfil this requirement, it is not necessarily the case with Zn^{2+}Im[12]aneN$_3$. For example, Atwood and Haake[25] have shown that Zn^{2+} promotes the hydrolysis of catechol cyclic monophosphate in aqueous acetonitrile, and that two to three imidazoles are required for coordination to Zn^{2+} for most effective catalysis.

If the basic buffer constituent acted as an external general base (Scheme 2), its catalytic efficiency should correlate with the pK_a value of the buffer acid. HEPES (pK_a = 7.48 at 298.2 K[18]) is approximately as strong a base as imidazole (pK_a = 7.10 at 298.2 K[19]). Accordingly, if imidazole serves as a general base catalyst, the catalytic effect of HEPES should be comparable to that of imidazole. As seen from Fig. 2, and also reported previously,[11] this is not the case. The Zn^{2+}-promoted hydrolysis of UpU is moderately retarded with the increasing concentration of HEPES buffer, in striking contrast to the bell-shaped dependence observed with the Zn^{2+}/Im system. The most straightforward explanation for this difference is that the metal ion and buffer base do not act in a mutually independent manner, but complexing of these species must be taken into account.

To prevent the precipitation of zinc hydroxide, the measurements with Zn^{2+}/Im and Zn^{2+}/HEPES systems discussed above were performed at pH 6.4 (at 298.2 K), *i.e.*, under conditions where the acidic buffer constituent predominates. To extend the studies to a higher pH range, where the proportion of the buffer base is more marked, Zn^{2+}[12]aneN$_3$

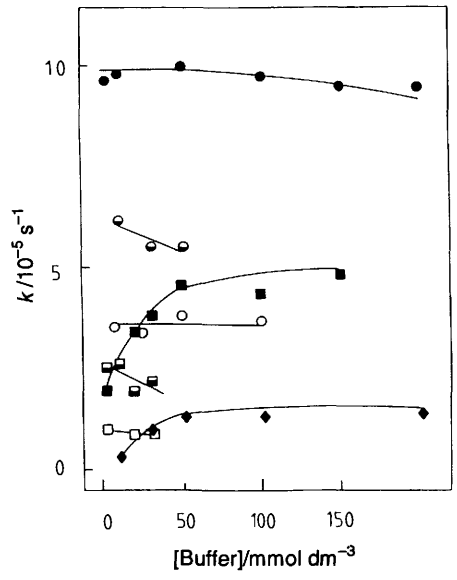

Fig. 2 First-order rate constants for the metal-ion-promoted hydrolysis of UpU in $HEPES^-Na^+$–HEPES and TEA–$TEAH^+ClO_4^-$ buffers at 363.2 K. (●) $[Zn^{2+}(12)aneN_3] = 3 \times 10^{-3}$ mol dm^{-3}, $[HEPES^-Na^+]:[HEPES] = 3:1$, pH 7.8, $I = 0.2$ mol dm^{-3}; (◒) $[Zn^{2+}(12)aneN_3] = 3 \times 10^{-3}$ mol dm^{-3}, [TEA]:$[TEAH^+ClO_4^-] = 1:3$, pH 7.3, $I = 0.2$ mol dm^{-3}; (■) $[Zn^{2+}(12)aneN_3] = 3 \times 10^{-3}$ mol dm^{-3}, $[HEPES^-Na^+]:[HEPES] = 1:3$, pH 6.8, $I = 0.2$ mol dm^{-3}; (◒) $[Zn^{2+}(12)aneN_3] = 3 \times 10^{-3}$ mol dm^{-3}, [TEA]:$[TEAH^+ClO_4^-] = 1:9$, pH 6.9, $I = 0.2$ mol dm^{-3}; (○) $[Zn^{2+}(12)aneN_3] = 2 \times 10^{-3}$ mol dm^{-3}, $[HEPES^-Na^+]:[HEPES] = 1:10$, pH 6.3, I not adjusted; (◆) $[Zn^{2+}(12)aneN_3] = 2 \times 10^{-3}$ mol dm^{-3}, $[HEPES^-Na^+]:[HEPES] = 1:10$, pH 6.3, $I = 0.1$ mol dm^{-3}; (□) $[Zn^{2+}] = 1 \times 10^{-2}$ mol dm^{-3}; $[HEPES^-Na^+]:[HEPES] = 1:10$; pH 6.3, $I = 0.1$ mol dm^{-3}. Ionic strength adjusted with $NaClO_4$. The pH values refer to 298.2 K.

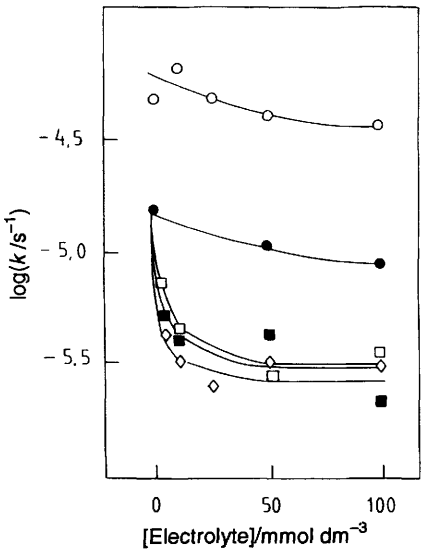

Fig. 3 Effect of electrolyte concentration on the $Zn^{2+}[12]aneN_3$-promoted hydrolysis of UpU in HEPES buffer at 363.2 K. $[Zn^{2+}(12)aneN_3] = 2 \times 10^{-3}$ mol dm^{-3}. (○) NaCl, pH 7.6, (●) $NaClO_4$, pH 6.3, (□) NaCl, pH 6.3, (■) NaBr, pH 6.3, (◇) CH_3CO_2Na, pH 6.3. The pH values refer to 298.2 K. At pH 7.6 $[HEPES^-Na^+]:[HEPES] = 0.065:0.035$ mol dm^{-3}. At pH 6.3 $[HEPES^-Na^+]:[HEPES] = 0.01:0.1$ mol dm^{-3}.

was employed as an electophile. As seen from Fig. 2, neither HEPES nor triethanolamine (TEA; $pK_a = 8.08$ at 298 K[26]) buffers accelerate the $Zn^{2+}[12]aneN_3$-promoted hydrolysis of UpU at pH 7.3 and 6.8 (at 298.2 K), respectively. In HEPES buffer only a barely noticeable dependence on the buffer concentration is observed, while the TEA buffer is moderately rate-retarding. This difference may be accounted for by the higher affinity of TEA to Zn^{2+} [27,28] In other words, no evidence for buffer catalysis is obtained.

In HEPES buffers at pH 6.8 and 6.3, a rate-acceleration is observed at low buffer concentrations, the increment levelling off to a constant value at 50 mmol dm^{-3} HEPES. There are, however, three reasons that argue against buffer catalysis as a source of this rate-acceleration. Firstly, the shape of the curves at pH 6.3 and 6.8 is virtually identical, in spite of the fact that the mole fraction of the buffer base at pH 6.8 is 2.5-fold that at pH 6.3. Secondly, the rate-increase is observed only with the chelate as a catalyst; the catalytic activity of Zn^{2+} aquo ion is decreased upon increasing the HEPES concentration (Fig. 2). Thirdly, the dependence of rate on the concentration of TEA or imidazole buffers is totally different in this pH range: instead of rate enhancement, a continuous rate retardation is observed. The studies of Kimura[2] on the complexing properties of Zn^{2+}-$[12]aneN_3$ offer a more attractive explanation. This chelate exhibits a rather strong tendency to bind anions. The logarithmic stability constants for the 1:1 complexes of Zn^{2+}-$[12]aneN_3$ with acetate, bromide and chloride ions are 2.6, 1.5 and 1.3, respectively. The anionic ligand lowers the acidity of the aquo ligand[2] and hence the catalyst becomes less efficient if the bifunctional mechanism depicted in Scheme 1 operates. The additional coordination may also be considered to reduce the ability of the catalyst to coordinate to the phosphodiester monoanion. Accordingly, the anion used to maintain the ionic strength as the buffer concentration is decreased may result in a rate-deceleration. In other words, the hydrolysis is not accelerated at high buffer concentrations but decelerated by negative salt effects in dilute buffers. To evaluate the validity of this tentative explanation, the effect of additional anions on the rate of the $Zn^{2+}[12]aneN_3$-promoted hydrolysis of UpU was studied. The results are shown in Fig. 3. It is seen that acetate, chloride and bromide ions reduce the catalytic activity of the chelate significantly in the concentration range 0–1.5×10^{-2} mol dm^{-3} and that the influence levels off to a constant value thereafter. Perchlorate anion exerts a less prominent, but still a significant effect. On examining the dependence of hydrolysis rate on the concentration of HEPES buffer ($[HEPES^-Na^+]$ + [HEPES]), the ionic strength was kept constant with $NaClO_4$. As the concentration of buffer was increased, that of $NaClO_4$ was decreased. Accordingly, the rate-enhancement observed on increasing the buffer concentration may be attributed to the decrease in the $NaClO_4$ concentration rather than the increase in the buffer concentration. Data supporting this conclusion is also shown in Fig. 2. One of the curves refers to experiments where the ionic strength of HEPES buffer was not adjusted. In this case the rate of the $Zn^{2+}[12]aneN_3$-promoted hydrolysis of UpU is independent of HEPES concentration and it is higher than in the same buffers containing $NaClO_4$.

It is worth noting that the apparent buffer catalysis of the $Zn^{2+}[12]aneN_3$-promoted hydrolysis is observed only with HEPES buffers. These buffers consist of neutral zwitterionic HEPES and the sodium salt of HEPES monoanion as buffer constituents. Accordingly, the ClO_4^- concentration is decreased with the increasing buffer concentration, when the ionic strength is adjusted with $NaClO_4$. With imidazole and triethanolamine buffers the ClO_4^- concentration remains unchanged, since these buffers consist of a neutral base and a perchlorate salt of either imidazolium or triethanolammonium ion.

The fact that the $Zn^{2+}[12]aneN_3$-promoted hydrolysis is apparently accelerated by HEPES buffers only at relatively low pH values is also noteworthy. Hydroxide ion is known to exhibit higher affinity to $Zn^{2+}[12]aneN_3$ than other anions.[2] The mole fraction of the hydroxo form of $Zn^{2+}[12]aneN_3$ is

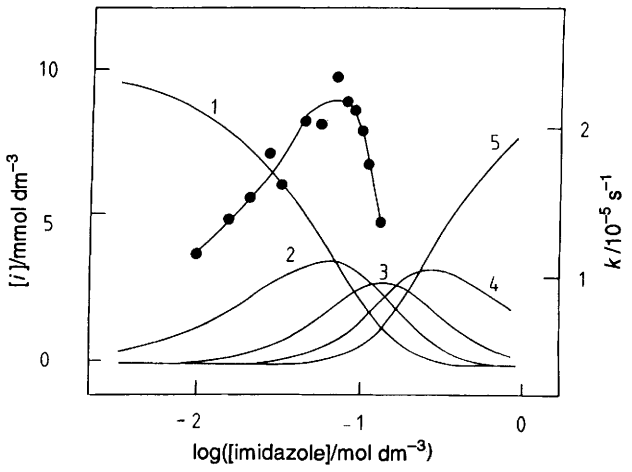

Fig. 4 Rate profile for the Zn^{2+}-promoted hydrolysis of UpU in imidazole buffers at 363.2 K and the distribution diagram of Zn^{2+}-Im_n complexes (n = 0–4) under the same conditions. [Im]:[ImH^+ $HClO_4^-$] = 1:1 ([Im] = total concentration of free Im and its Zn^{2+} complexes), [Zn^{2+}] = 1 × 10^{-2} mol dm^{-3}, I = 0.1 mol dm^{-3} ($NaClO_4$). The distribution curves indicated refer to: (1) Zn^{2+} aquo ion, (2) $Zn^{2+}Im$, (3) $Zn^{2+}Im_2$, (4) $Zn^{2+}Im_3$ and (5) $Zn^{2+}Im_4$. The kinetic data are presented with filled circles (refer to the y-axis on the right hand side).

increased with increasing pH (pK_a of the aquo ligand of the chelate at 278.2 K is 7.3 [2]) and hence complex formation with other anions competes less severely. As seen from Fig. 3, chloride ion retards the catalytic activity of the Zn^{2+}[12]aneN_3 more markedly at pH 6.3 than at pH 7.6. At the lower pH, the catalytic activity of the chelate is decreased to one tenth upon addition of 0.01 mol dm^{-3} NaCl. By contrast, at the higher pH, the addition of 0.1 mol dm^{-3} NaCl reduces the rate by only 25%.

On the basis of the foregoing it appears clear that the metal ion and buffer base do not promote the hydrolysis of UpU in a mutually independent manner. If the buffer influences the catalytic activity of the metal ion, the latter has to complex with the buffer base and this complexing may either be rate enhancing or rate retarding. Additionally, interaction with other electrolytes in the reaction solution may affect the catalytic activity of the metal ion species.

The complex formation between the buffer base and metal ion also offers an alternative explanation for the bell-shaped dependence of the hydrolysis rate of UpU on imidazole concentration at a constant concentration of Zn^{2+} ion. The log K values for the 1:1, 1:2, 1:3 and 1:4 complexes of Zn^{2+} with imidazole have been reported to be 2.57, 2.36, 2.22 and 2.01, respectively.[29] Fig. 4 shows the distribution of different complexes as a function of imidazole concentration at [Zn^{2+}] = 1 × 10^{-2} mol dm^{-3} and 363.2 K. The rate constants obtained for the hydrolysis of UpU in these solutions are also presented in Fig. 4. Comparison of the rate profile with the distribution curves reveals that under conditions where the Zn^{2+} concentration decreases, the hydrolysis rate is increased, suggesting that the catalyst is not a mere Zn^{2+} aquo ion but an imidazole complex of it. Comparison of the kinetic data with the distribution diagram also suggests that the most active catalyst would be the 1:1 complex, while the $Zn^{2+}Im_4$ complex is catalytically considerably less active. As already mentioned, Atwood and Haake[25] have shown that Zn^{2+}/Im complexes promote the hydrolysis of catechol cyclic monophosphate in aqueous acetonitrile and suggested that the catalytically most active species are the 1:2 and 1:3 complexes. The accuracy of the distribution diagram in Fig. 4 does not allow firm conclusions on the relative catalytic efficiency of the $Zn^{2+}Im$, $Zn^{2+}Im_2$ and $Zn^{2+}Im_3$ to be drawn. The stability constants used to construct the diagram were obtained by extrapolation from 298.2 K to 363.2 K, assuming that the dependence of log K_i (i = 1–4) on T is the same as in the temperature range 273 to 298 K.[29] This undoubtedly gives rise to considerable uncertainity, but the main conclusions, according to which the $Zn^{2+}Im$ complex is catalytically more active than Zn^{2+} and $Zn^{2+}Im_4$, appear to be reasonably well founded.

It seems probable that binding to imidazole ligands increases the acidity of the aquo ligand of Zn^{2+}. For comparison, the aquo ligand of Zn^{2+} in carbonic anhydrase, where Zn^{2+} is coordinated to three imidazoles, has a pK_a of only about 7,[2] *i.e.*, two units lower than that of uncomplexed Zn^{2+} aquo ion. Since it has been shown[11] that the rate promoting effect of aza complexes of Zn^{2+} correlates with the pK_a value of their aquo ion, a logical explanation for the apparent imidazole catalysis is the formation of Zn^{2+}/Im complexes having aquo ligands more acidic than that of Zn^{2+} aquo ion.

In summary, the results of the present paper strongly suggest that the metal-ion-promoted hydrolysis of UpU is not susceptible to catalysis by external general bases. In other words, the previously suggested mechanism depicted in Scheme 2 appears unlikely. The data obtained is consistent with the rather widely accepted[8–11] bifunctional mechanism described in Scheme 1. Accordingly, the metal ion would not only act as an electrophile, but its hydroxo ligand would also serve as an intracomplex general base. However, the present data do not allow one to strictly exclude a mechanism where the metal ion would facilitate the nucleophilic attack of 2′-hydroxy function by coordinating simultaneously to 2′-OH and the phosphoryl oxygen ligand. Although we have previously[1] regarded this mechanistic alternative less attractive, more conclusive evidence is undoubtedly needed to decide whether the hydroxo ligand deprotonates the 2′-hydroxy function, or whether the metal ion facilitates the deprotonation by direct coordination. Finally, the present results suggest a tentative mechanism for the observed[7] sequence-selective RNase activity of imidazole tethered oligodeoxyribonucleotides in the presence of Zn^{2+}. Most likely, the imidazole group of the oligonucleotide conjugate hybridized with the target RNA sequence offers a coordination site for Zn^{2+} and hence brings the catalytically active metal ion into proximity with one of the phosphodiester bonds. The actual hydrolysis would in principle proceed as depicted in Scheme 1, the Zn^{2+} ion being simultaneously coordinated to the imidazole group of the cleaving agent, *i.e.*, the oligodeoxyribonucleotide conjugate and the phosphodiester bond of the target RNA.

Experimental

Materials

The 1,5,9-triazacyclododecane ligand (Aldrich) was purchased as a free base. The buffer constituents and electrolytes were of reagent grade. The nucleosides and nucleoside monophosphates used as reference materials were the products of Sigma.

Uridylyl(3′,5′)uridine was prepared in a fully protected form from 5′-*O*-(4-monomethoxytrityl)-2′-*O*-tetrahydropyranyluridine and 2′,3′-di-*O*-acetyluridine by the phosphotriester method in solution using 2-chlorophenylphosphorodichloridate as a phosphorylating agent and 1-hydroxybenzotriazole as an activator.[30] Deblocking with aqueous ammonia and aqueous acetic acid gave a product that was, after RP-HPLC purification (Hypersil ODS column, 250 × 10 mm, 5 μm, acetic acid–sodium acetate buffer, pH 4.3, containing 0.1 mol dm^{-3} NH_4Cl and 10% acetonitrile) and desalting (the same column), chromatographically pure (>98%) and identical with the commercial product of Sigma.

Kinetic measurements

The principle of the HPLC techniques used to follow the progress of reactions has been described earlier.[31] The pH of the reaction solutions was measured at room temperature before and after the kinetic run and the values were observed to agree within 0.1 pH unit. Moreover, the constancy of pH within each series of buffers was checked by pH measurement at 348 K. The reaction solutions were allowed to stand at least overnight before starting the kinetic runs. Aliquots withdrawn from the reaction solution were cooled in an ice-bath, and analysed on a Hypersil RP-18 column (250 × 4 mm i.d., 5 μm) with a 97:3 mixture of acetate buffer (pH 4.3, 0.1 mol dm^{-3} NH_4Cl) and acetonitrile as an eluent.

Calculation of the rate constants

The disappearance of the starting material (and its possible isomerization product 2′,5′-UpU) was followed by HPLC and the rate constants were calculated from the decrease of the total concentration of both isomers as a function of time *via* the first-order rate equation. The method is valid, since the rate of the hydrolysis of both isomers has been shown to be equal in the presence of metal ions.[11] Furthermore, the formation and hydrolysis of 2′,5′-UpU is of minor importance, since the rate of the hydrolysis is significantly faster than that of the isomerization, except with Mg^{2+}-promoted reactions.

The pH values of the imidazole buffers were extrapolated to 363.2 K (in order to construct the distribution diagram) by the known temperature dependence of the pK_a value.[32]

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References

1 For a recent review, see S. Kuusela and H. Lönnberg, *Metal Ions Biol. Syst.*, 1996, **32**, in the press.
2 E. Kimura, *Progr. Inorg. Chem.*, 1994, **41**, 443.
3 A.-M. Pyle, *Science*, 1993, **261**, 709.
4 J. K. Bashkin, E. I. Frolova and U. Sampath, *J. Am. Chem. Soc.*, 1994, **116**, 5981.
5 D. Magda, R. A. Miller, J. L. Sessler and B. L. Iverson, *J. Am. Chem. Soc.*, 1994, **116**, 7439.
6 K. Matsumura, M. Endo and M. Komiyama, *J. Chem. Soc., Chem. Commun.*, 1994, 2019.
7 J. Hovinen, A. Guzaev, E. Azhayeva, A. Azhayev and H. Lönnberg, *J. Org. Chem.*, 1995, **60**, 2205.
8 M. K. Stern, J. K. Bashkin and E. D. Sall, *J. Am. Chem. Soc.*, 1990, **112**, 5357.
9 V. M. Shelton and J. R. Morrow, *Inorg. Chem.*, 1991, **30**, 4295.
10 J. R. Morrow, L. A. Buttrey and K. A. Berback, *Inorg. Chem.*, 1992, **31**, 16.
11 S. Kuusela and H. Lönnberg, *J. Phys. Org. Chem.*, 1993, **6**, 347.
12 D. Wahnon, R. C. Hynes and J. Chin, *J. Chem. Soc., Chem. Commun.*, 1994, 1441.
13 H. Ikenaga and Y. Inoue, *Biochemistry*, 1974, **13**, 577.
14 R. Breslow and D.-L. Huang, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 4080.
15 R. Breslow, D.-L. Huang and E. Anslyn, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 1746.
16 T. P. Prakash and K. N. Ganesh, *J. Chem. Soc., Chem. Commun.*, 1994, 1357.
17 T. P. Prakash, S. S. Kunte and K. N. Ganesh, *Tetrahedron*, 1994, **50**, 11 699.
18 N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, *Biochemistry*, 1966, **5**, 467.
19 *Stability Constants, Supplement No. 1*, The Chemical Society, London 1971, p. 280.
20 *Handbook of Chemistry and Physics*, 62th edn., CRC Press, 1982, Boca Raton, USA, p. F-175.
21 IUPAC, *Stability Constants of Metal-Ion Complexes, Part A: Inorganic Ligands*, Pergamon Press, Oxford, England.
22 C. W. Childs, *Inorg. Chem.*, 1970, **9**, 2465.
23 C.-P. Askolin, S. Kuusela, J. Mattinen and H. Lönnberg, unpublished results.
24 R. B. Martin and Y. H. Mariam, *Metal Ions Biol. Syst.*, 1979, **8**, 57.
25 L. Atwood and P. Haake, *Bioorg. Chem.*, 1976, **5**, 373.
26 Ref. 19, p. 470.
27 M. J. Benitez, M. Company, A. Arevalillo and J. S. Jiménez, *Antimicrob. Agents Chemother.*, 1991, **35**, 1517.
28 *Stability Constants of Metal-Ion Complexes*, The Chemical Society, 1964, London, England, p. 525.
29 Ref. 28, p. 388.
30 J. H. van Boom and C. T. J. Wreesman in *Oligonucleotide Synthesis. A Practical Approach*, ed. M. J. Gait, IRL Press, Oxford 1984, pp. 153–182.
31 M. Oivanen and H. Lönnberg, *J. Org. Chem.*, 1989, **54**, 2556.
32 S. P. Datta and A. K. Grzybowski, *J. Chem. Soc. B*, 1966, 136.

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