ChemPubSoc Europe

DOI: 10.1002/ejoc.201800300

EurJOC European Journal of Organic Chemistry

Full Paper

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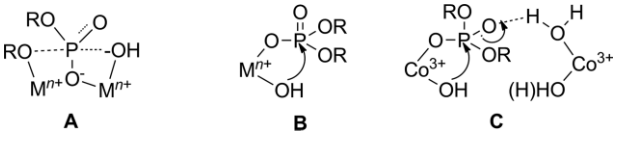
Distance between Metal Centres Affects Catalytic Efficiency of Dinuclear Co^{III} Complexes in the Hydrolysis of a Phosphate Diester

Eva Szusanna Bencze,[a] Cristiano Zonta,[a] Fabrizio Mancin,[a] Leonard J. Prins,[a] and Paolo Scrimin*[a]

Abstract: Dinuclear Co^{III} complex catalysed hydrolysis of bis-*p*-nitrophenylphosphate, a DNA model substrate, is reported. The catalysts were designed in such a way that the two Co^{III} ions cannot be contemporaneously involved in the complexation of the substrate or transition state. Experimental evidence of the involvement of such a remote metal centre in the catalysis of the hydrolysis of a phosphate diester is provided. This contribution amounts to a ca. 64-fold rate acceleration for the second-order rate constants of the best dinuclear complex over the mononuclear one, which is apparently due to general acid or H-bonding catalysis. Furthermore, there is significant distance dependence for this catalytic contribution and, in this case, it appears that the best distance (as estimated by DFT calculations) is ca. 7.7 Å. This may indicate that the presence of metal centres in close proximity, as required for mechanism proposals in which all metals are directly involved in transition-state coordination, is not the only option for rate acceleration in natural phosphate hydrolysis.

Introduction

Nucleophilic cleavage of the P–O bond in phosphate diesters is a very challenging reaction. With extraordinary efficiency, enzymes accelerate this process by up to 17 order of magnitude.[1] Experimental evidence indicates that most of these enzymes present in their active site catalytically relevant divalent metal ions.[2] The most plausible mechanisms for them require two of these ions to operate in a concerted fashion, such as depicted in Scheme 1, **A**.[2–4] This conventional wisdom has been questioned with the suggestion that only one metal ion is really catalytically necessary.[5] To make this picture more complex, phosphate-cleaving enzymes with more than two metal centres are also known.[6] Not all metals in these systems are close enough to interact directly with the substrate or the nucleophilic species involved in the cleavage. Previous work from our group[7] and others[2b,8,9] had estimated a distance between the two metal centres to be less than 6.5 Å to take advantage of such close interaction by studying model substrates. Many crystallographic studies reveal that, compared to the metal ions considered critical for catalysis, the "remote" (in most cases the third) metal ion is too far away from the others to interact with the transition-state complex.[6d] This appears to be the case for the DNA repair enzyme endonuclease IV, T5 flap endonuclease, and alkaline phosphatase, to provide a few examples, for which three metals are present and have been suggested as catalytically relevant, although possibly to different extents.[6] One explanation that was suggested in these cases is that that isolated metal ion moves within the protein to follow the changes of the substrate towards the transition state of the reaction.[6b] In this scenario the distance between metal centres would change in reaching the transition state. This, however, does not account for all possible mechanistic options and the question of the catalytic relevance of these "remote" metal ions, even without a rearrangement of the protein in the course of the catalytic process, is still open. Interestingly, also artificial catalysts appear to be more effective in diphosphate cleavage when based on a cluster of metal ions,[10] although in these systems a relevant statistical contribution to binding has been suggested.[11] Herein, we address the possible role of a remote metal ion in the catalysis of the hydrolysis of a phosphate diester with a good leaving group and provide experimental evidence that such a metal ion can be catalytically active without getting



Scheme 1. **A** represents a putative transition state for the cleavage of a phosphate diester catalysed by two transition metal ions. The extent of the formation of the bonds between the nucleophile, the leaving group and the phosphorus atom defines whether the mechanism is dissociative, associative or concerted. **B** represents the accepted mechanism for mononuclear Co^{III} complex-catalysed cleavage of a phosphate diester. **C** represents the suggested mechanism for the best dinuclear catalysts discussed here. The coordinating cyclen derivatives and the spacing units have been omitted for clarity.

[a] *Department of Chemical Sciences, University of Padova, Via Marzolo, 1-35131 Padova, Italy*
E-mail: paolo.scrimin@unipd.it
http://www.chimica.unipd.it/paolo.scrimin/pubblica/
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close enough to the catalytic site to be involved in the coordination of the transition state.

Results and Discussion

To simplify our approach, we have reduced the transition-state coordinating site to a single ion, choosing a Co^{III} complex as a model. The mechanism of action of Co^{III} complexes in phosphate cleavage is fairly well known[12] and requires (Scheme 1, **B**) the coordination of the phosphate to the metal ion and nucleophilic attack by a deprotonated water molecule bound to the same metal in a pseudo-intramolecular process. This Co^{III} ion plays the double role of activating the phosphate (Lewis acid catalysis) and delivering the nucleophilic species. In many dinuclear phosphate-cleaving enzymes such a double role is typically played by two different metal ions (like Zn^{II}, Mg^{II} or Ca^{II}). Thus the behaviour of a single Co^{III} ion is reasonably similar to that of many dinuclear catalysts but for the assistance in leaving group departure. In model studies this latter is usually addressed by using a substrate with a very good leaving group. Although Co^{III} is not found in the catalytic site of phosphate-cleaving enzymes, it has the advantage of a well-defined coordination geometry, thus allowing a better analysis of the system than with other, more biologically relevant, metal ions such as Zn^{II} or Mg^{II}.

To assess the role of the second Co^{III} ion (it would be the third in a trinuclear catalyst in which two metals are directly involved in the stabilisation of the transition state[13]) we have varied systematically its distance from the first one from 7.1 to 9.3 Å (based on DFT calculations), monitoring the effect on the cleavage of the phosphate diester bis-*p*-nitrophenyl phosphate (BPNPP). A comparison with neutral *p*-nitrophenyl-diphenyl-phosphate (PNPDPP), a phosphate triester was also performed.

The Catalysts

We have designed our catalysts by selecting relatively rigid aromatic spacers and, as ligand subunits, we selected 1,4,7,10-tetraaza-cyclododecane (cyclen) for its symmetry and, hence, ease of functionalisation and for the relatively fast rate of phosphate coordination (anation) of its Co^{III} complexes compared with that of other Co^{III} complexes with tetraaza ligands.[3b,3d] If the phosphate binding process were the rate-determining step in the process it would be impossible to obtain mechanistic information on the hydrolysis. Ligands **1**–**3** were obtained by reacting the 1,7-benzyloxycarbonyl (*Z*)-diprotected cyclen with the appropriate dibromomethyl derivative of the aromatic system used as the spacer. Mononuclear ligand **4** was also synthesised for comparison. The structure of the four ligands and the synthetic route followed for their synthesis is reported in Scheme 2. Their Co^{III} complexes were obtained by following procedures reported for similar complexes.[12,14]

Structure of the Complexes

To better understand the structures of the complexes involved in the reaction, in the absence of suitable crystals for a diffractometric analysis, we performed calculations using the unrestricted DFT method, UB3LYP, with the 6-31+G(d,p) basis set. This gave us the possibility to determine the Co–Co distances for the tetrahydrated dinuclear Co^{III} complexes of ligands **1**–**3**. The presence of the flexible methylene groups allows two stable conformations for each dinuclear complex (*syn* and *anti*) but the distance between the two ions in each of the two varies only slightly: 7.07 and 7.15 for **1**-$Co^{III}_2(H_2O)_4$, 7.64 and 7.75 Å for **2**-$Co^{III}_2(H_2O)_4$, and 9.55 and 9.17 Å for **3**-$Co^{III}_2(H_2O)_4$, for the *syn* and *anti* isomers, respectively. The minimised structures of the *syn* and *anti* isomers **2**-$Co^{III}_2(H_2O)_2$ are reported in Figure 1 and those of complexes **1**-$Co^{III}_2(H_2O)_2$ and **3**-$Co^{III}_2(H_2O)_2$ are reported in the Supporting Information. Although we have not performed calculations for structures with the Co^{III} ions pointing outward with respect to the cavity, available crystal struc-

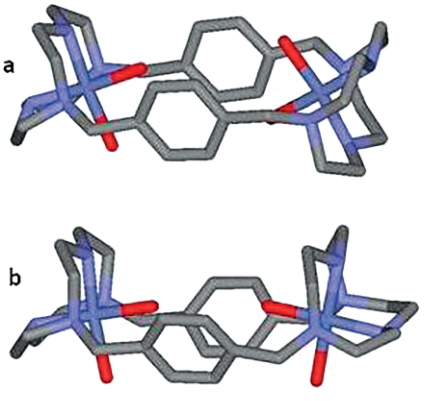
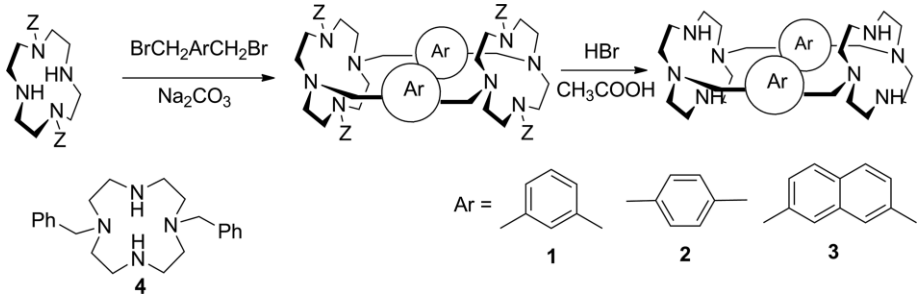


Figure 1. Structure obtained from the DFT calculations for the *anti* (a) and *syn* (b) isomers of complex **2**-$Co^{III}_2(H_2O)_4$. Hydrogen atoms have been omitted for clarity.



Scheme 2. Synthesis of ligands **1**–**3** and their structure as well as that of **4**.

tures for Co^{III} complexes of cyclen[15] indicate that the substituents on the nitrogen atoms are oriented in the same direction as the metal ion (i.e., inward in the dinuclear complex). Such a binding mode appears, accordingly, rather improbable. Furthermore, the Co–Co distances rule out a possible mechanism involving a bridging of the pentacoordinate[16] phosphorane transition state or intermediate among the two metal centres, or the involvement of an OH- bound to the second metal ion as the nucleophile.[17] Experimentally, this is confirmed by the ^{31}P NMR spectra of BPNPP with **2**-Co^{III}_2 and **4**-Co^{III}, which revealed that very little substrate is bound to the metal centres irrespective of whether the catalyst is mono- or dinuclear, thus indicating that the second metal ion is not involved in the binding as this would have resulted in larger binding constants.[18]

Rate of Phosphate Hydrolysis

The observed rate constants for the cleavage of BPNPP for the three dinuclear complexes and the mononuclear complex at 40 °C and pH 6.6 are reported in Table 1. For correct comparison, kinetics experiments were run at the same nominal concentration of Co^{III} (1.0×10^{-3} M; namely, the concentration of the mononuclear catalyst is double that of the dinuclear catalysts. At this concentration, the possibility for **4**-Co^{III} to dimerise to give a more reactive dinuclear complex[19] should be minimised. On the contrary, the conformation of the dinuclear complexes (see above) makes such an event impossible. The very low binding constant of the substrate for the complexes[18] and the concentration of catalysts used ensure that, at 2×10^{-5} M substrate concentration, the kinetics studies were performed well below the saturation conditions. Analysis of Table 1 reveals that the most active catalyst is **2**-Co^{III}_2 (31-fold better than the mononuclear complex) whereas the worst one is **3**-Co^{III}_2. The activity of this latter is lower than that of mononuclear **4**-Co^{III}. This likely implies that each dinuclear catalyst can interact with a single substrate molecule. It is remarkable that by slightly changing the distance between the two Co^{III} ions in the dinuclear complexes, the observed rate constants change so significantly. In fact, just by increasing the distance from ca. 7.1 Å (**1**-Co^{III}_2) to 7.7 Å (**2**-Co^{III}_2), k_{obs} becomes 11-fold faster whereas it drops 44-fold by increasing the distance to ca. 9.3 Å (**3**-Co^{III}_2).

Table 1. Observed rate constants[a] k_{obs} (s^{-1}) for the cleavage of BPNPP and PNPDPP by catalysts **1**–**3**-Co^{III}_2 and **4**-Co^{III} at 40 °C.

Catalyst[b]	Substrate	10^6, k_{obs} [s^{-1}] (rel. rate)	Substrate [e]	10^6, k_{obs} [s^{-1}] (rel. rate)
1-Co^{III}_2	BPNPP	7.2 (2.9)	PNPDPP	6.0 (1.7)
2-Co^{III}_2	BPNPP	77.8 (31.1)	PNPDPP	9.9 (2.8)
2-Co^{III}_2	BPNPP	55.0[d] (22.0)	PNPDPP	n. d.[f]
3-Co^{III}_2	BPNPP	1.8 (0.7)	PNPDPP	n. d.[f]
4-Co^{III}[c]	BPNPP	2.5 (1)	PNPDPP	3.5 (1)

[a] At pH 6.6 and [substrate] = 2×10^{-5} M. [b] [Catalyst] = 5.2×10^{-4} M. [c] [Catalyst] = 1.0×10^{-3} M. [d] In D_2O. [e] Solutions contained 5 % methanol (v/v) to ensure solubility of the substrate. [f] Not determined.

The activity of the dinuclear Co^{III} complexes of ligands **1** and **2** and that of mononuclear complex with ligand **4** as a function of pH is reported in Figure 2. All three complexes show a bell-shaped profile with maximum activity in the 6.5–7 pH interval. By fitting the kinetic data with Equation (1):

$$k_{obs} = k[C_o]_{tot}/(1 + [H]/K_{a11} + K_{a12}/[H]) \quad (1)$$

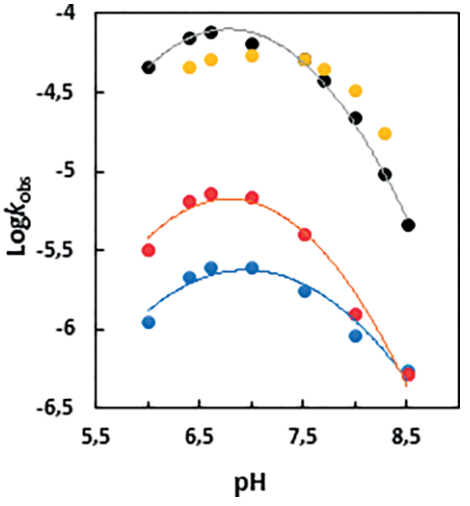


Figure 2. Dependence on pH of the observed rate of cleavage of BPNPP by dinuclear complexes **1**-Co^{III}_2 (red), **2**-Co^{III}_2 (black) and mononuclear **4**-Co^{III} (blue). The yellow points represent the observed rate constants for **2**-Co^{III}_2 in D_2O. The solid lines represent the fitting of the experimental points by using Equation (1) for **4**-Co^{III} and **1**-Co^{III}_2 or Equation (2) for **2**-Co^{III}_2.

(where K_{a11}, K_{a12} represent the acidity constants of the two water molecules bound to the metal ion) we obtain pK_a 6.1 and 7.8 (Table 2) for the two H_2O molecules bound to **4**-Co^{III} assuming the mechanism shown in Scheme 1, **B**. These values compare reasonably well with those reported[12] for cyclen-Co^{III} (5.6 and 8.0). Since this mechanism[12] requires the replacement of one of the two water molecules bound to the metal with the phosphate and the pseudo-intramolecular attack of the other one in its deprotonated form as in Scheme 1, **B**, the curve shows a bell-shaped profile. In the low pH regime, the phosphate replaces one of the water molecules bound to Co^{III} while the second one is deprotonated and acts as a nucleophile; in the downward part of the curve, at higher pH, the second water molecule is also deprotonated, thus favourably competing with the phosphate for coordination to the metal ion. It is well known that a Co^{III}-coordinated water molecule is easily replaced by the phosphate contrary to an hydroxyl ion. At first glance, the plots of Figure 2 suggest that also for both dinuclear catalysts **1**-Co^{III}_2 and **2**-Co^{III}_2 a mechanism similar (or kinetically equivalent) to that described above for mononuclear **4**-Co^{III} is

Table 2. Values of pK_a obtained from the interpolation of kinetic data and second-order rate constants for the active catalyst obtained from the interpolation of the kinetic data in Figure 2 using Equation (1) for **4**-Co^{III} and **1**-Co^{III}_2 or Equation (2) for **2**-Co^{III}_2.

Catalyst	pK_a11	pK_{a12}	pK_{a21}	k (M^{-1} s^{-1})
1-Co^{III}_2	6.15 ± 0.15	7.2 ± 0.1	[a]	2.2×10^{-2}
2-Co^{III}_2	6.05 ± 0.05	7.6 ± 0.05	8.3 ± 0.1	2.05×10^{-1}
4-Co^{III}[c]	6.1 ± 0.15	7.8 ± 0.15	–	3.2×10^{-3}

[a] Fitting of the data with Equation (2) gives an unreliable value for pK_{a21} (8.9), see discussion.

operative. However, a less superficial examination reveals that the downward part of the bell-shaped plot at the higher pH regime, particularly for the dinuclear catalyst **2**-Co^{III}_2, is much steeper than that required for such a mechanism. This hints at the loss, by increasing the pH, of a further proton, providing an extra contribution to catalysis as compared to **4**-Co^{III}. Indeed, it is impossible to fit the data points for **2**-Co^{III}_2 with Equation (1). The situation for **1**-Co^{III}_2 appears borderline (see below).

The Role of the Second, Remote Co^{III} Ion

The most obvious way of thinking is to consider the involvement of one of the water molecules bound to the second Co^{III} ion. Considering a third acid dissociation constant as kinetically relevant for the hydrolytic process catalysed by **2**-Co^{III}_2, requires (see also the Supporting Information) the data to be interpolated by Equation (2):

$$k_{obs} = k[C_o]_{tot}/(1 + [H]/K_{a11} + K_{a12}/[H] + K_{a12}K_{a12}/[H]^2) \quad (2)$$

(where, again, K_{a11}, K_{a12} represent the acidity constants of the two water molecules bound to the first metal ion and K_{a21} represents the acidity constant of one of the water molecules bound to the second metal ion). The best fit of the experimental points gives values for pK_{a11}, pK_{a12}, and pK_{a21} of 6.05, 7.6, and 8.3, respectively (Table 2). The fitting obtained implies that only one of the two water molecules bound to the second Co^{III} ion plays a significant role in the catalytic process.

A possible explanation for this behaviour is a strong H-bond or general acid catalysis involving this water molecule that stabilises the negative charge developing on the phosphate oxygen following the attack of the OH^- bound to the first Co^{III} ion (Scheme 1, **C** and the Supporting Information). As a reviewer pointed out, one might expect a general acid to have a catalytic effect, but not an effect on binding; a hydrogen bond would seem likely to be able to form in both the ground state and transition state. Accordingly, the fact that we do not see substantial differences in the binding of the substrate to the catalysts[18] might favour the general acid catalysis hypothesis. It has to be considered, however, that a new charge develops during the reaction and this affects the interaction of the transition state with the water molecule bound to the second Co^{III} ion.

It is reasonable to assume that the pK_a values of the two water molecules bound to the second Co^{III} ion do not differ much from those of the first one. The pK_a value we obtain from the interpolation of the kinetic data for this molecule is ca. 0.5 units higher than that of the least acidic H_2O molecule bound to **4**-Co^{III}. However, it is expected that a proton, bridging between the oxygen atoms of water and of the phosphate (Scheme 1, **C** and the Supporting Information), to be less acidic than that not involved in this process. Thus, this higher pK_a appears reasonably in line with the above mechanistic picture.

The alternative explanation of a simple electrostatic stabilization of the dianionic transition state by the additional positive charge brought about by the second ion does not appear so relevant, because this effect would favour the complex with the smaller intermetallic distance, which is not the case. Electrostatic contribution to binding has negligible geometrical dependence and decreases with the inverse square of the distance. On the contrary, the peculiar dependence of the rate acceleration on the distance between the two ions discussed above suggests a catalytic contribution with stringent geometrical requirements.

When we use Equation (2) to fit the kinetic data of complex **1**-Co^{III}_2 we obtain values for pK_{a11}, pK_{a12} and pK_{a21} of 6.15, 7.35 and 8.9, respectively. In this case, however, the data fit equally well just assuming the involvement of two acidic species [Equation (1), see Table 2]. Indeed, the particularly high value of pK_{a21} obtained from Equation (2) suggests that the third deprotonation does not provide any significant effect in the pH range explored (in other worlds, pK_{a21} is a superfluous parameter in the fit). Accordingly, we believe that, in the case of **1**-Co^{III}_2, the contribution of one of the water molecules bound to the second Co^{III} ion should be much less relevant than in the case of complex **2**-Co^{III}_2. On the contrary, the rate acceleration brought about by the electrostatic stabilisation of the transition state could be more significant than that present with **2**-Co^{III}_2 because of the shorter distance between the two Co^{III} ions in **1**-Co^{III}_2.

Experimental support for the mechanism suggested above for **2**-Co^{III}_2 comes from the solvent kinetic isotope effect (SKIE) obtained by carrying out the reaction in D_2O (Figure 2). In this solvent, the maximum rate for **2**-Co^{III}_2 is 1.4-fold slower while the maximum of the curve shifts to a higher pH (from 6.6 to 7.0) as expected. This relatively large SKIE is in striking contrast to the 0.8 value we have observed in catalysts with an ammonium group in close proximity to the metal catalytic site.[20] This has likely to do with the different pK_a of the species involved: the pK_a of the ammonium group was 9.5, whereas those of the two water molecules bound to the second Co^{III} are significantly lower. The calculated pK_a for the second oxygen of a putative phosphorane intermediate (the first one is bound to the Co^{III} ion) is 7.9, which is very close to the pK_a of the above water molecules but lower than that of the ammonium. Accordingly, thermodynamics favour H-bonding (or even proton transfer) to this phosphorane oxygen.

Notably, catalysts **1**-Co^{III}_2 and **2**-Co^{III}_2 perform quite similarly to **4**-Co^{III} when the substrate is *p*-nitrophenyl-diphenylphosphate (PNPDPP; see Table 1), a neutral phosphate triester for which the single developing negative charge in the transition state is stabilised by direct coordination to the first metal ion and, hence, stabilisation of other non-bridging oxygens is little relevant. This also excludes assistance in leaving group departure by the second Co^{III}, a quite unlikely event in view of the very low pK_a of *p*-nitrophenol.[21] Equations (1) and (2) allow one to obtain second-order rate constants for the processes catalysed by mononuclear **4**-Co^{III} and dinuclear **1**-Co^{III}_2 or **2**-Co^{III}_2. They are 3.2×10^{-3} M^{-1} s^{-1}, 2.2×10^{-2} M^{-1} s^{-1} and 2.05×10^{-1} M^{-1} s^{-1}, respectively (Table 2). Thus, the kinetic advantage brought about by this second, remote Co^{III} ion amounts to up to a 64-fold rate acceleration with **2**-Co^{III}_2. This is a quite significant contribution to the catalytic process considering it is also not involved in the coordination of the substrate in the delivery of a nucleophilic species.

Conclusions

We have provided experimental evidence for the involvement of a remote metal centre in the catalysis of the hydrolysis of a phosphate diester without direct involvement of this metal ion in transition-state stabilisation. In the case of a Co^{III}-based catalyst, this contribution amounts to a 64-fold rate acceleration and is apparently due to general acid or H-bonding catalysis.[22] Furthermore, there is significant distance dependence for this catalytic contribution and, in this particular case, it appears that the best distance (as estimated by DFT calculations) is ca. 7.7 Å. This indicates that three metal centres in close proximity, as required for mechanism proposals in which all three metals are directly involved in transition state coordination, is not the only option for rate acceleration in natural phosphate hydrolysis. Thus, it appears, as it has been suggested,[3a] that metal ions may not have unique roles and several mechanisms are possible in phosphate cleaving enzymes.[23,24] Our model system indicates that even a remotely located metal ion, when positioned at a suitable distance, can be kinetically beneficial. The difference between the metal ions present in the catalytic site of phosphate-cleaving enzymes (Zn^{II} and Mg^{II}) and Co^{III} used in the present investigation is the presence of a precise coordination geometry for the latter. It is thus not possible to extend the conclusion we have obtained here as for the optimum distance of a remotely-placed but catalytically relevant metal ion when Zn^{II} or Mg^{II}, devoid of a field effect, are the metal ions. With this caveat in mind it is nevertheless undeniable that a kinetic contribution of such remote metal centres is a concrete possibility also in natural enzymes.

Experimental Section

General: Solvents were purified by standard methods. All commercially available reagents and substrates were used as received. TLC analyses were performed using Merck 60 F254 precoated silica gel glass plates. Column chromatography was carried out on Macherey–Nagel silica gel 60 (70–230 mesh). NMR spectra were recorded with a Bruker AC250F spectrometer operating at 250 MHz for ^1H and 62.9 MHz for ^{13}C and a Bruker AV300 operating at 300 MHz for ^1H and 121.5 MHz for ^{31}P. Chemical shifts are reported relative to internal Me_4Si. ESI-MS mass spectra were obtained with an Agilent Technologies LC/MSD Trap SL mass spectrometer. UV/Vis spectra and kinetic traces were recorded with Perkin–Elmer Lambda 16 and Lambda 45 spectrophotometers equipped with thermostatted multiple cell holders. Kinetics were followed by monitoring the change of absorbance of formed *p*-nitrophenol at 317 nm (pH < 6.2) or *p*-nitrophenolate at 400 nm (pH > 6.2). They were started by adding 20 μL of substrate in CH_3CN to the cuvette containing 2 mL of buffered solution with the desired amount of catalyst. The buffer components were used as supplied by the manufacturers: acetic acid (Aldrich), 2-morpholinoethanesulfonic acid (MES, Fluka), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPES, Sigma), 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES, Sigma), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS, Sigma). The substrate bis-*p*-nitrophenyl phosphate sodium salt (BPNPP) was an Aldrich product, used as received. The substrate *p*-nitrophenyl diphenylphosphate (PNPDPP) was synthesised by following a reported procedure.[25] All kinetics were performed under pseudo-first-order conditions with excess of catalyst over substrate. Elemental analyses were performed by the "Laboratorio di Microanalisi" in our Department.

1,7-Bis(benzyloxycarbonyl)-1,4,7,10-tetraazacyclododecane was synthesised according to a reported procedure.[26] The product was purified using rotating-disk chromatography using as eluent $CHCl_3$/MeOH (100:2). Yield: 72 %. ^1H NMR (250 MHz, $CDCl_3$): δ = 7.34 (10 H, C_6H_5), 5.15 (s, 4 H, OCH_2), 3.42 (br., 8 H, NCH_2), 2.85 (br., m, 8 H, NCH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 155.99 (CO), 135.72, 128.55, 128.30, 127.89 (aromatic carbons), 67.66 (OCH_2), 50.43, 50.35, 50.8, 49.83, 49.48, 49.11, 48.88 (NCH_2CH_2N) ppm. ESI-MS: *m/z* = 441.1 [M + H^+], 462.1 [M + Na^+].

2,7-Bis(bromomethyl)naphthalene was prepared by following a reported procedure[27,28] with slight modifications. 2,7-Dimethyl naphthalene (1.14 g, 7.31 mmol) was dissolved in freshly distilled acetone (200 mL) and NBS (2.2 equiv., 16.08 mmol) was added to the solution. N_2 was bubbled through the solution for 1 h, maintaining a week stream of N_2 during the reaction time. When solids were completely dissolved, a catalytic amount of benzoyl peroxide was added to the reaction mixture. The solution was irradiated with a water-cooled Hg lamp (120W) until the product formation was completed (4–5 h, monitored by TLC). The solvent was then evaporated, the solid residue was taken up with CH_2Cl_2 (30 mL) and washed with NaOH 1 N solution (2 × 25 mL) and H_2O (25 mL). The residue was purified by rotating disc chromatography (petroleum ether ether/ethyl acetate = 95:5). Yield: 50 %. ^1H NMR (250 MHz, $CDCl_3$): δ = 7.84, 7.80, 7.53 (d), 7.50 (d, 6 H, aromatic protons), 4.65 (s, 4 H, CH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 135.8, 132.94, 132.64, 128.57, 127.85, 127.48 (aromatic carbons), 33.72 (CH_2) ppm.

Synthesis and Characterization of Compounds 1–4

1,7-Dibenzyl-1,4,7,10-tetraazacyclododecane (4), Tetrahydrobromide Salt: 1,7-Bis(benzyloxycarbonyl)-4,10-bis(benzyl)-1,4,7,10-tetraazacyclododecane was prepared by following a reported procedure[26] with some modifications. Thus, to a refluxing suspension of 1,7-bis(benzyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (340 mg, 0.77 mmol) and Na_2CO_3 (410 mg, 3.86 mmol) in anhydrous MeCN (6 mL) was added dropwise benzyl bromide (193 μL, 1.62 mmol). After 1.5 h, the reaction mixture was cooled and filtered. The solvent was evaporated, the residue was purified using rotating disc chromatography (eluent: 97:3 = petroleum ether ether/ethyl acetate) to give the pure compound. Yield: 260 mg (55 %). ^1H NMR (250 MHz, $CDCl_3$): δ = 7.15 (20 H, C_6H_5, OC_6H_5), 4.92 (s, 4 H, CH_2), 3.60 (s, 4 H, OCH_2), 3.43 (br., 8 H, NCH_2), 2.68 (br., 8 H, NCH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 156.23 (CO), 136.64, 128.28, 128.13, 127.90, 127.75, 126.93 (aromatic carbons), 66.86 (OCH_2), 54.5 (CH_2), 47.20 (br., NCH_2CH_2N) ppm. ESI-MS: *m/z* = 621.4 [M + H^+], 643.4 [M + Na^+].

The above compound was dissolved in a minimum volume of chloroform and HBr in acetic acid (4 M) was added in excess to the solution. The solution was left stirring for 1 h at room temperature, while a white precipitate formed. The white solid was filtered off, washed with Et_2O and dried in dessicator over P_2O_5. Yield: 84 %. ^1H NMR (250 MHz, D_2O): δ = 7.39, 7.36, 7.28, 7.26 (m, 10 H, C_6H_5), 3.66 (s, 4 H, CH_2), 3.06 (br., 8 H, NCH_2), 2.78 (m, 8 H, NCH_2) ppm. ^{13}C NMR (62.860 MHz, D_2O): δ = 136, 129.5, 128.9, 128.3, 58.15, 48.18, 42.53 ppm. ESI-MS: *m/z* = 353.4 [M + H^+]. $C_{22}H_{32}N_4$·4HBr·2H_2O (712.19): calcd. C 37.10, H 5.66, N 7.87; found C 36.98, H 5.71, N 7.90.

Tetrabenzyloxycarbonyl Derivatives of Macrocycles 1–3, General Procedure: To 1,7-bis(benzyloxycarbonyl)-1,4,7,10-tetraazacyclododecane (1.07 g, 2.4 mmol) dissolved in anhydrous acetonitrile

(500 mL) was added anhydrous Na_2CO_3 (0.65 g). The solution was brought to reflux and then a solution of the dibromoderivative [1,3-bis(bromo-methyl)benzene for **1**, 1,4-bis(bromomethyl)benzene for **2** and 2,7-bis(bromomethyl)naphthalene for **3**] (2.7 mmol) in anhydrous acetonitrile (35 mL) was added dropwise over a period of 2.5 h. The reaction mixture was kept under reflux for further 40 h. The solution was cooled and filtered, the residue was purified by chromatography on silica gel column (petroleum ether/ethyl acetate, 60:40 to 40:60 for **1**; petroleum ether/ethyl acetate/ethanol, 50:50:5, for **2**; petroleum ether/ethyl acetate, 60:40 to 35:65 for **3**) to give the final products as oils.

Tetrabenzyloxycarbonyl Derivatives of Macrocycle 1: Yield: 455 mg (35 %). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.52–7.15 (m, 28 H, aromatic protons), 5.06 (d, 8 H, OCH_2), 3.53 (br., 16 H, NCH_2), 3.12 (d, 8 H, CH_2), 2.74 (br., 16 H, NCH_2) ppm. ESI-MS: *m/z* = 1085.2 [M + H$^+$], 1107.2 [M + Na$^+$].

Tetrabenzyloxycarbonyl Derivatives of Macrocycle 2: Yield: 264 mg (20 %). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.35, 7.29 (m, 28 H, aromatic protons), 5.04 (br., 8 H, OCH_2), 3.50–3.26 (br., m, 24 H, CH_2, NCH_2), 2.66 (br., 16 H, NCH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 156.56 (CO), 138.29, 136.66, 128.99, 128.34, 127.81 (aromatic carbons), 66.93 (OCH_2), 60.06 (CH_2), 54.97, 47.83 (NCH_2CH_2N) ppm. ESI-MS: *m/z* = 1085.2 [M + H$^+$], 1107.2 [M + Na$^+$].

Tetrabenzyloxycarbonyl Derivatives of Macrocycle 3: Yield: 112 mg (10 %). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.75–7.24 (m, 32 H, aromatic protons), 5.08, 4.96 (br., d, 8 H, OCH_2), 3.80 (br), 3.47 (d), 3.18 (d), 2.86 (br, 40 H, NCH_2 + CH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 156.36 (CO), 136.70, 136.31, 133.27, 131.85, 128.35, 127.85, 127.77, 127.48 (aromatic carbons), 66.89 (OCH_2), 60.33 (CH_2), 53.58, 47.36 (NCH_2CH_2N) ppm. ESI-MS: *m/z* = 1185.7 [M + H$^+$], 1207.7 [M + Na$^+$].

Synthesis of the HBr Salts of Macrocycles 1–3, General Procedure: The tetrabenzyloxycarbonyl derivative of the corresponding macrocycle was dissolved in a minimum volume of chloroform, and HBr in acetic acid (4 M) was added in excess to the solution, which was left stirring for 2 h at room temperature. The white precipitate obtained was filtered off, washed with Et_2O and dried in a dessicator over P_2O_5. Trimacrocycles **1** and **2** have been already synthesised by following a different route to that used here.[27] Our physical data are consistent with those already reported.

Trimacrocycle 1, Heptahydrobromide: Yield: 59 %. ^{1}H NMR (250 MHz): δ = 7.34–7.12 (m, 8 H, aromatic protons), 3.88 (s, 8 H, CH_2), 2.80 (br), 2.64 (s, 32 H, NCH_2) ppm. ^{13}C NMR (62.860 MHz, $CDCl_3$): δ = 140.06, 128.20, 128.12, 127.15 (aromatic carbons), 60.78 (CH_2), 53.05, 47.01 (NCH_2CH_2N) ppm. ESI-MS: *m/z* = 549.6 [M + H$^+$]. $C_{32}H_{52}N_8$·7HBr·3H_2O (1169.24): calcd. C 32.87, H 5.60, N 9.58; found C 32.83, H 5.63, N 9.52.

Trimacrocycle 2, Heptahydrobromide: Yield: 64 %. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.42 (s, 8 H, aromatic protons), 3.53 (s, CH_2), 2.71–2.48 (br., m, 36 H, NH, NCH_2) ppm. ^{1}H NMR (400 MHz, D_2O): δ = 7.57 (s, 8 H, aromatic protons), 3.81 (s, 8 H, CH_2), 3.25 (t, 16 H, NCH_2), 3.13–2.89 (br., m, 16 H, $HNCH_2$) ppm. ^{13}C NMR (100.61 MHz, D_2O/CH_3OH): δ = 137.61, 129.67 (aromatic carbons), 58.86 (CH_2), 49.09, 42.78 (NCH_2CH_2N) ppm. ESI-MS: *m/z* = 549.4 [M + H$^+$], 571.4 [M + Na$^+$]. $C_{32}H_{52}N_8$·7HBr·2H_2O (1151.22): calcd. C 33.39, H 5.52, N 9.73; found C 33.44, H 5.48, N 9.69.

Trimacrocycle 3, Hexahydrobromide: Yield: 76 %. ^{1}H NMR (400 MHz, D_2O): δ = 8.30 (s, 4 H), 8.18 (d, 4 H), 7.65 (d, 4 H), 3.93 (s, 8 H, CH_2), 3.26–2.94 (br. m, 32 H, NCH_2) ppm. ^{13}C NMR (100.61 MHz, $CDCl_3$): δ = 136.3, 133.3, 131.8, 128.4, 127.8, 127.7, 60.3, 56.4, 47.3 ppm. ESI-MS: *m/z* = 649.5 [M + H$^+$], 671.5 [M + Na$^+$]. $C_{40}H_{56}N_8$·6HBr (1134.4): calcd. C 42.35, H 5.51, N 9.88; found C 42.27, H 5.55, N 9.84.

Preparation of the Cobalt(III) Complexes: Sodium tris(carbonato)cobaltate(III) trihydrate, $Na_3Co(CO_3)_3$·3H_2O was synthesised by using a reported procedure.[14a] In the preparation of the Co^{III} complexes, reported synthetic procedures were followed[12,14] and the properties of the complexes were compared with literature data of similar complexes.[28–30] The dinuclear Co^{III} complexes of the trimacrocycles **1**–**3** and the mononuclear one of compound **4** were obtained as NO_3 salts by following a procedure in which they were first converted into the carbonates and, subsequently, treated with concentrated HNO_3 to yield the final products. This two-step procedure was also used previously by other authors.[15] The NO_3 salts were used in all kinetic experiments.

Synthesis of (4)Co(NO$_3$)$_3$·2H$_2$O: Into a vial containing **4** (80 mg, 0.12 mmol) and H_2O (10 mL) was added $Na_3Co(CO_3)_3$·3H_2O (57 mg, 0.14 mmol). The solution was placed in an oil bath (70 °C, ca. 2 h) until the vigorous effervescence ceased. During the heating time the solution turned to deep purple. MeOH (10 mL) was added and the solution was filtered to remove unreacted sodium tris(carbonato)cobaltate(III). The solution was concentrated to half its volume on a rotary evaporator and some more precipitate was removed. Afterwards, acetone (30 mL) was added, removing the precipitate that formed immediately. Addition of further acetone (10 mL) resulted in the formation, overnight, of a dark-violet precipitate. After filtration and vacuum drying, the carbonate salt (42 mg, 64 %) was obtained. $C_{23}H_{32}BrCoN_4O_3$ (551.36): calcd. C 50.10, H 5.85, N 10.16; found C 49.96, H 5.89, N 10.09. This carbonate salt was dissolved in a very small amount of cold water and stirred vigorously until the complex had dissolved completely, then ca. 5 mL of HNO_3 (6 M) was added. After a few hours, a precipitate could be observed. This was allowed to stand at room temperature overnight. The solid was then filtered off, washed with ice-cold HNO_3 (0.3 M) and then with ethanol. The treatment with HNO_3 was repeated twice to ensure complete replacement of the carbonate by the nitrate. The obtained reddish powder (44 mg, 78 %) was dried with P_2O_5. $C_{22}H_{36}CoN_7O_{11}$ (633.49): calcd. C 41.71, H 5.73, N 15.48; found C 41.83, H 5.79, N 15.43.

Synthesis of (1)Co$_2$(NO$_3$)$_6$·4H$_2$O, (2)Co$_2$(NO$_3$)$_6$·4H$_2$O, and (3)Co$_2$(NO$_3$)$_6$·4H$_2$O: These compounds were obtained by following a procedure identical to that reported above for **(4)Co(NO$_3$)$_3$·2H$_2$O** with yields: 58.5 %, 61 %, 33 %, respectively.

(1)Co$_2$(NO$_3$)$_6$·4H$_2$O: Intermediate carbonate salt: IR: $\tilde{\nu}$[Co(O–C=O)] = 1669, 1628, ν(C=O) = 1455 cm^{-1}. UV: λ_{max} (H_2O) = 372, 563 nm. $C_{34}H_{52}Br_2Co_2N_8O_6$ (946.50): calcd. C 43.14, H 5.54, N 11.84; found C 43.07, H 5.59, N 11.79. Title compound: UV: λ_{max} (H_2O) = 375, 550 nm. $C_{32}H_{60}Co_2N_{14}O_{22}$ (1110.77): calcd. C 34.60, H 5.44, N 17.65; found C 34.66, H 5.40, N 17.59.

(2)Co$_2$(NO$_3$)$_6$·4H$_2$O: Intermediate carbonate salt: IR: $\tilde{\nu}$[Co(O–C=O)] = 1675, 1635, ν(C=O) = 1458 cm^{-1}. UV: λ_{max} (H_2O) = 370, 568 nm. $C_{34}H_{52}Br_2Co_2N_8O_6$ (946.50): calcd. C 43.14, H 5.54, N 11.84; found C 43.21, H 5.53, N 11.91. Title compound: UV: λ_{max} (H_2O) = 375, 550 nm. $C_{32}H_{60}Co_2N_{14}O_{22}$ (1110.77): calcd. C 34.60, H 5.44, N 17.65; found C 34.71, H 5.51, N 17.56.

(3)Co$_2$(NO$_3$)$_6$·4H$_2$O: Intermediate carbonate salt: IR: $\tilde{\nu}$[Co(O–C=O)] = 1660, 1615, ν(C=O) = 1455 cm^{-1}. UV: λ_{max} (H_2O) = 357, 574 nm. $C_{42}H_{56}Br_2Co_2N_8O_6$ (1046.62): calcd. C 48.20, H 5.39, N 10.71; found C 48.31, H 5.30, N 10.77. Title compound: UV: λ_{max} (H_2O) = 375, 550 nm. $C_{40}H_{64}Co_2N_{14}O_{22}$ (1210.88): calcd. C 39.68, H 5.33, N 16.19; found C 39.66, H 5.39, N 16.11.

Computational Methods: Density functional theory (DFT) calculations were performed with Gaussian 09 Revision B.01. Minima and transition structures were optimised using the unrestricted DFT method, UB3LYP, with the 6-31+G(d,p) basis set. Frequency analyses were carried out on stationary points to verify that they are minima.

Acknowledgments

This work was supported by the EU under contract HPRN-CT1999-00008, ENDEVAN (fellowship to E. S. B.) and by the Ministero dell'Università e della Ricerca (MIUR) of Italy (PRIN 2011).

Keywords: Enzyme models · Metalloenzymes · Hydrolysis · Phosphate cleavage · Cobalt · Dinuclear

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Received: February 20, 2018