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Hydrolysis of Phosphodiesters by Diiron Complexes: Design of Nonequivalent Iron Sites in Purple Acid Phosphatase Models

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New μ-oxo-diferric complexes have been designed for hydrolysis of phosphodiesters. To mimic the diiron active site of purple acid phosphatase, a combinatorial method has been used to select complexes containing two distinct iron coordination spheres. The introduction of a bidentate ligand, a substituted phenanthroline (L) into complex **1**, $[Fe_2O(bipy)_4(OH_2)_2](NO_3)_4$, generates in solution the complex $[Fe_2O(bipy)_3(L)(OH_2)_2](NO_3)_4$ as shown by ESI/MS and ^{1}H NMR studies. The latter complex was found to be 20-fold more active than complex **1**. On the basis of kinetic studies, we demonstrated that the complex $[Fe_2O(bipy)_3(L)(OH)(OH_2)](NO_3)_3$ was the active species and the reaction proceeded through the formation of a ternary complex in which one iron binds a hydroxide and the second, the substrate. At nonsaturating concentrations of the substrate, the increased activity with increased methyl substituents in L was due to an increased affinity of the complex for the substrate. The activity of $[Fe_2O(bipy)_3(33'44'Me_2Phen)(OH_2)_2](NO_3)_4$ [33′44′Me$_2$Phen = 3,3′,4,4′-dimethyl-1,10-phenanthroline] was found to be comparable to that reported for Co(III) or Ce(IV) complexes.

Introduction

The importance of dinuclear centers is clearly seen through their involvement in numerous epoxidation and hydroxylation biocatalysts, and one may recognize the importance of the dinuclearity for activity.[1] For example, it has been recently shown that the presence of two iron sites in a catalytic complex is crucial for control of the stereoselectivity during sulfoxidation reactions.[2] Another example of the importance of the dinuclear center[3,4] is found in hydrolytic metalloenzymes such as alkaline phosphatase,[5] purple acid phosphatase,[6] Ser/Thr protein phosphatase,[7] or phosphotriesterase.[8] In the case of purple acid phosphatases (PAPs), these dinuclear centers are asymmetric, with either the same metal in different redox states, such as the $Fe^{3+}-Fe^{2+}$ center in mammalian PAPs,[9] or with different metal ions, such as the Fe−Zn or Fe−Mn centers in plant PAPs.[6,10] Other phosphatases have two Zn centers which are in different coordination environments.[11,12] It is generally believed that the ion with the largest Lewis acidity serves to stabilize a hydroxide (either monodentate[13] or bridging[14]) at physiological pH whereas the second ion serves to reversibly bind the

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phosphoester substrate (either monodentate or bridging).[15] The second ion also serves to increase the electrophilic susceptibility of the substrate and neutralize the anionic substrate, thus moderating the electrostatic repulsion of the attacking nucleophile. The reaction then proceeds through an intramolecular nucleophilic attack of the substrate (P−O bond cleavage) by the adjacent hydroxide.[16]

In recent years, bioinspired phosphatases and nuclease mimics[17] have incorporated dinuclear active sites to achieve activity. In particular, trivalent Co[18] and Ln binuclear centers[19] have been found to be very active for phosphoester hydrolysis and for DNA cleavage as a consequence of their strong Lewis acidity. Biomimetic model systems based on Zn[20] and Cu complexes[21] have also been described; however, iron has been much less studied for its hydrolytic reactions.[22,23] This is due to the fact that Fe-dependent DNA cleavage is often considered to proceed via redox reactions, as in the case of Fe-bleomycin-dependent reactions.[24]

To reproduce the hydrolytic activity of PAP, we have concentrated our effort on the design of diiron mimics. In 1997, we demonstrated that a μ-oxo diferric complex, $[Fe_2O(Phen)_4(H_2O)(HO)]^{3+}$ [Phen = 1, 10- phenanthroline], **2**, was able to hydrolyze phosphodiesters in pure water via an intramolecular process in which the hydroxide ligand to one ferric center reacted with a phosphodiester bound to the second metal center.[25] Since then, we have focused our attention on the stereoelectronic parameters which control the hydrolytic properties of this type of complex. Here, we report that the substitution of a bipyridine ligand by another bidentate ligand in the compound $[Fe_2O(bipy)_4(H_2O)(HO)]^{3+}$ [bipy = 2,2′-bipyridine], complex **1**, can cause a large acceleration of the rate of hydrolysis of a phosphodiester. Among the 25 different ligands tested, the largest effect was observed for methylated phenanthrolines. These effects are discussed, and a reaction mechanism is proposed.

Experimental Section

Materials. Most of the reagents were of the best commercial grade and were used without further purification. BDNPP was synthesized as previously described.[26]

$(CD_3)_2$-4,4′-dimethyl-1,10-phenanthroline (D-44′Me$_2$Phen). 4,4′-Dimethyl 1,10-phenanthroline (100 mg) was suspended in 600 μL of D_2O (99.9% D). The mixture was heated under vacuum in a Schlenk tube for 24 h at 210 °C, and after cooling, a light brown powder is obtained. The yield of deuteration was over 90%, and the sample was used without purification. Only a trace of $(CHD_2)_2$-1,10-phenanthroline was observed. ESI-MS: *m*/*z* 215 (100%). ^{2}H NMR (δ ppm/C*D*Cl$_3$, H$_2$O): −1.57 (C*D*$_3$). ^{1}H NMR (CDCl$_3$, δ ppm): 9.0 (2); 8.01 (2); 7.5 (2). ^{13}C NMR: 149 (s), 146 (s), 144 (s), 123 (s),122 (s), 18 (m).

Synthesis. Complex **1** was prepared as follows: To 2 g of $Fe(NO_3)\cdot 9H_2O$ (4.95 mmol) dissolved in 2 mL of water was added 1.54 g of 2,2′-bipyridine (9.87 mmol) previously solubilized in 50 mL of acetonitrile. The resulting brown solution was stirred for 15 min, affording a green precipitate. The powder of **1** was filtrated and washed with diethyl ether. Yield = (73%). UV−vis [H$_2$O, λ_{max} (ϵ)]: 600 nm (200 M^{-1} cm^{-1}), 360 nm (10000). ^{1}H NMR (300 MHz, D$_2$O, δ in ppm): 32 (*H*αpy); 19.8 (6), 17.5 (6), 15.1 (2), 12.1 (2) (*H*βpy); 8 (*H*δpy); 8.3 and 7.8 (*H*γpy).

Anal. Calcd for $[Fe_2O(bipy)_4(H_2O)_2](NO_3)_4\cdot 4H_2O$ ($C_{40}H_{44}Fe_2N_{12}O_{19}$): C, 43.36; H, 4.00; Fe, 10.08; N, 15.17. Found: C, 43.12; H, 3.64; Fe, 10.15; N, 15.42.

Complex **2** was synthesized as previously described.[27] UV−vis [H$_2$O, λ_{max} (ϵ)]: 575 nm (160 M^{-1} cm^{-1}), 350 nm (10000). ^{1}H NMR (300 MHz, D$_2$O, δ in ppm): 30.5 (*H*αpy), 16.8, 13.5, (*H*βpy); 11.4, 10.2(*H*δpy); 8.3 and 7.8 (*H*γpy).

The complexes $[Fe_2O(44'Me_2Phen)_4(H_2O)_2](NO_3)_4$ and $[Fe_2O(4Me_2Phen)_4(H_2O)_2](NO_3)_4$ were synthesized following the protocol of the synthesis of complex **2**. $[Fe_2O(4Me_2Phen)_4(H_2O)_2](NO_3)_4$ (yield = 50%): UV−vis [H$_2$O, λ_{max} (ϵ)]: 580 nm (160 M^{-1} cm^{-1}), 350 nm (10000). ^{1}H NMR (300 MHz, D$_2$O, δ in ppm): 30 (*H*αpy), 16.8, 12.9 (*H*βpy), 11.6, 10.5 (*H*δpy); 8.6, 7.8 and 7.3 (*H*γpy); HC*H*3 2.8, 4.9 and 6.1. $[Fe_2O(44'Me_2Phen)_4(H_2O)_2](NO_3)_4$ (yield = 60%): UV−vis [H$_2$O, λ_{max} (ϵ)]: 600 nm (160 M^{-1} cm^{-1}), 355 nm (10000). ^{1}H NMR (300 MHz, D$_2$O, δ in ppm): 29 (*H*αpy); 16.8, 12.9 (*H*βpy); 12.2, 10.6 (*H*δpy); HC*H*3 2.8, 4.9 and 6.1.

Measurement of Initial Rates. In 3 mL of buffer solution preequilibrated at 50 °C was added 10 μL of 0.02 M DMSO solution of the dinitrogen ligand L2, followed by 10 μL of a 0.02 M aqueous solution of complex **1** ([**1**]$_f$ = 67 μM). The mixture is equilibrated for 5 min at 50 °C, and the phosphodiester BDNPP is added (10 μL of 0.02 M DMSO solution) for a 67 μM final concentration. The reaction is followed by the measurement of the absorbance at 400 nm (absorption of DNP for ϵ = 19100 M cm s^{-1}), and the values reported corresponded to the average of three experiments performed the same day. The rates at low pH have been corrected taking into account the pH-dependent equilibrium of DNP (pK_a = 3.95). The errors observed were within 10% of the reported value. The rates were found to be buffer-dependent, and for our pH profile, corrections have been made by changing the buffer concentration: 14 mM acetate buffer (pH ≤ 5.5); 20 mM

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MES ($5.6 \leq pH \leq 6.8$); 50 mM HEPES ($6.9 \leq pH \leq 8.1$); 20 mM CHES ($pH \leq 8.3$).

A control experiment with $Fe_3O_2(Phen)_6(OAc)_2(ClO_4)_3$ as hydrolytic species shows no activity under standard conditions.

Physical Methods. ^1H NMR (300 MHz) spectra were recorded on a DPX300 Brüker spectrometer, and T_1 measurement were measured by the inversion/recovery method. It was possible to titrate the released bipy ligand concentration versus the one introduced in the complex. To reduce imprecision due to the broadness of the considered diamagnetic peak and its exchange in solution, we have measured the additional peak area of one resonance attributed to a free bipy obtained in a spectrum where 1 equiv of bipy has been added to a **1**/44′Me$_2$phen 1:1 mixture and took it as the area for 1 bipy. The error has been estimated to be less than 10%.

^2H NMR experiments were performed on a Varian 400 MHz, and the chemical shifts were referenced to an internal reference $CDCl_3$ ($\delta = 4.0$ ppm). Visible absorption spectra were recorded on a Varian Cary1Bio.

EPR measurements (10 K) were performed on a Bruker ESP 300 equipped with an Oxford cryostat. The impurity reported in the main text has a signal comparable to a trinuclear species, $Fe_3O_2(Phen)_6(CH_3CO_2)_2(ClO_4)_3$.[28] The spin quantification of the mixtures was performed using this complex as reference.

Resonance Raman spectroscopy experiments were performed at the Consortium des Moyens Technologiques Communs (University of Grenoble) using a Dilor XY spectrophotometer on a frozen sample ($T = 100$ K).

Elemental Analyses were obtained from Laboratoire de Microanalyze CNRS, Lyon, France.

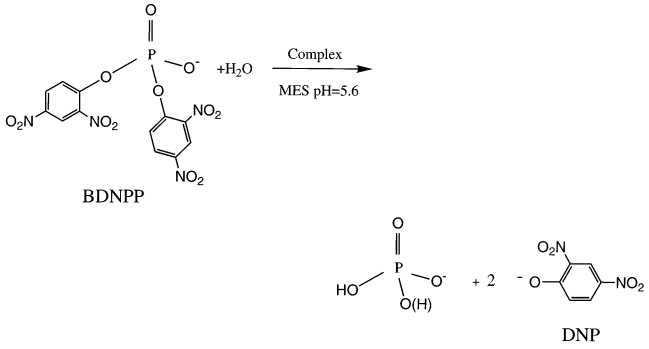
ESI-MS Method. ESI-MS spectra have been obtained using an LCQ trap ion spectrometer with a temperature fixed at 50 °C. Each spectrum recorded for each **1**/44′Me$_2$Phen mixture contained a lot of fragments attributed to molecular peaks and their fragmentation of different diiron species. We could easily extract the molecular peaks attributed to $[Fe_2O(bipy)_{4-n}(44'Me_2Phen)_n(OH)(NO_3)]^{2+}$ and $[Fe_2O(bipy)_{4-n}(44'Me_2Phen)_n(OH)_2]^{2+}$. The other peaks corresponded to fragmentation of these peaks, and other ones, to the fragmentation with bipy losses of **1** and $[Fe_2O(bipy)_{4-n'}(44'Me_2phen)_n(NO_3)_3]^+$ (Figure 3).

On the basis of the molecular peaks from $[Fe_2O(bipy)_{4-n}(44'Me_2Phen)_n(OH)(NO_3)]^{2+}$, a yield of each species could be approached by calculating the ratio between all species observed by ESI-MS considering the following expression:

$$\text{complex \%} = I_c/I_\Sigma$$

where I_c is the intensity of the fragment $[Fe_2O(bipy)_{4-n'}(44'Me_2phen)_{n'}(OH)(NO_3)_3]^{2+}$ considered and I_Σ the sum of all the intensities of the observed $[Fe_2O(bipy)_{4-n'}(44'Me_2phen)_{n'}(OH)(NO_3)]^{2+}$ fragment (see main text). The ratio measured is only indicative because the fragmentation of each species $[Fe_2O(bipy)_{4-n'}(44'Me_2phen)_{n'}(OH)\ (H_2O)(NO_3)_3]$ could have a different fragmentation yield. Nevertheless, we assumed that these species are chemically close enough to have similar fragmentation properties. The experimental intensities were directly read on the ESI-MS spectrum. It has to be noted that the negative mode did not provide any useful fragmentation.

Scheme 1 Hydrolysis of the BDNPP Phosphodiester by Complex **1**



Results

Methodology for Screening Diferric Complexes as Catalysts of Phosphodiester Hydrolysis. From the observation that one or several aromatic dinitrogen ligands L such as bipyridine in dinuclear iron complexes $Fe_2O(L)_4(H_2O)_2(NO_3)_4$ can be replaced by another ligand L2, we set up a combinatorial method for the optimization of such dinuclear iron complexes as catalysts for phosphodiester hydrolysis. The screening procedure was based on a spectrophotometric assay using bis(2,4-dinitrophenyl)phosphate (BDNPP) as a substrate because the hydrolytic product, 2,4-dinitrophenolate anion (DNP) (Scheme 1), is colored ($\lambda_{max} = 400$ nm; $\epsilon = 19100\ M^{-1}\ cm^{-1}$), allowing the reaction to be easily monitored by recording the optical density in the test solution.

In a typical experiment, each well of a 96-well plate was filled first with complex **1** (67 μM) and BDNPP (final concentration 67 μM) followed by the addition of an increasing number of equivalents of L2 (from 1 to 4). With such a plate, several L2 molecules and several pH conditions (from 4.6 to 9.1) could be tested in a single experiment. The closed plate was then placed in a drying oven (50 °C), and the reaction was stopped after 1 h incubation. Control experiments with BDNPP alone in the absence of catalysts or with BDNPP in the presence of the ligand L2 only (no iron) were always included. They showed that no reaction occurred under these conditions.

Complex **1** has been used as starting material and incubated with 1 equiv of 25 different L2 ligands at various pHs. The L2 ligands included carboxylic acids such as acetic, benzoic, or polycarboxylic acids (malonic acid); aliphatic diamines such as ethylenediamine or *N*,*N*′ tetra(methyl)-ethylenediamine; imidazoles and substituted imidazoles such as histidine (L-Hist) and 2-pyridylimidazole (pym); pyridines such as dipyridyl ketone, picolinic acid, or various substituted phenanthrolines and bipyridines; catechol, substituted catechols, and 8-hydroxyquinoline. These ligands were selected on the basis of their ability to stabilize μ-oxo-diferric complexes.[29,30]

The screening method described earlier in the text allowed us to identify the L2 ligands which stimulated the activity of **1** (Figure 1). It has to be noted that the reported activities were those obtained at optimal pH (see figure caption). As

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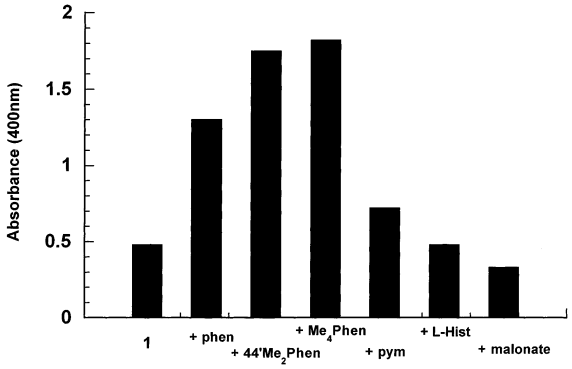

Figure 1. Absorbance of DNP anion at 400 nm as a function of selected L2 ligands in the presence of complex **1**. Experimental conditions: [**1**] = [BDNPP] = [L2] = 67 μM. Optimal pH for **1**/L2 = 5.6. [L-Hist = L-histidine; pym = (2-pyridyl)imidazole; Me_4phen = 33′44′Me_4Phen, and see text for the other abbreviations.]

shown in Figure 1, complex **1** was markedly stimulated by several ligands in the following order: pym < phen < 44′Me_2Phen < 33′44′Me_4Phen with the latter giving almost a 4-fold increase of the activity.

Carboxylates (malonate for example) inhibited the hydrolytic reaction with all the complexes tested. On the other hand, addition of imidazole such as L-histidine had no influence on the reactivity. Finally, the mixture containing the diamines or the catechols led to a degradation of the starting diferric unit with the observation of the spectroscopic signatures for $Fe(L)_3$ complexes [L = 22′-bipyridine or 1,10-phenanthroline].

Then, the combinations containing complex **1** and substituted phenanthroline ligands such as 4,4′-methyl-1,10-phenanthroline (44′Me_2Phen), 3,3′,4,4′-dimethyl-1,10-phenanthroline (33′44′Me_4Phen), or 4-methyl-1,10-phenanthroline (4MePhen) were further studied in order to define the best catalytic system for BDNPP hydrolysis.

Solution Study of Complex 1/*n*L2 mixture where L2 = Phen or Substituted Analogue. Evidence for Ligand Exchange and Formation of an Asymmetric μ-Oxo-Diiron(III) Unit

UV−Vis and EPR Spectroscopy. The ligand exchange reaction has been followed by electronic spectroscopy. Spectra of μ-oxo-diferric species, such as **1**, are dominated by strong CT bands between 300 and 400 nm and, depending upon the number and the structure of bridges between the metal centers, by a weak CT band in the visible region ranging from 510 to 750 nm.[31]

When 1 equiv of 44′Me_2Phen was added to a solution of **1**, the resulting UV−vis spectrum showed a loss in intensity of the CT band in the 300−400 nm region (the ϵ value based on two iron atoms decreased from 10 to 8.5 mM cm^{-1}) and a slight shift of the forbidden CT band from 600 to 580 nm. This showed that the ligand substitution did not affect the Fe_2O core of complex **1** significantly. Moreover, no signature for the ferrous complex $[Fe(L)_3]$ (L = bipy or Phen), a degradation product of complex **1**, could be observed.

The presence of the μ-oxo bridge has been also demonstrated by resonance Raman (RR) spectroscopy. The RR spectrum with λ_{exc} at 514 nm of complex **1** in MES-buffered solution displayed Fe−O vibrations of an Fe_2O core at 400 and 840 cm^{-1}, which were not significantly affected when 1 equiv of 44′Me_2Phen was added to the solution of complex **1**.[32]

X-band EPR spectroscopy confirmed the conservation of the EPR silent diferric unit upon ligand substitution. The EPR spectrum of **1** in pH = 5.6 buffered solution displayed a very weak signal, characterized by g values at 8.5, 5.5, and 3.3. This signal is similar to that of an $S = {}^5/_2$ trinuclear μ-oxo species and accounted for less than 10% of the total complex **1** concentration.[28] The addition of 1 equiv of 44′Me_2Phen in complex **1** solution did not affect the amount of this impurity.

^{1}H NMR Study. The ^{1}H NMR spectrum of **1** displayed resonances between 0 and 40 ppm in D_2O in accordance with the presence of strong antiferromagnetic coupling between the iron atoms via the oxo bridge. The resulting smaller paramagnetism compared to that of a mononuclear ferric species explains the characteristic spectrum with a small chemical shift range and reasonable T_1 values (1−20 ms) for protons in close contact with the paramagnetic ferric centers. The proton resonances for **1** have been assigned previously: the α protons of the pyridine ring were found at 30 ppm; signals between 12 and 20 ppm and the ones between 6 and 9 ppm were assigned to the β and γ protons, respectively.[33]

It should be noted that the diamagnetic region of the spectrum displayed additional weak sharp resonances, assigned to very small amounts of "free" ligand, 2,2′-bipyridine, in a protonated form in nonbuffered solution as shown by resonances at 9 ppm for the α proton (data not shown). It has been shown that these signals were always present whatever the preparation was and in fact revealed the presence of ligand exchange in aqueous solution. Accordingly, the T_1 values of these resonances were found 1 order of magnitude lower than the ones found for the diamagnetic molecule (in the absence of metal), demonstrating that they were in fact in slow exchange on the NMR scale.

The addition of L2 ligand such as 4,4′-dimethyl-1,10-phenanthroline (44′Me_2Phen) caused a great change in the paramagnetic 30−10 ppm region, and the appearance of resonances in the diamagnetic region was attributed to the protons of released bipyridine ligands expelled from the coordination sphere (Figure 2). On the other hand, no characteristic resonances of free 44′Me_2Phen could be detected under these conditions in the diamagnetic region. The introduction of the 44′Me_2Phen ligand into the coordination sphere in place of a bipy ligand could be monitored from the appearance of an intense peak at 12 ppm, a resonance observed in a pure sample of $[Fe_2O(44'Me_2phen)_4$-

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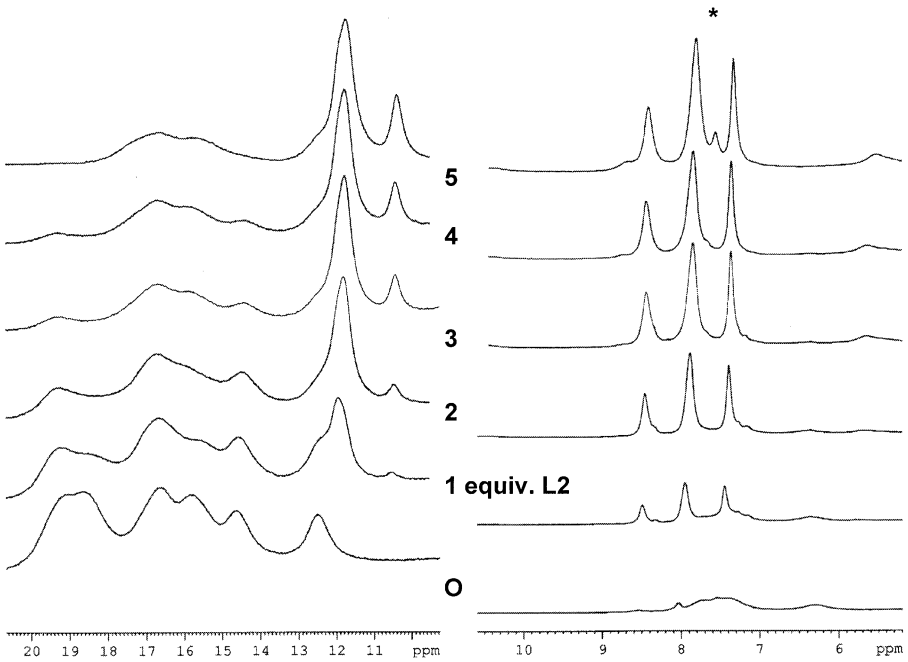

Figure 2. ^{1}H NMR of **1**/44′Me$_2$Phen mixtures ([**1**] = 5 mM in 50 mM MES buffered D_2O solution) from 0 to 5 equiv of 44′Me$_2$Phen. Asterisk refers to resonances of free 44′Me$_2$Phen. The diamagnetic region is dominated by the resonances of released 22′-bipyridine and the paramagnetic region by the ones of coordinated 22′-bipy and 44′Me$_2$Phen.

$(OH_2)_2](NO_3)_4$, prepared independently, under identical pH conditions (see Experimental Section). This also showed that during substitution the Fe_2O core was maintained.

By integrating the new peaks at 8.5 and 7.4 ppm characteristic for released bipy (Figure 2), we determined that 1 equiv of bipy was displaced by each equivalent of 44′Me$_2$phen added ligand. Accordingly, after addition of 4 equiv of the substituted phenanthroline, the paramagnetic region of the resulting spectrum was superimposable on the one of a pure sample of $[Fe_2O(44'Me_2phen)_4(OH_2)_2](NO_3)_4$ under the same pH conditions. Only when a fifth equivalent was added could the resonances of free 44′Me$_2$phen, marked by an asterisk in Figure 2, be observed.

The same observations have been made with Phen, 4MePhen, and 33′44′Me$_4$Phen, as L2 ligands, attesting that these ligands were able to substitute bipy ligands of complex **1** quantitatively.

The same experiment was carried out with a deuterated version of the 44′Me$_2$phen ligand (deuteriation of the methyl groups) and the reaction monitored by ^{2}H NMR spectroscopy. That the added ligand (named D-44′Me$_2$Phen) was fully incorporated into the complex was shown by the absence of the resonance at −1.57 ppm characteristic for the molecule free in solution. Two new resonances with very different intensities were observed at 2.25 and 1.28 ppm corresponding to the deuterated ligand within Fe(D-44′Me$_2$-Phen)$_2$ and Fe(bipy)(D-44′Me$_2$Phen) coordination, respectively (data not shown). The first system was almost negligible after addition of 1 equiv of D-44′Me$_2$Phen (it corresponds to less than 2−3%) and became significant only after addition of more equivalents of D-44′Me$_2$Phen. On the other hand, this experiment could not differentiate the two possible complexes (bipy)(D-44′Me$_2$Phen)Fe−O−Fe(bipy)-(D-44′Me$_2$Phen) and (bipy)$_2$Fe−O−Fe(D-44′Me$_2$Phen)-(bipy), both containing the Fe(bipy)(D-44′Me$_2$Phen) moiety.

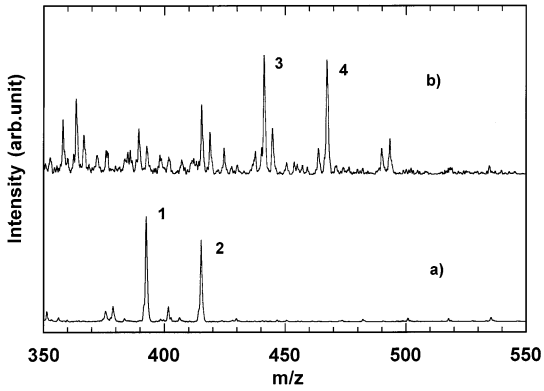

Figure 3. ESI-MS spectra of 1 mM complex **1** (a) and 1:1 mixtures of **1**/44′Me$_2$phen (b). See text for the attribution of peaks 1−4.

ESI-MS Study. The ligand substitution reaction could also be studied by mass spectrometry in positive mode in order to determine the metal speciation as a function of the number of equivalents of 44′Me$_2$Phen added to a water solution of complex **1**.[34] The ESI-MS spectrum of complex **1** is dominated by two fragments at *m*/*z* 393 (100) (noted 1 in Figure 3a) and 415.5 (80) (noted 2) attributed to $[Fe_2O(bipy)_4(OH)_2]^{2+}$ and $[Fe_2O(bipy)_4(OH)(NO_3)]^{2+}$, respectively, in agreement with theoretical isotopic patterns (Figure 3). An MS/MS experiment on the peak at *m*/*z* 415.5 showed the appearance of the *m*/*z* 337.5 peak assigned to the fragment $[Fe_2O(bipy)_4(OH)(NO_3)\text{-bipy}]^{2+}$ whereas the same experiment on the peak at *m*/*z* 393 afforded the fragment $[Fe_2O(bipy)_4(OH)_2\text{ -bipy}]^{2+}$.

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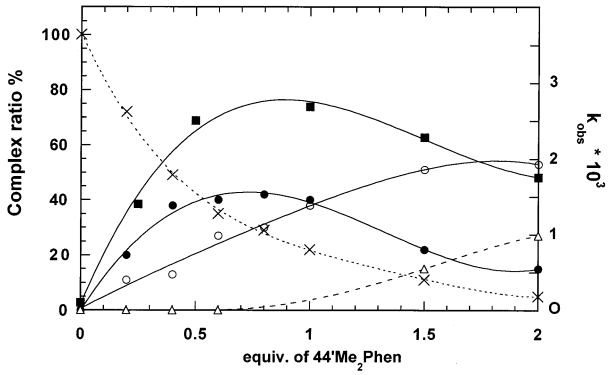

Figure 4. Complex distribution as estimated by ESI-MS in positive mode as a function of the number of 44′Me_2phen equivalents: (×) complex **1**; (●) $[Fe_2O(bipy)_3(44'Me_2phen)(NO_3)_4]$; (○) $[Fe_2O(bipy)_2(44'Me_2phen)_2(NO_3)_4]$; (△) $[Fe_2O(bipy)(44'Me_2phen)_3(NO_3)_4]$; (■) k_{obs} measured under standard conditions (see main text).

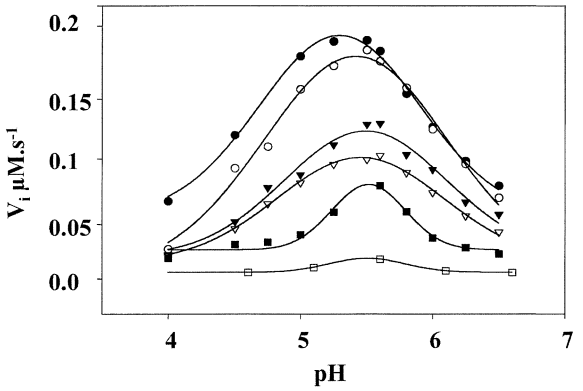

Figure 5. Initial rates of DNP formation at 50 °C as a function of pH for 1:1 (□) **1**/L2 mixtures. [L2 species are identified as the following: (■) = Phen; (▽) = 5MePhen(6); (▼) = 4MePhen, (○) = 44′Me2Phen, (●) = 33′44′Phen.] [**1**] and [BDNPP] = 67 μM.

When 1 equiv of 44′Me_2phen was added to the solution of **1** (Figure 3b), the resulting spectrum in the *m*/*z* 350−550 range became complex with an increased number of fragments, dominated by those corresponding to $[Fe_2O(bipy)_{4-n}(44'Me_2phen)_n(OH)(NO_3)]^{2+}$ and $[Fe_2O(bipy)_{4-n}(44'Me_2phen)_n(OH)_2]^{2+}$ ions. The major peaks at *m*/*z* 441 (100%) (noted 3 in Figure 3) and 467.5 (90) (noted 4) are assigned to $[Fe_2O(bipy)_3(44'Me_2phen)(OH)(NO_3)]^{2+}$ and $[Fe_2O(bipy)_2(44'Me_2phen)_2(OH)(NO_3)]^{2+}$, respectively, and the minor ones at *m*/*z* 419(20) and 445(25), to $[Fe_2O(bipy)_{4-n}(44'Me_2phen)_n(OH)_2]^{2+}$ fragments (n = 1 or 2). It has to be noted that the fragments characteristic for complex **1** are still observable and a peak started to appear at 493(10%) corresponding to traces of $[Fe_2O(bipy)(44'Me_2phen)_3(OH)(NO_3)]^{2+}$. The fragments present under *m*/*z* 400 are the results of the fragmentation of the described peaks in which 1 or 2 bipy molecules have been lost.

This simple analysis indicated that the addition of 1 equiv of 44′Me_2phen to complex **1** led to the partial conversion of complex **1** mainly to $[Fe_2O(bipy)_3(44'Me_2phen)(H_2O)_2](NO_3)_3$ and $[Fe_2O(bipy)_2(44'Me_2phen)_2(OH_2)_2](NO_3)_4$ (Figure 3), in equilibrium with their conjugated base.

Mass spectrometry was thus used to evaluate the relative proportion of the various complexes during titration of complex **1** with increased equivalents of 44′Me_2Phen (see Experimental Section for calculations). The results are reported in Figure 4. They show that 2 equiv of 44′Me_2Phen are required to convert complex **1** almost completely. During titration (below 1 equiv), $[Fe_2O(bipy)_3(44'Me_2phen)(H_2O)_2](NO_3)_4$ and $[Fe_2O(bipy)_2(44'Me_2phen)_2(OH_2)_2](NO_3)_4$ are formed with the former being the major species. Then, in the range 1−2 equiv, $[Fe_2O(bipy)_3(44'Me_2phen)(H_2O)_2](NO_3)_4$ is converted to $[Fe_2O(bipy)_2(44'Me_2phen)_2(OH_2)_2](NO_3)_4$ which continues to accumulate. $[Fe_2O(bipy)(44'Me_2phen)_3(OH_2)_2](NO_3)_4$ starts to form significantly only after addition of 1.5 equiv of 44′Me_2Phen.

In conclusion, spectroscopic data show that during the addition of 0−1 equiv of 44′Me_2Phen to complex **1**, (i) the μ-oxo-diiron core is maintained, while bipy is substituted by the added dinitrogen molecule, and (ii) a substitution occurs resulting in the formation of a mixture of $[(bipy)(44'Me_2Phen)Fe-O-Fe(bipy)_2]^{4+}$ and $[(bipy)(44'Me_2Phen)Fe-O-Fe(bipy)(44'Me_2Phen)]^{4+}$, the former being the major species. The complex $[(44'Me_2Phen)_2Fe-O-Fe(bipy)_2]^{4+}$ is excluded on the basis of ^2H NMR experiments.

Hydrolysis of BDNPP Catalyzed by Compound 1/*n*44′Me_2phen Mixtures. All standard reactions were performed at 50 °C with 67 μM initial concentrations for both complex **1** and BDNPP at pH 5.6 MES buffered solutions. Total conversion of BDNPP to DNP (Scheme 1), monitored spectrophotometrically, was achieved after 1 h of reaction, under optimal conditions. The final product of the reaction was found to be inorganic phosphate on the basis of ^{31}P NMR and is relevant to the yield of the reaction as 2 equiv of DNP are released per equivalent of BDNPP.

Complex **1** alone promoted the release of DNP by a pH-dependent reaction. The initial rate value was 9×10^{-3} μM s^{-1} at the optimal pH value of 5.2 (Figure 5). The initial rate was linearly dependent on BDNPP concentration up to 5 mM with no observable saturation behavior, probably as a consequence of weak substrate binding (see later). It has to be noted that the rate was at least 1 order of magnitude higher than that for the uncatalyzed reaction.

Addition of 1 equiv of all substituted phenanthrolines to a solution of complex **1** caused a great acceleration of the phosphoesterase activity at pH = 5.6 whereas the addition of bipy was ineffective. Figure 5 compares the effects of the addition of 2 equiv of various L2 ligands as a function of pH. It is shown that the optimal pH is not dramatically affected and that the rate acceleration is in the following order: 33′44′Me_4Phen > 44′Me_2Phen > 4MePhen > 5MePhen > phen. This result showed that the more basic the ligand, the more efficient the catalyst, with the highest initial rate with 33′44′Me_4Phen being 0.19 μM s^{-1} at pH 5.6.

In the case of 44′Me_2Phen, illustrating the general case, the addition of a second equivalent of the dinitrogen ligand 44′Me_2Phen caused a noticeable decrease of the reaction rate from 0.18 to 0.12 μM s^{-1} with no significant change of the optimal pH value (Figure 6). The decrease of the reaction rate was more pronounced, and the optimal pH shifted to higher values with the addition of 3 or 4 equiv of L2 (1.6 ×

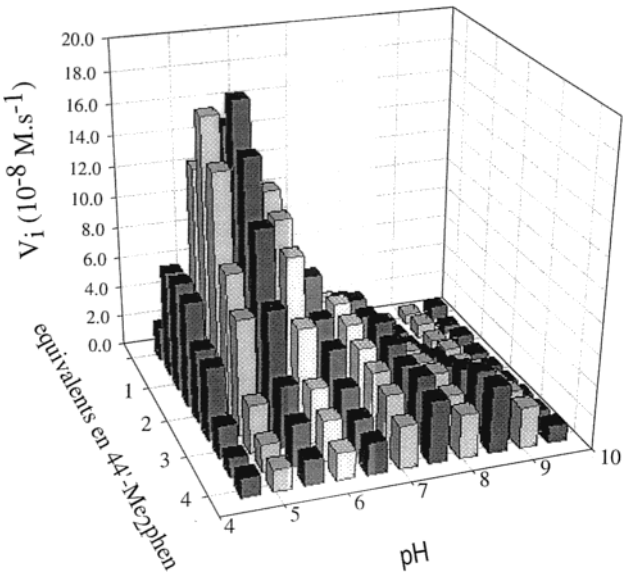

Figure 6. Initial rate evolution as a function of pH and number of 44′Me_2Phen equivalents. [**1**] and [BDNPP] = 67 μM; T = 50 °C.

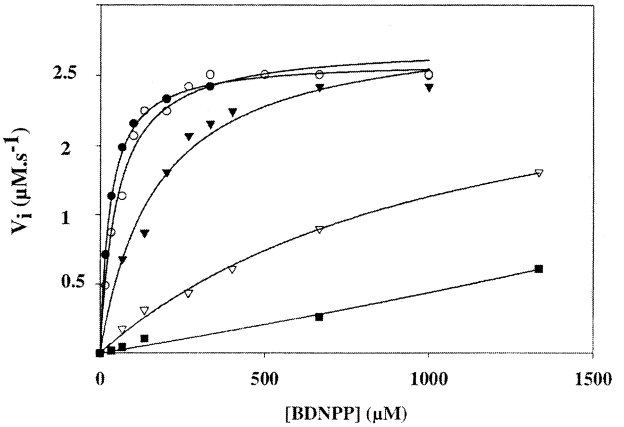

Figure 7. Initial rates as a function of [BDNPP] for 1:1 (■) **1**/L mixtures. [L2 species are identified as the following: (▽) = Phen; (▼) = 4MePhen, (○) = 44′Me2Phen, (●) = 33′44′Phen.] [**1**] = 67 μM at 50 °C; pH = 5.6.

10^{-2} μM s^{-1}, pH 7.3, and 1.0 × 10^{-2} μM s^{-1}, pH 8.5, respectively) (Figure 6). The latter values obtained were comparable to those obtained for the $[Fe_2O(4,4'Me_2phen)_4(OH_2)_2](NO_3)_4$ complex prepared independently. The optimal activity was thus obtained for the addition of 1 equiv of phenanthroline to complex **1**. This is also shown in Figure 4, with the remarkable correlation between activity and the proportion of $[Fe_2O(bipy)_3(4,4'Me_2phen)(OH)(OH_2)]^{3+}$ complex present in solution. Altogether, our data strongly support the notion that this monosubstituted complex displays the highest activity.

Kinetic Study. In Figure 7, it is shown that in the case of a mixture of complex **1** and L2 in a 1:1 ratio the initial reaction rate [L2 = Phen, 4MePhen, 44′Me_2Phen, 44′33′Me_4Phen] increased as the concentration of BDNPP increased but reached a plateau at high substrate concentration. The observed saturation kinetics and the fact that the initial rates followed typical Michaelis−Menten kinetics, with double reciprocal plots (see Supporting Information) of the results giving a straight line, likely implies the binding of the phosphodiester to the active species during the reaction.

Table 1. Kinetic Parameters for BDNPP Hydrolysis Catalyzed by Diiron Complexes

complex or 1/*n*L mixtures	pK_{a1} L (ref 40)	K_D (μM)	V_{sat} (M 10^{-7} s^{-1}) (pH max)
	1 + 1 equiv L		
1		*a*	
1/Phen	4.9	1090 ± 150	2.37(0.5) (5.6)
1/4MePhen		165 ± 30	2.37(0.7) (5.6)
1/44′Me_2Phen	5.95	50 ± 5	2.21(0.45) (5.6)
1/33′44′Me_4Phen	6.4	30 ± 1	2.12(0.15) (5.6)
	1 + *n* equiv of L		
1/2 44′Me_2Phen		50 ± 5	1.78 (5.6)
1/3 44′Me_2Phen		300 ± 25	3 (7.3)
	$[Fe_2O(L)_4(H_2O)_2]^{4+}$		
$Fe_2O(Phen)_4$ **(2)**		667 ± 50[b]	0.86 (6.0)
$Fe_2O(4MePhen)_4$		380 ± 40	2.9 (7.0)
$Fe_2O(44'Me_2Phen)_4$		410 ± 40	0.96 (9)

[a] Not measured. [b] This value was found one order of magnitude higher than than in ref 25, and this is attributed to different reaction conditions.

Then, we could interpret each kinetic curve using a simple model adapted from the Michaelis−Menten formalism (because we are not under catalytic conditions) shown here:

$$\text{complex} + \text{BDNPP} \overset{K_D}{\rightleftharpoons} \text{complex·BDNPP} \xrightarrow{V_{sat}} \text{complex} + \text{products.}$$

where K_D referred to the concentration of the substrate required to reach half of V_{sat} and V_{sat} the maximal velocity.

Then, K_D values for the substrate extracted from the experimental results were found to be highly dependent on the nature of the added dinitrogen ligand (Table 1), ranging from 1090 to 30 μM as the basicity of the added ligand increased (bipy ≫ phen > 4MePhen > 44′Me_2Phen > 44′33′Me_4Phen). On the other hand, V_{sat} was clearly found independent of the nature of the ligand under standard conditions (Table 1).

The K_D value was also found to increase as a function of an increased number of equivalents of a given L2 ligand as shown, for example, from the kinetic parameters measured for the 1:2 and 1:3 **1**/44′Me_2Phen mixtures and $[Fe_2O(44'Me_2phen)_4(H_2O)_2](NO_3)_4$ (Table 1).

Evaluation of the k_{obs} Maximal Value. The maximal k_{obs} value corresponds to the maximal velocity constant obtained using an excess of catalysts with regard to the substrate and has been used to compare our system with the ones reported in the litterature. The maximal value that can be measured was determined with a concentration of complex of 0.67 mM (A 10-fold excess with respect to the substrate), because at higher concentrations, the absorption of the catalyst interfered with our colorimetric assay. We showed that the more efficient system was the 1:1 **1**/44′Me_2Phen mixture with a k_{obs} value of 0.25 s^{-1} and that for a given L ligand this k_{obs} value was much larger than that for complex **1** and the related tetrasubstituted Fe_2OL_4 complexes. This corresponds to a 3 × 10^6 acceleration of the uncatalyzed reaction (k_{obs} = 6.7 × 10^{-7} s^{-1}),[35] in the range of the best systems exemplified by Co(III),[36] Ce(IV),[37] or dinuclear Ni[38] complexes reported so

(35) Chin, J.; Zou, X. *J. Am. Chem. Soc.* **1988**, *110*, 223.
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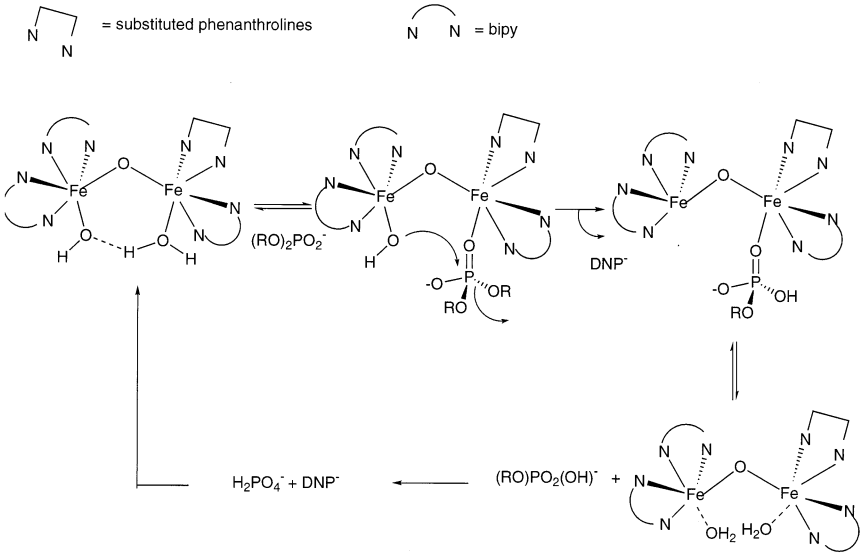
Scheme 2 Proposed Mechanism for Hydrolysis of Phosphodiesters at pH 5.6 by $[Fe_2O(bipy)_3(L2)(H_2O)_2](NO_3)_4$



far. However, under catalytic conditions, for example with a BDNPP/complex in 10:1 excess, no more than one turnover could be achieved.

Discussion

To design new artificial nucleases and elucidate their reaction mechanism, our laboratory has evaluated the reactivities of various diiron complexes and found that they have the potential to catalyze hydrolysis of phosphodiesters.[25] In this study, we have investigated diferric complexes in which the ferric ions are differently coordinated.

Synthesis of binuclear complexes containing distinct iron sites is not trivial, and we wanted to avoid the time-consuming and complex synthesis of such species.[23] We therefore took advantage of the easy substitution of the 2,2′-bipyridine in complex **1** to generate a variety of complexes in water. By controlled ligand substitution, we could compare the reactivity of a number of different complexes in which the Fe_2O core was maintained but the coordination spheres of each iron were gradually modified. By the simple screening method, using the BDNPP hydrolysis reaction in water at a given pH, we found that increased activities were obtained when bipy was replaced by more electron-donating bidentate ligands such as substituted phenanthrolines.

At a fixed pH, we observed that monosubstitution by substituted phenanthrolines (L2) ($[Fe_2O(bipy)_3(L2)(OH_2)_2](NO_3)_4$) resulted in an increased BDNPP hydrolysis activity. For example, when L2 was 44′Me_2Phen, the activity was 20-fold larger than that of complex **1**. It is important to note that even though the addition of 1 equiv of 44′Me_2Phen resulted in a mixture of complexes in solution as shown by NMR and mass spectrometry, the activity correlated well only with the concentration of $[Fe_2O(bipy)_3(44'Me_2Phen)(OH)(OH_2)](NO_3)_3$, suggesting that this single species was responsible for the rate acceleration (Figure 4). It is remarkable that this complex is more efficient not only than complex **1** but also than the tetrasubstituted species $[Fe_2O(44'Me_2Phen)_4(H_2O)(OH)](NO_3)_4$, even at its optimal pH. This strongly suggests that the acceleration cannot only be explained by the increase of the basicity of the ligands and points to the importance of an asymmetric complex with two different metal sites.

It has been previously shown that the pH dependence in this class of complexes is linked to the fact that the diferric complex exists in three different forms, $Fe(OH_2)$-$Fe(OH_2)$, $Fe(OH_2)$-Fe(OH), and Fe(OH)-Fe(OH).[25] Only the second one carrying both nucleophilic hydroxide and an exchangeable site displays activity. The first one can bind the substrate, but the iron aquo ligand is not a reactive enough nucleophile whereas the third one cannot bind the substrate, because the hydroxo ligand is much less exchangeable than the aquo ligand. The pH optimum for BDNPP hydrolysis slightly varies with the basicity of the dinitrogen ligands. It is thus not surprising, for example, that it is increased in $[Fe_2O(44'Me_2Phen)_4(H_2O)_2](NO_3)_4$ with respect to complex **1**. On the other hand, no significant variations of the pH optimum upon substitution of only 1 bipy ligand of **1** by 44′Me_2Phen could be observed, indicating that a single substitution has a moderate effect on the Lewis acidity of the corresponding ferric iron.

The most likely mechanism involves a first step during which the substrate binds to form a ternary **1**/L2-OH-substrate complex followed by a second intramolecular step during which the nucleophile OH^-, bound to one iron, attacks the phosphodiester bound to the second iron (Scheme 2). This mechanism is consistent with the fact that initial rates saturate with increasing substrate concentration.

Careful analysis of the kinetic data, in particular the concentration dependence of the initial rates of BDNPP hydrolysis (Figure 7), revealed that in fact the monosubstitution had no effect on the V_{sat} value. The reason that, at a fixed nonsaturating substrate concentration, the activity of the complex was greatly increased was most exclusively due

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(38) Yamaguchi, K.; Akagi, F.; Fujinami, S.; Suzuki, M.; Shionoya, M.; Suzuki, S. *Chem. Commun.* **2001**, 375.

to a larger affinity of the complex for BDNPP. Thus, we show here that this affinity, expressed by K_D (Table 1), increased with an increased number of methyl substituents at phenanthroline L2.

The ability for the phosphodiester to bind to one iron atom of complex **1** depends on the exchangeability of the aquo ligand and thus the accessibility to the coordination site. It is likely that in the complexes studied here the aquo and hydroxo ligands interact through a hydrogen bond which stabilizes the overall structure and makes exchange of ligands more difficult. Several examples of such a hydrogen bonded species have been recently reported.[39] The introduction of substituted phenanthrolines in the coordination spheres might result in the weakening of this interaction because of both steric and electronic effects, thus favoring the substitution of the aquo ligand by the phosphodiester substrate. This result points to the importance of an asymmetric complex for phosphodiester hydrolysis, in the case of bis-diaquo-diferric centers. In the metallohydrolases, this effect is maximized by the presence of either two different metal atoms or the same atom in different redox states, with very different Lewis acidity properties.

Conclusion

The method presented here, based on ligand monosubstitution, allows the generation of novel complexes for phosphodiester hydrolysis in water. It is simple and inexpensive, and it allows subtle structural and electronic variation of the complex. This work also confirms the potential of binuclear ferric complexes as robust hydrolytic catalysts which will be studied further in our laboratory in particular for DNA hydrolysis.

Supporting Information Available: Figure S1 depicting saturation curve and its reciprocal plots for **1**/44Me_2Phen extracted from Figure 7. This material is available free of charge via the Internet at http://pubs.acs.org.

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