Purple acid phosphatases (PAPs) belong to the family of binuclear metallohydrolases and catalyze the hydrolysis of a variety of phosphoester substrates within the pH range of 4-7. They are the only binuclear metallohydrolases where the necessity for a heterovalent active site (Fe\textsuperscript{III}-M\textsuperscript{II}, where M = Fe, Zn, or Mn) for catalysis has been clearly established. To date, the crystal structures of PAPs from red kidney bean (rkbPAP),\textsuperscript{2a} rat,\textsuperscript{2b} pig,\textsuperscript{2d} human,\textsuperscript{2e} and sweet potato\textsuperscript{2f} have been reported. In the structure of rkbPAP,\textsuperscript{2a} the Fe\textsuperscript{III} ion is coordinated by a tyrosine, a histidine, and an aspartate, and a Zn\textsuperscript{II} ion is coordinated by two histidines and an asparagine. The Fe\textsuperscript{III}/Zn\textsuperscript{II} ions are bridged by two oxygen atoms, one from the carboxylate group of an aspartate and the other from a modeled μ-(hydr)oxo group. Two oxygen atoms from a μ-1.3 phosphate group complete the coordination spheres of the Zn\textsuperscript{II} and Fe\textsuperscript{III} ions.

Despite the availability of detailed structural data, the catalytic mechanism of PAPs remains a matter of controversy. For rkbPAP, a mechanism in which, in the first step of the catalytic cycle, the substrate binds in a monodentate fashion to the Zn\textsuperscript{II} ion has been proposed.\textsuperscript{2a} The enzyme–substrate complex is oriented in such a way that a terminal Fe\textsuperscript{III}-bound hydroxide can efficiently attack the phosphorus atom of the substrate, leading to the release of the alcohol product.\textsuperscript{2a} The monodentate binding of the substrate to Zn\textsuperscript{II} is corroborated by the fact that the addition of phosphate to the spectroscopic properties of the Fe\textsuperscript{III} ion at the pH of optimal activity (pH 6.5).\textsuperscript{3} However, for pig\textsuperscript{4a} and sweet potato PAP,\textsuperscript{2f,4b} an alternative mechanism in which the substrate forms a (hydr)oxo bridge in an ideal position to act as the reaction-initiating nucleophile, has also been proposed.

Homo- and heterodinuclear Fe\textsuperscript{III}M\textsuperscript{II} complexes which are capable of reproducing the structural, spectroscopic, and functional properties of PAPs can be very informative to evaluate the mechanism(s) of reproducing the structural, spectroscopic, and functional properties of PAPs. The crystal structure of 1 reveals the presence of a terminal Fe-bound water molecule (Fe-O3 = 2.054(6) Å), which occupies a position equivalent to that of the proposed nucleophile in rkbPAP (vide supra).\textsuperscript{2a}

Potentiometric titration of 1 in water/CH\textsubscript{3}CN (50:50) showed the neutralization of 3 mol of KOH/mol of complex in the pH range of 2-10. Fitting the data with the BEST7 program (Figure S1) resulted in the following deprotonation constants: pK\textsubscript{a1} = 2.93, pK\textsubscript{a2} = 4.81, and pK\textsubscript{a3} = 8.30. These pK\textsubscript{a} values are consistent with the following equilibria: [(OH)\textsubscript{2}Fe(μ-OH)Zn(OH\textsubscript{2})] (a) ⇌ [(OH)\textsubscript{2}Fe(μ-OH)Zn(OH\textsubscript{2})] (b) ⇌ [(OH)\textsubscript{2}Fe(μ-OH)Zn(OH\textsubscript{2})] (c) ⇌ [(OH)\textsubscript{2}Fe(μ-OH)Zn(OH\textsubscript{2})] (d). Species b is the one determined by X-ray crystallography, with an additional Zn-coordinated water molecule. The solution structure of 1 was further investigated using X-ray absorption fine structure (XAFS) spectroscopy. The comparison of the 10 K solid state and (frozen) solution Fe and Zn XAFS data indicates that both metal centers are six-coordinate in solution (Figure S2). Importantly, a Fe...Zn distance of ~3.04 Å is found, in full agreement with the X-ray structure of 1 (see above).
and indicating the retention of the μ-OH bridge in solution. This hypothesis is supported by the lack of intensity change in the Fe pre-edge features in going from solid to solution, indicating that the centrosymmetric environment is maintained.6

The phosphatase-like activity of 1 was determined using the activated substrate 2,4-bis(dinitrophenyl)phosphate (BDNPP) by following spectrophotometrically the absorbance increase of the liberated 2,4-dinitrophenolate anion (λ_{max} = 400 nm), under conditions of excess substrate. The ability of 1 to cleave BDNPP is strongly influenced by the pH of the reaction mixture with a bell-shaped pH rate profile (Figure S3) and an optimum at pH 6.5, which is similar to that found for rkbPAP.21 Sigmoidal fits reveal pK_a values of 5.3 and 8.1. which are in good agreement with the pK_a2 = 4.81 and pK_a3 = 8.30 obtained from the potentiometric titration experiments, demonstrating that the catalytically active species is of the type [(OH)Fe(μ-OH)Zn(OH_2)]. The pK_a value of 5.3 obtained from the acid limb of the V_0 versus pH profile is due to the protonation equilibrium in the catalyst–substrate complex, while the corresponding pK_a2 of 4.81 is associated with the free catalytic enzyme.

The determination of the initial rates at pH 6.5 as a function of substrate concentration reveals saturation kinetics with Michaelis–Menten-like behavior (Figure S4). The kinetic parameters (k_cat = 9.13 \times 10^{-4} s^{-1} and K_M = 4.20 \times 10^{-3} mol L^{-1}) were obtained after Lineweaver–Burk linearization of the initial rates (V_0 vs [BDNPP]; Figure S4). Compared to the uncatalyzed reaction 1 accelerates the turnover rate 4.8 \times 10^3-fold. Furthermore, the measured kinetic isotope effect of kH/kD = 1.34 (Figure S5) suggests that no proton transfer is involved in the rate-limiting step and thus supports an intramolecular nucleophilic attack by an Fe^III-bound hydroxide.

In order to elucidate the mode of interaction between BDNPP and the dinuclear catalytic center in 1, we followed the spectral change of the reaction mixture at the optimum pH over a period of 24 h in the presence of excess substrate (Figure S6). The absorption maximum (λ_{max} = 486 nm) and the intensity of the phenolate–Fe^III charge-transfer band are only slightly affected, thus strongly suggesting monodentate binding of the substrate to the Zn^II. This hypothesis is strongly supported by EPR spectroscopic data of 1, measured in the absence and presence of BDNPP (Figure S7), which indicate that the substrate does not interact with the Fe^III center. On the other hand, the absorption at 400 nm continues to increase (Figure S6) due to the formation of the product 2,4-dinitrophenolate, with 182 turnovers in 24 h. Interestingly, after 2 weeks at room temperature, a crystal suitable for X-ray diffraction was formed in this solution. The crystal structure (Figure S8) of this complex (2) reveals a tetranuclear cation in which two dinuclear [LFe^III/Zn^II] structural units without the OH^- bridge are linked through a phosphate anion. The Zn^II is pentacoordinated, while the hexacoordination around Fe^III is completed by a 2,4-dinitrophenolate anion. Therefore, a second sequential reaction must be taken into account in which the product of the first reaction cycle, the 2,4-dinitrophenylphosphate (DNPP) monoester, is further hydrolyzed.

We tested the activity of 1 for the hydrolysis of the monoester DNPP directly with an excess of substrate, and over a period of ~5 h, only the background reaction was observed. After this time, BDNPP was added to the reaction mixture and immediately the absorbance at 400 nm started to increase (Figure S9), indicating the recovery of the catalyst and hydrolysis of the diester substrate. The coordination of DNPP bridging both metal ions is proposed based on the hypsochromic shift observed after ligation of DNPP to 1. Therefore, we may conclude that the μ-hydroxide is a significantly poorer nucleophile than the terminally Fe^III-bound OH^- group, and that hydrolysis of the monoester to form inorganic phosphate and 2,4-dinitrophenolate occurs exclusively due to the background reaction.

In summary, the combined data support a mechanistic model whereby the [(OH)Fe(μ-OH)Zn(OH_2)] species of 1 is the catalytically relevant system (Scheme 1).

![Scheme 1](image-url)

In brief, monodentate binding of the substrate to Zn^II (K_{a}) is followed by a nucleophilic attack by the terminal, Fe^III-bound hydroxide and the concomitant release of 2,4-dinitrophenolate. The μ-1,3-coordinated DNPP intermediate undergoes substitution by two water molecules and regenerates the active site for the next catalytic cycle. Additionally, in a significantly slower reaction, some of the complex dimerizes to the stable tetranuclear end product (inset in Scheme 1). Further studies involving the synthesis of substituted phenolate–R (R = Me, NO_2, Br) derivatives of 1 and their interactions with phosphodiester bonds in model substrates and DNA are underway and will be the subject of future reports.

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Supporting Information Available: Crystallographic data of 1 and 2 have been deposited at the Cambridge Structural Database CCDC 637120 and CCDC 637121. Synthesis and characterization for 1 and Figures S1–S9 in PDF format. This material is available free of charge via the Internet at http://pubs.acs.org.

References