J|A|C|S COMMUNICATIONS

Published on Web 07/23/2009

Insights on Nuclease Mechanism: The Role of Proximal Ammonium Group on Phosphate Esters Cleavage

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Nucleases, enzymes hydrolyzing phosphodiester bonds in nucleic acids, play a fundamental role in the processing of genetic information. Moreover, they achieve record-level rate accelerations (up to 10^{18}) in the cleavage of one of the most stable bonds in nature.[1,2] As in most cases of enzymatic catalysis, mechanisms of action of nucleases are still not fully understood and their better comprehension, also obtained through model studies, could give important information on chemical reactivity. A crucial role in most nucleases is played by at least one metal ion (Mg(II), Ca(II), and Zn(II)) present in the active site, which activates the fissile phosphate group toward nucleophilic attack and favors the deprotonation of a coordinated water molecule or alcoholic hydroxyl (Ser) to provide a reactive nuclophile.[1,2] However, fundamental contributions are also provided by other functional groups present in the active site. In several metallonucleases a key residue is a highly conserved Lys group located just behind the water molecule best placed to attack the phosphate.[2] Early hypotheses suggested that the positively charged Lys ammonium group could stabilize the developing negative charge on the transition state.[2,3] More recently, it has been proposed that the role of this group may be that of cooperating with the metal ion in helping the deprotonation of the attacking water molecule.[3] An unanswered question that arises when these two mechanisms are considered concerns the effect of the pK_a decrease of the attacking nucleophile on its reactivity. Undoubtedly metal coordination increases the amount of nucleophile available at physiological pH, but what about its reactivity? And is further activation by external charged centers needed, or could it be detrimental for reactivity?

Measuring β_{nuc} values of nucleophiles toward metal-activated phosphates is hampered by the difficulty in dissecting this contribution to activity from other ones.[4] We reasoned that, in the case of metal-bound alkoxide nucleophiles, such as our previously studied and highly reactive Zn(II) complex **1** (Chart 1),[5a,b] chemical modification of the alcoholic arm could allow modulation of its acidity and evaluation of its effect on reactivity. The high hydrolytic activity of complexes based on the bis(2-amino-pyridinyl-6-methyl)amine (BAPA)[5,6] should allow easy investigation of the substituent effect. Thus, we designed **2**, where an ammonium group is located behind the active nucleophile similarly to the Lys group in nucleases, and **3**, where the electron-withdrawing CF_3 group is introduced to mimic the effects of the ammonium group on the acidity of the alcoholic residue but without concomitant electrostatic effects due to the positive charge. Although Zn(II) is not the most diffused divalent ion in nucleases as compared to Mg(II) and Ca(II), it allows realization of stable complexes with a well-defined geometry that allow us to draw sound conclusions from their study.

Chart 1. Complexes and Substrates Used (for Each Substrate, the Most Accepted Mechanism of Its Metal Ion-Promoted Cleavage Is Reported)

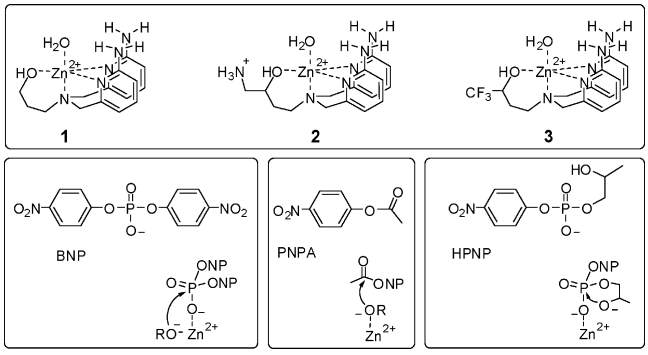

Table 1. Ligand (pK^n) and Zn(II) Complexes (pK^n_a) Deprotonation Constants and Zn(II) Complexation Constants (log K_f) for **1**−**3** As Obtained from Potentiometric Titrations (NaCl 0.1 M, 25 °C, Errors Are within 5%)

	H_nL^{3+}				$[(L)Zn]^{2+}$			
complex	pK^1	pK^2	pK^3	pK^4	log K_f	pK^1_a	pK^2_a	pK^3_a
1[a]	–	7.72	5.33	1.95	6.68	7.94	9.96	–
2	9.09[b]	7.13	5.36	<2	6.08	7.64	8.07	9.44[b]
3[c]	10.72[d]	7.23	5.49	<2	6.00	7.86	8.16	–

[a] Data from ref 5b. [b] Ammonium group. [c] $NaClO_4$ 0.1 M. [d] Alcoholic group.

Potentiometric titrations confirmed the similarity of the pK_a of the two alcoholic groups of **2** and **3**. Table 1 reports for the complex formation constants (log K_f) and the pK_a of the metal-bound species (pK^n_a). The ionization behavior of the complexes is quite complex. Previous investigation with **1** lead to attributing the two deprotonation events observed respectively to a metal-bound water molecule (pK^1_a) and to the alcoholic group (pK^2_a).[5b] In **2**, as proposed for the Lys residue in enzymes, the presence of the ammonium strongly affects the acidity of the neighboring metal-bound alcoholic hydroxyl, lowering the pK^2_a by ∼2 units. Likely, such increased acidity is due to intramolecular H-bonding or electrostatic stabilization of the deprotonated alcoholic residue. The acidity of the alcoholic group in **3** is close to that for **2**, confirming the effects of the CF_3 group on the alcoholic residue are similar to those of the ammonium.

The possibility of **2** to mimic a nuclease active site is also confirmed by DFT calculation on its complex with the DNA model substrate bis-*p*-nitrophenyl phosphate (BNP, Chart 1). The minimized structure (Figure 1 (left), details in Supporting Information (SI)) reveals an extended network of intracomplex interactions. Besides the expected intramolecular H-bonds between the pyridine amino groups and the phosphate oxygen bound to Zn^{2+}, two other relevant H-bonds, both from the NH_3^+ group, are detected.

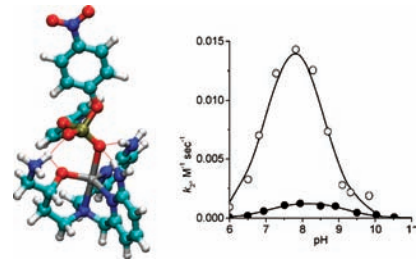

Figure 1. (Left) Solution-phase (PCM model) optimized structure of **2**-BNP in its monodeprotonated form (H-bonds evidenced by dashed red lines; colors: Zn, gray; P, yellow; C, cyan; O, red; N, blue; H, white). (Right) pH dependence of second-order rate constant for the reaction between BNP and Zn^{2+} complexes **2** (○) and **3** (●) at 25 °C ([buffer] = 5.0×10^{-2} M).

10.1021/ja9033236 CCC: $40.75

Table 2. Kinetic pK_a Values[a] (pK^n_a) and Second-Order Rate Constants (k_{SUB}) for Reaction of Monodeprotonated Zn^{2+} Complexes with BNP, PNPA, and HPNP[b]

complex	pK^1_a	pK^2_a	k_{BNP} (M^{-1} s^{-1})	k_{PNPA} (M^{-1} s^{-1})	k_{HPNP} (M^{-1} s^{-1})
1[c]	7.9	10.2	0.097	1.20	0.26
2	7.3	8.4	0.022	0.26	0.70
3	7.7	8.6	0.0015	0.34	0.22

[a] From the fitting of BNP profiles. [b] 25 °C, [buffer] = 5.0×10^{-2} M, errors are within 10%. [c] Data from ref 5b.

The first points to the alkoxyde group, and the second to the phosphate peripheral oxygen not bound to the metal ion. The presence of these two H-bonds indicates that the ammonium group can, in principle, participate in the reaction playing both roles proposed for the Lys residue in enzymes: assistance to nucleophile deprotonation and transition state stabilization.

Figure 1 (right) reports the pH dependence of the apparent second-order rate constants for the cleavage of BNP in the presence of **2** and **3**. The profiles are bell-shaped, as previously reported for **1**, indicating that the reactive species are the monodeprotonated complexes.[5,6b] The reactivity maximum is reached at ~pH 8 for both Zn complexes which is in line with the pK_a values of the metal-bound species (maximum activity at pH 9 was, on the contrary, observed for **1**).[5] The pH profiles were fitted with a kinetic model involving two deprotonation equilibria for the metal complex (eq 1, SI). The pK_a values obtained are in good agreement with those determined from potentiometric titrations. The second-order rate constants for the reaction of BNP with the monodeprotonated complexes are reported in Table 2. Both **2** and **3** are less reactive than parent **1**, but **2** is sensibly more reactive than **3**. Such differences are not related to different substrate affinities. In fact, competitive inhibition experiments carried out with dimethyl phosphate (DMP) yield almost identical binding constants: 72, 75, and 78 M^{-1} respectively for **1**, **2**, and **3**. Finally, the solvent kinetic isotope effect (skie, k_{H_2O}/k_{D_2O}) measured for reaction with **2** is 0.8 (SI). Similar skie's have been reported for intramolecular nucleophilic attack of Zn(II)-bound methoxide on phosphate triesters[7a] and support nucleophilic catalysis with no movement of protons in the rate-determining step.[7b]

The major effects of the ammonium group in **2** are hence the shift of the optimum reactivity toward lower pH values and a reactivity decrease with respect to **1**. However, the reactivity of **2** is 15-fold larger than that of **3** notwithstanding the similar pK_a's of the alcoholic groups. This suggests the role played by the ammonium group is subtler than a simple nucleophilicity decrease due to the reduced basicity of the nucleophile.

Valuable insights into the mode of action of the two complexes were provided by the study of their reactivity toward two additional substrates: *p*-nitrophenyl acetate (PNPA) and 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP, Chart 1). PNPA is cleaved by hydrolytic metal complexes with sole nucleophilic catalysis (Chart 1);[8] hence the reactivity toward this substrate yields information on the nucleophilicity of the alkoxide.[8b] The second-order rate constants for the monodeprotonated complexes (Table 2) indicate a similar nucleophilicity for both **2** and **3** and a lower activity with respect to **1**, as expected based on the pK_a values.

HPNP reaction with metal complexes is an intramolecular transesterification where the nucleophile is the substrate's hydroxyl groups (Chart 1). As a consequence, HPNP reactivity is not affected by the activity (or even by the absence) of metal-bound nucleophiles and provides a good indication of the ability of the catalyst to stabilize the reaction transition state.[6,9] Note that, with this substrate (Table 2), the presence of the ammonium group leads to the larger reactivity of **2** compared to **1** and **3**. Since ground state effects can be ruled out based on the DMP binding values obtained and general acid catalysis by the ammonium group is excluded by the skie effect observed, stabilization of the developing negative charge in the transition state by electrostatic or H-bonding interaction with the ammonium group must be the key point.

In summary, the ammonium group in **2** produces several effects, and not all positive, on the reactivity toward phosphate diesters. First, as proposed for the Lys residue in enzymes, it helps the formation of the alkoxyde nucleophile by decreasing its pK_a by 2 orders of magnitude. Thus, the maximum reactivity pH is shifted closer to physiological values. The important point that emerges from the results reported here, and that is apparently underestimated when enzyme mechanisms are discussed, is that this pK_a benefit is heavily paid in terms of reactivity loss as demonstrated by the 65-fold lower reactivity of **3** with respect to **1**. The second effect of the ammonium group, again in line with the proposed enzymatic mechanism, is the increased activity of the system, due to the electrostatic effects exerted by its positive charge in stabilizing the reaction transition state.[10] In the model system studied here, electrostatic stabilization brings about a substantial benefit with HPNP, producing the most active monometallic Zn^{2+} complex toward this substrate so far reported.[11] However with BNP this effect is not strong enough to compensate for the reactivity decrease due to nucleophile deactivation. For the Lys groups in enzymes, if the two roles proposed, i.e., decreased basicity of the nucleophile and stronger transition state stabilization, were mutually exclusive, the second should be preferred since it is the only one producing a net positive effect. However, in a less polar environment such as the active site of the enzymes, electrostatic interaction can be much stronger.[12] If this were the case, the combined effect of both contributions could lead to a substantial reactivity gain at physiological pH.

Supporting Information Available: Cartesian coordinates, synthesis of the ligands, and kinetic details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA9033236