Asp by a neutral residue destabilizes the transition state by approximately 4–6 kcal/mol.[65] Experimentally, one hydrogen bond of the oxyanion hole contributes about 5 kcal/mol to stabilization of the tetrahedral intermediate.[66]

Summary

We present both semiempirical molecular orbital and molecular mechanical calculations to study the serine protease catalyzed hydrolysis of amides and esters and the effect of the protein environment and dynamics on this process. The lowest energy pathway for formation of the tetrahedral intermediate was for serine to approach the substrate, followed by coupled heavy atom movement and protein transfer to complete the reaction. The reaction pathway calculations were performed with a truncated active site model. We do not mean to suggest with the use of such a reduced representation of the serine protease active site that the protein is unimportant in catalysis, especially in light of evidence indicating that specific side chain interactions between substrate and enzyme sites remote from the active site can alter both the rate of catalysis and the mechanism.[5] Due to system

size limitations, though, one is forced to employ truncated systems even with semiempirical methods. It is interesting, though, that the environment itself, excluding the key active site residues and interactions, was important in stabilizing the substrate and did so better in the covalent than noncovalent complex. Hence, other residues besides those that we included in the semiempirical calculations are important, but we find it gratifying that such a simple model can reproduce many aspects of serine protease catalysis. Inclusion of the protein environment and surrounding solvent in a more sophisticated way will, no doubt, be necessary for quantitative agreement with experiment, but we find these initial results encouraging and informative. Our studies suggest modes of proton transfer and nucleophilic attack that are consistent with the experimental results and, to our knowledge, novel. In the future, we hope to pursue studies combining both quantum mechanics and molecular dynamics directly to more fully account for environmental effects.

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Roles of Zinc(II) Ion in Phosphatases. A Model Study with Zinc(II)–Macrocyclic Polyamine Complexes

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Abstract: The zinc(II) complexes of 1,5,9-triazacyclododecane (L$_4$, [12]aneN$_3$) and 1,4,7,10-tetraazacyclododecane (L$_2$, [12]aneN$_4$) promote the hydrolysis of tris(4-nitrophenyl) phosphate, TNP^0 (a neutral phosphotriester), and bis(4-nitrophenyl) phosphate, BNP$^-$ (a monoanionic phosphodiester). Kinetic studies show that the reactive species are commonly L–Zn^{II}–OH$^-$ and the kinetically obtained pK_a values are almost identical to those obtained thermodynamically (pK_a(H$_2$O)). The comparative reactivities of OH$^-$, L$_4$–Zn^{II}–OH$^-$, and L$_2$–Zn^{II}–OH$^-$ with different pK_a(H$_2$O) values of 15.7, 7.3, and 8.0 have been studied to understand the role of Zn^{II} in alkaline phosphatases. For the neutral phosphotriester TNP^0, the second-order rate constants (M^{-1} s^{-1}) at 25 °C and $I = 0.20$ are 10.7, 7.0, and 3.7, respectively, indicating free OH$^-$ ion (the most basic OH$^-$ species) is the best promoter. On the other hand, toward the anionic phosphodiester BNP$^-$, the L$_4$–Zn^{II}–OH$^-$ becomes more efficient than free OH$^-$ ion: The second-order rate constants ($\times 10^5$, M^{-1} s^{-1}) are 2.4, 8.5, and 2.1, respectively, at 35 °C and $I = 0.20$. A bifunctional ("hybrid") mechanism of Zn^{II} (especially the Zn^{II}–L$_4$ system) toward phosphates (especially the anionic phosphate), in which the Zn^{II}-bound OH$^-$ acts as a nucleophile, while the vacant Zn^{II} coordination site anchors the substrate P=O or P—O$^-$ group, is well disclosed by comparing these kinetic results with the second-order rate constants (at 25 °C and $I = 0.10$) of 4-nitrophenyl acetate hydrolysis, 9.5, 4.1 $\times$ 10^{-2}, and 1.1 $\times$ 10^{-1} M^{-1} s^{-1}, respectively, where all of the OH$^-$ species act merely as nucleophiles to the carbonyl group, as indicated by a linear relationship between the pK_a(H$_2$O) values and the rate constants. Phosphate anion affinity constants K(A$^-$) (M^{-1}) with the Zn^{II}–L$_4$ complex determined by pH titration are 10$^{3.5}$ for phenyl phosphate, PP^{2-}, and 10$^{3.1}$ for 4-nitrophenyl phosphate, NP^{2-}, which are ca. 25 times larger than those with the less acidic Zn^{II}$_{aq}$ ion.

Introduction

Zinc(II) ion plays a vital role in Zn^{II}-containing phosphate esterases.[1] Although there have been numerous reports of the esterase model systems[2] using well-defined metal (e.g., Co^{III},[3] Cu^{II},[4,5] Ni^{II},[5] Zn^{II},[5,6a] La^{III},[6b] and Ir^{III} [7]) complexes, most were addressed to attaining better acceleration of the hydrolysis rates

and their applications. Only a few Zn^{II} model complexes[8,9] were studied on the reactivities in hydrolysis of neutral phosphotriesters.

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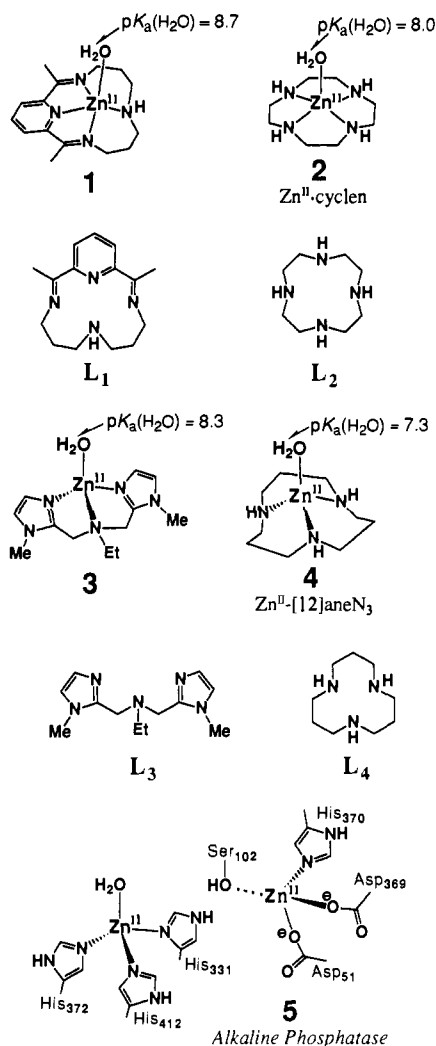[‡] Institute for Molecular Science.

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Chart I

pKa(H2O) = 8.7

pKa(H2O) = 8.0

1

2
Zn^{II}·cyclen

L$_1$

L$_2$

pKa(H2O) = 8.3

pKa(H2O) = 7.3

3

4
Zn^{II}-[12]aneN$_3$

**L$_3$**

L$_4$

5
Alkaline Phosphatase

The structurally well-defined Zn^{II} complexes thus far used in the model studies were made of four-coordinating ligands such as macrocyclic tetraamines L$_1$ for 1[8] and L$_2$ (cyclen) for 2[9] (Chart I). Probably, only these rigid, four-coordinate ligands can keep holding Zn^{II} firmly up to alkaline conditions. Both of these H$_2$O-bound Zn^{II} complexes succeeded in generating L-Zn^{II}-OH$^-$ species with pKa(H$_2$O) values of 8.7[10] and 8.0,[11] respectively. The

variation of the observed rate constants for hydrolysis of neutral phosphotriesters with pH implicated the L-Zn^{II}-OH$^-$ complexes as active species in the mechanistic scheme. Although these models drew intriguing and convincing pictures about the essential role of the Zn^{II} ion, the fatal drawbacks were these Zn^{II} ions being five-coordinate against the four-coordinate Zn^{II} system in alkaline phosphatase (see 5).[12] Furthermore, in alkaline phosphatases that are well studied,[1,12] two Zn^{II} ions in different ligand fields seem to work cooperatively. The role of each Zn^{II} is not clarified.

In 1989, a Zn^{II} complex (3) with three-coordinate ligand L$_3$ was introduced as an alkaline phosphatase model,[13] where the L$_3$-Zn^{II}-OH$^-$ species with a pKa(H$_2$O) value of 8.3 is also an active form in hydrolysis of a phosphotriester. Although the kinetic behavior of 3 was similar to those previously shown by 1 and 2, the thermodynamic and the structural studies still remained to be defined, the reason probably being the labile nature of the linear N$_3$ ligand. Thus, it seemed more appropriate to construct a phosphatase model with a more rigid N$_3$ ligand.

Recently, we discovered that a Zn^{II} complex (4) with a tridentate ligand, 1,5,9-triazacyclododecane (L$_4$, [12]aneN$_3$), is structurally as well as chemically the best model for carbonic anhydrase (CA).[11,14] This complex generates the first well-characterized, crystalline Zn^{II}-OH$^-$ species from 4 having a pKa(H$_2$O) value of 7.3, which is almost the same as that for CA. Moreover, 4 (as OH$^-$ species) catalyzes the hydration of acetaldehyde and hydrolyses of carboxylic esters. We thus thought that 4 would make a good catalyst for phosphate hydrolysis. A remarkable advantage of 4 over 1 and 2 is that it is coordinatively less saturated and/or sterically more open for phosphate substrates, whereby the electrophilic coordination site is more available for P=O (or P—O$^-$).

In the past model studies using Zn^{II} complexes, the substrates were mostly *neutral* phosphotriesters such as diphenyl 4-nitrophenyl phosphate (DNP^0),[8] diethyl 2,4-dinitrophenyl phosphate (DDP^0),[9a] and tris(4-nitrophenyl) phosphate (TNP^0).[13] A comparative study of TNP^0 and *monoanionic* bis(4-nitrophenyl) phosphate (BNP$^-$) hydrolysis with 4 would help us more clarify the function of Zn^{II} in the phosphatase enzymes.

DNP^0

DDP^0

TNP^0

BNP·
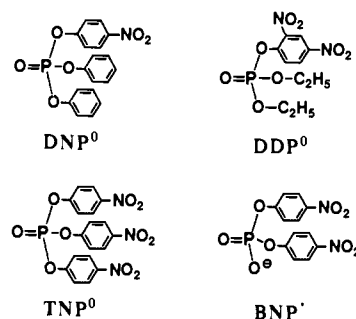
Experimental Section

General Information. Kinetic study was carried out by a UV–visible spectral method using a Hitachi U-3200 spectrophotometer equipped with a stopped flow injection attachment and a thermostated cell compartment. Proton NMR spectra were recorded on a JEOL GX·400 (400 MHz) spectrometer, and were obtained in D$_2$O, with the DSS signal being used as the internal standard at 25 °C.

Materials. All reagents and solvents used were of analytical grade. Disodium 4-nitrophenyl phosphate (Sigma), bis(4-nitrophenyl) phosphate (Nacalai), tris(4·nitrophenyl) phosphate (Nacalai), disodium phenyl phosphate (Nacalai), diphenyl phosphate (Aldrich), and Dojin Chemical buffers MES (2·(N-morpholino)ethanesulfonic acid), HEPES (N-(2-hydroxyethyl)piperazine-N'-3·propanesulfonic acid), CHES (2·(cyclo-

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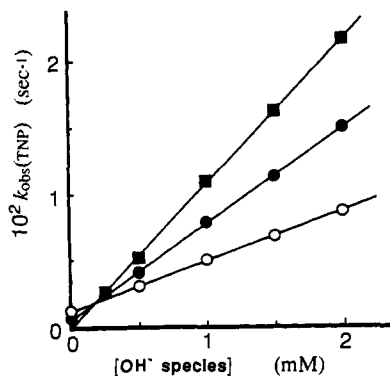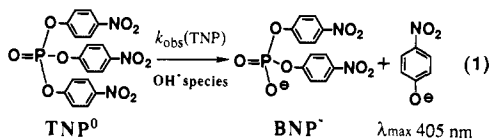(14) Kimura, E.; Koike, T. *Comments Inorg. Chem.* 1991, *11*, 285.

Roles of Zinc(II) Ion in Phosphatases

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Figure 1. Dependence of k_{obs}(TNP) for the hydrolysis of tris(4-nitrophenyl) phosphate on OH⁻ species concentration at 25 °C, $I = 0.20$ (NaClO₄): (■) free OH⁻ ion; (●) L₄-Zn^{II}-OH⁻ at pH 8.7 (10 mM TAPS buffer); (O) L₂-Zn^{II}-OH⁻ at pH 9.3 (10 mM CHES buffer).

hexylamino)ethanesulfonic acid), and TAPS (3-[[tris(hydroxymethyl)·methyl]amino]propanesulfonic acid) were purchased from commercial sources and used without further purification. Macrocyclic polyamines L₂ and L₄ were synthesized by the Richmann–Atkins procedure.[15] Crystalline Zn^{II} complex with L₄ ((L₄-Zn^{II}-OH⁻)₃(ClO₄)₃·HClO₄) was prepared using the same method as described in a previous paper.[11] A stock solution (50 mM) of the 1:1 Zn^{II}-L₂ complex (2) was prepared by a method similar to that developed by Norman.[9a]

Potentiometric pH Titration. The preparation of the test solutions and the calibration of the electrode system (Orion pH meter 811) were described earlier.[16] The pH value in 33% EtOH aqueous solution at 25.0 ± 0.1 °C was determined by subtracting 0.09 units from the pH meter reading (pH′) according to the method of Bates.[17] By a similar method using solutions of 1, 2, and 4 mM HCl, we determined the pH in 10% (v/v) CH₃CN aqueous solution as a value of pH′ − 0.03 at $I = 0.10$ (NaClO₄) and 25.0 ± 0.1 °C. The calculation methods for the deprotonation constant, pK_a(H₂O) of the L-Zn^{II}-OH₂, and the phosphate anion affinity constants are the same ones as described in a previous paper.[11] For the determination of these constants, at least three independent pairs of titrations were always made.

Kinetics of Tris(4-nitrophenyl) Phosphate Hydrolysis. The hydrolysis of tris(4-nitrophenyl) phosphate (TNP⁰) in 33% EtOH solution was followed by an increase in absorption maximum at 405 nm (assigned to 4-nitrophenolate anion; e.g., ε 19 000 at pH 8.7, 20 000 at pH 9.3, and 20 200 at pH 11) at 25.0 ± 0.2 °C and $I = 0.20$ (NaClO₄) (see reaction 1). The initial TNP⁰ solutions with 5, 10, and 20 μM concentrations

$$\text{TNP}^0 \xrightarrow[\text{OH}^-\text{species}]{k_{obs}(\text{TNP})} \text{BNP}^- + \text{NO}_2 \quad \lambda_{max}\ 405\ nm \quad (1)$$

were prepared from a dry THF solution containing 1.0 mM TNP⁰. Buffered reaction solutions containing 10 mM buffer (MES (pH 6–7), HEPES (pH 7–8), TAPS (pH 8–9), and CHES (pH >9)) or NaOH (0.25–2.00 mM) were used. The typical procedure is as follows: Immediately after rapid injection of 15 μL of 1.0 mM TNP⁰ in dry THF into a buffer solution (3.0 mL, 10 mM TAPS, pH 8.7) of 2.0 mM Zn^{II}-L₄ (the reference experiment did not contain the Zn^{II} complex), the absorption increase was recorded until 90% hydrolysis was completed (within 5 min); an end point was determined after $\geq 5t_{1/2}$. The succeeding hydrolysis of the produced bis(4-nitrophenyl) phosphate was too slow to be detected under these conditions (see next paragraph). This first hydrolysis showed a good first-order behavior. The pseudo-first-order rate constants, k_{obs}(TNP) (s⁻¹), were obtained by a log plot method (correlation coefficients >0.99). From the slopes of all the linear plots of k_{obs}(TNP) against [OH⁻ species] (Figure 1) were obtained the second-order rate constants k(TNP) (M⁻¹ s⁻¹). The intercepts, k_0(TNP) (s⁻¹) (at [OH⁻ species] = 0), in these plots were zero for free OH⁻, 6.1 × 10⁻⁴ for L₄-Zn^{II}-OH⁻, and 1.2 × 10⁻³ for L₂-Zn^{II}-OH⁻. The latter

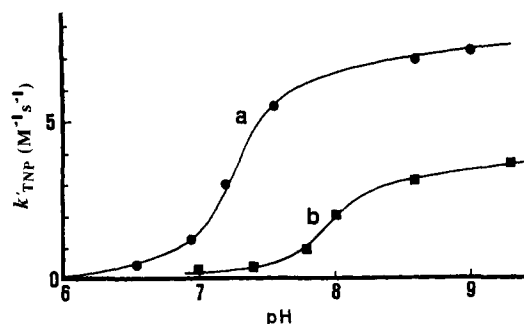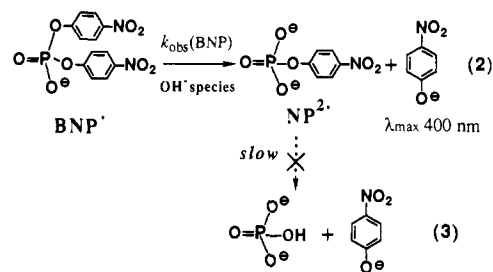**Figure 2.** pH–rate profile for the Zn^{II}-L-promoted hydrolysis of tris(4-nitrophenyl) phosphate (TNP⁰) at 25 °C and $I = 0.20$ in 33% EtOH aqueous solution: (a) 2 mM **4** (●); (b) 2 mM **2** (■).

two k_0(TNP) values are mainly due to the attack of free OH⁻ anion and the buffer ion used.

Kinetics of Bis(4-nitrophenyl) Phosphate Hydrolysis. The hydrolysis of bis(4-nitrophenyl) phosphate (BNP⁻) in aqueous solution was measured by following the increase in 400-nm absorption (e.g., ε 17 800 at pH 8.6, 18 300 at pH 9.2) of the released 4-nitrophenolate at 35.0 ± 0.2 °C and $I = 0.20$ (NaClO₄) (see reaction 2). In order to prevent com-

$$\text{BNP}^- \xrightarrow[\text{OH}^-\text{species}]{k_{obs}(\text{BNP})} \text{NP}^{2-} + \text{NO}_2 \quad \lambda_{max}\ 400\ nm \quad (2)$$

$$slow \quad \xrightarrow{} \quad + \quad \text{NO}_2 \quad (3)$$

plications due to the buffer catalysis, the desired pH was maintained by a pH·stat method with 10 mM NaOH solution. The reaction solution contained 5.0, 10.0, or 20.0 mM BNP⁻, and 0–20 mM hydroxide species. Ionic strength was maintained at 0.20 with corresponding perchlorate salts. The absorption increase giving a straight line was recorded until 1% hydrolysis of BNP⁻ (ca. 2 h at pH 8.6, [L₄-Zn^{II}-OH⁻] = 2 mM, [BNP⁻] = 10 mM). The hydrolysis products were determined by proton NMR analysis as follows: After the reaction solution produced ca. 5% 4-nitrophenolate, the solvent was evaporated. The residue was dissolved in D₂O at pD 10, and then the solution was subjected to the proton NMR analysis. The aromatic signals (δ) were assigned to those of BNP⁻ (7.38, 8.27 (J = 9.3 Hz)), 4-nitrophenyl phosphate, NP²⁻ (7.33, 8.23 (J = 9.3 Hz)), and 4-nitrophenolate (6.52, 8.46 (J = 9.5 Hz)), and the concentration ratio of [NP²⁻]/[4-nitrophenolate] was almost 1. These facts indicate that the hydrolysis of BNP⁻ to NP²⁻ occurred with practically no further hydrolysis of the first product NP²⁻ under the experimental conditions (see reaction 3).[18]

From the obtained slope ([produced 4-nitrophenolate]/time) and the concentration of BNP⁻, we determined the pseudo-first-order rate constant k_{obs}(BNP) (s⁻¹). The rates of 4-nitrophenolate production were directly proportional to each concentration of BNP⁻ and L-Zn^{II}-OH⁻ (or hydrated OH⁻ anion). We then obtained second-order rate constants k(BNP) (M⁻¹ s⁻¹) by the same method as that used for the methyl acetate hydrolysis kinetics reported in a previous paper.[11]

Kinetics of 4-Nitrophenyl Acetate Hydrolysis. The hydrolysis rate of 4-nitrophenyl acetate (NA) was measured by following the increase in 400-nm absorption of 4-nitrophenolate in 10% (v/v) CH₃CN aqueous solution. We obtained the second-order rate constants k(NA) (M⁻¹ s⁻¹) for the L₂-Zn^{II}-OH⁻ complex by the same initial slope method as that used for the L₄-Zn^{II}-OH⁻ complex,[11] except here for the use of 50 mM CHES buffer at pH 9.2 and $I = 0.10$ (NaClO₄). For the rate–pH profile determination, buffered reaction solutions containing 50 mM buffer (HEPES (pH 7.0, 7.5), TAPS (pH 8.0, 8.6), and CHES (pH 9.2, 9.5)) were used.

Results

Hydrolysis of Tris(4-nitrophenyl) Phosphate, TNP⁰. Because TNP⁰ is insoluble in pure water, we selected EtOH as a cosolvent.

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Table I. Second-Order Rate Constants, k(TNP) (M^{-1} s^{-1}), for Hydrolysis of Neutral Tris(4-nitrophenyl) Phosphate (TNP^0) in 33% EtOH Aqueous Solution at I = 0.20 and 25 °C

nucleophile	k(TNP)	pK_a(H$_2$O)
OH$^-$	10.7 ± 0.2^a	15.7^e
L$_4$-Zn^{II}-OH$^-$	7.0 ± 0.2^b	7.2 ± 0.1^f
L$_2$-Zn^{II}-OH$^-$	3.7 ± 0.2^c	7.9 ± 0.1^f
L$_3$-Zn^{II}-OH$^-$	3.7^d	8.3^d

^{a} Based on pseudo-first-order rate constants with 0.25, 0.50, 1.0, 1.5, and 2.0 mM NaOH. ^{b} Based on pseudo-first-order rate constants with 0, 0.50, 1.0, 1.5, and 2.0 mM **4** at pH 8.7 (10 mM TAPS buffer). ^{c} Based on pseudo-first-order rate constants for 0, 0.50, 1.0, 1.5, and 2.0 mM **2** at pH 9.3 (10 mM CHES buffer). ^{d} From ref 13. ^{e} A calculated value by using log K_w (=-14.0). ^{f} Determined by potentiometric pH titration in 33% EtOH aqueous solution at 25 °C and I = 0.20 (NaClO$_4$).
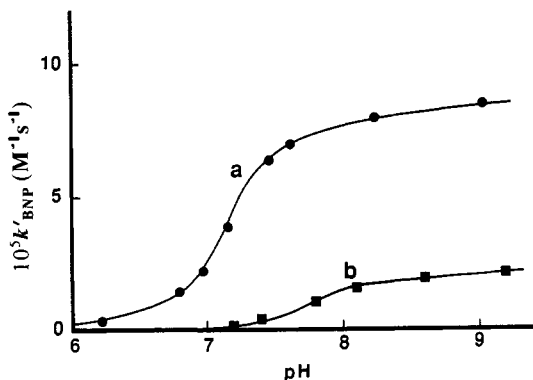
Figure 3. pH–rate profile for the Zn^{II}–L-promoted hydrolysis of bis(4-nitrophenyl) phosphate (BNP$^-$) at 35 °C and I = 0.20 (NaClO$_4$): (a) 5 mM **4** (●); (b) 5 mM **2** (■).

In 33% EtOH aqueous solution, thermodynamic deprotonation constants of Zn^{II}-bound H$_2$O (pK_a(H$_2$O)), 7.2 ± 0.1 for L$_4$-Zn^{II}-OH$_2$ (**4**) and 7.9 ± 0.1 for L$_2$-Zn^{II}-OH$_2$ (**2**), were determined by potentiometric pH titration at I = 0.20 (NaClO$_4$) and 25 °C, which are almost the same as the reported values (7.3 and 8.0, respectively) in 100% aqueous solution.[11]

The Zn^{II} complexes **4** and **2** promote the hydrolysis of TNP^0 to produce an equimolar amount of BNP$^-$ and 4-nitrophenolate in 33% EtOH aqueous solution. The pH dependence of the TNP^0 hydrolysis with **4** over the pH range 6.5–9.1 (six different points) is shown in Figure 2a, where apparent second-order rate constants, k'(TNP), were derived as (k_{obs}(TNP) – k_0(TNP))/[total Zn^{II} complex]. A value of k_0(TNP) is a conditional first-order rate constant for the hydrolytic processes mediated only by buffer and OH$^-$ ions. Figure 2a reveals a point of inflection at pH 7.3, almost the same as the pK_a(H$_2$O), and hence, we concluded that the L$_4$-Zn^{II}-OH$^-$ species must play a critical role in this reaction. The same pH–rate profile for **2** in the pH range 7.0–9.3 (six different points) gave an inflection point at pH 7.9, identical with the pK_a(H$_2$O) value of **2** (see Figure 2b). In all the hydrolyses, the active species are Zn^{II}-bound hydroxides (or kinetic equivalents), as reported in acetaldehyde hydration catalyzed by L$_4$-Zn^{II}-OH$^-$,[11] and in phosphotriester hydrolysis by L$_1$-Zn^{II}-OH$^-$,[8] and L$_3$-Zn^{II}-OH$^-$.[13]

Free OH$^-$ ion, L$_4$-Zn^{II}-OH$^-$, and L$_2$-Zn^{II}-OH$^-$ species all commonly promote the TNP^0 hydrolysis with first order in each component. The resulting second-order rate constants, k(TNP), at 25 °C and I = 0.20 are 10.7 ± 0.2, 7.0 ± 0.2, and 3.7 ± 0.2 (M^{-1} s^{-1}), respectively (see Table I). Toward the neutral substrate TNP^0, the free OH$^-$ ion is the best promoter, but its hydrolysis efficiency is not far superior to those for the L–Zn^{II}-OH$^-$ species. For comparison, see the reported k(TNP) value with the L$_3$-Zn^{II}-OH$^-$ complex under the same condition in Table I.

Hydrolysis of Bis(4-nitrophenyl) Phosphate, BNP^0. In aqueous solution, free OH$^-$ ion, L$_4$-Zn^{II}-OH$^-$ and L$_2$-Zn^{II}-OH$^-$ promote the hydrolysis of BNP$^-$ with first order in each component to produce NP^{2-} and 4-nitrophenolate at I = 0.20 (NaClO$_4$) and

Table II. Comparison of the Second-Order Rate Constants, k(BNP) (M^{-1} s^{-1}), for Hydrolysis of Bis(4-nitrophenyl) Phosphate (BNP$^-$) in the Absence and Presence of Cations at I = 0.20 and 35 °C

nucleophile	cation added	10^5 k(BNP)
L$_4$-Zn^{II}-OH^{-a}	0.20 M Na$^+$	8.5 ± 0.2^c
	0.05 M Na$^+$, 0.05 M Mg^{2+}	12.7 ± 0.3^c
	0.05 M Na$^+$, 0.05 M Ca^{2+}	11.4 ± 0.3^c
	0.10 M Na$^+$, 0.10 M (CH$_3$)$_4$N$^+$	7.3 ± 0.2^c
OH$^-$	0.20 M Na$^+$	2.4 ± 0.1^d
L$_2$-Zn^{II}-OH^{-b}	0.20 M Na$^+$	2.1 ± 0.2^e

^{a} pK_a(H$_2$O) value determined by potentiometric pH titration is 7.06 ± 0.03 at I = 0.10 (NaClO$_4$) and 35 °C. ^{b} pK_a(H$_2$O) value determined by potentiometric pH titration is 7.71 ± 0.03 at I = 0.10 (NaClO$_4$) and 35 °C. ^{c} Based on pseudo-first-order rate constants for 1.0, 2.0, 5.0, and 10 mM **4** at pH 8.6. ^{d} Based on pseudo-first-order rate constants for 2.0, 5.0, 10, 15, and 20 mM NaOH. ^{e} Based on pseudo-first-order rate constants for 5.0, 10, and 20 mM **2** at pH 9.2.

Table III. Second-Order Rate Constants, k(NP) (M^{-1} s^{-1}), for Hydrolysis of 4-Nitrophenyl Acetate at 25 °C

nucleophile	k(NP)	pK_a(H$_2$O)
OH$^-$	9.5^a	15.7
L$_2$-Zn^{II}-OH$^-$	$(1.1 \pm 0.2) \times 10^{-1 b}$	7.90 ± 0.05^d
L$_4$-Zn^{II}-OH$^-$	$4.1 \times 10^{-2 c}$	7.20 ± 0.05^d

^{a} From ref 19. ^{b} This work at pH 9.2 (50 mM CHES buffer and 10% (v/v) CH$_3$CN) and I = 0.10 (NaClO$_4$). ^{c} From ref 11 at 25 °C and I = 0.10 (NaClO$_4$). ^{d} Determined by potentiometric pH titration in 10% (v/v) CH$_3$CN aqueous solution at 25 °C and I = 0.10 (NaClO$_4$).

35 °C. A proton NMR study of the hydrolysis product indicates that the subsequent hydrolysis of NP^{2-} is much slower than that of BNP$^-$ under the experimental conditions (see Experimental Section).

Here again, plots of the apparent second-order rate constants k'(BNP) for the Zn^{II}-OH$^-$ complexes vs pH gave two sigmoidal curves like the TNP hydrolysis cases (Figure 3). Those midpoint pH values (7.2 and 7.8) correspond almost the same as the pK_a(H$_2$O) values of 7.06 ± 0.03 for **4** and 7.71 ± 0.03 for **2**, which were determined by potentiometric pH titrations at I = 0.20 (NaClO$_4$) and 35 °C. Like in the TNP^0 hydrolysis, L–Zn^{II}-OH$^-$ species must play a critical role in the BNP$^-$ hydrolysis.

The obtained second-order rate constants k(BNP) are far smaller than k(TNP) (M^{-1} s^{-1}), (8.5 ± 0.2) × 10^{-5} with L$_4$-Zn^{II}-OH$^-$, (2.4 ± 0.2) × 10^{-5} with free OH$^-$ ion, and (2.1 ± 0.2) × 10^{-5} with L$_2$-Zn^{II}-OH$^-$ (see Table II). Toward the anionic phosphate substrate BNP$^-$, L$_4$-Zn^{II}-OH$^-$ is more reactive than free OH$^-$ ion or L$_2$-Zn^{II}-OH$^-$. In order to determine the external cation effect on the hydrolysis efficiency, we ran a kinetic study in the presence of Mg^{2+}, Ca^{2+}, or N(CH$_3$)$_4^+$ ions in the place of Na$^+$ ion. The obtained conditional k(BNP) values are listed in Table II.

Hydrolysis of 4-Nitrophenyl Acetate Ester. The pK_a(H$_2$O) value for **2** was determined by potentiometric pH titration in 10% (v/v) CH$_3$CN aqueous solution at 25 °C and I = 0.10 (NaClO$_4$). The obtained pK_a(H$_2$O) value of 7.90 ± 0.05 is almost equal to the reported value (8.02) in 100% aqueous solution.[11]

L$_2$-Zn^{II}-OH$^-$ species promoted the hydrolysis of 4-nitrophenyl acetate in the same mechanistic manner as shown with L$_4$-Zn^{II}-OH$^-$ species.[11] The kinetically obtained pK_a value of 7.9 for **2** is the same as the pK_a(H$_2$O) value obtained potentiometrically. The resulting second-order rate constant k(NP) for L$_2$-Zn^{II}-OH$^-$ is listed in Table III, together with the reported k(NP) for OH$^-$ ion[19] and L$_4$-Zn^{II}-OH$^-$,[11] and the pK_a(H$_2$O) values.

Phosphate Anions Affinity to 4. In a previous paper,[11] we have pointed out that our model complex **4** has a unique anion (e.g., acetate, halogen ions) binding trend which is almost comparable with that of CA^{20} (equilibrium 4). Phosphate anions are often

(19) Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 2622.
(20) Pocker, Y.; Stone, J. T. *Biochemistry* **1968**, *7*, 2936.

$$4 + A^- \xrightleftharpoons{K(A^-)} 6 \quad (4)$$

BPP⁻   PP²⁻
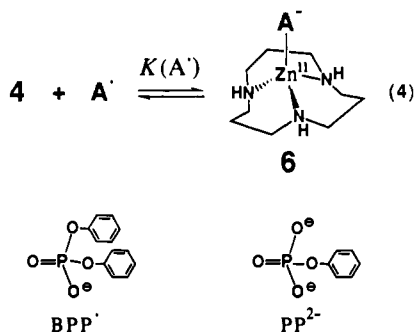
good inhibitors for Zn^{II}–enzymes.[21] In order to check the substrate or product phosphate anion binding with 4, we have conducted the potentiometric pH titration with 1 mM 4 in the presence of a large excess amount (10, 20, and 30 mM) of a phosphate anion (NP²⁻), phenyl phosphate (PP²⁻), BNP⁻, or bis(phenyl) phosphate (BPP⁻).

Elaborate calculations[11] with the titration data have established equilibrium 4 with the 1:1 affinity constants $K(A^-)$ for NP²⁻ and PP²⁻. However, the affinity of the monoanionic phosphodiesters BNP⁻ and BPP⁻ was too weak to be determined accurately. The insufficient solubilities (up to ca. 50 mM) in aqueous solution prevented the use of higher concentrations of these anions. Table IV summarizes the log $K(A^-)$ values, along with the literature values for hydrated Zn^{II} ion.[22]

Discussion

The Macrocyclic N$_3$ Complex 4 as the Best Zn^{II}-Containing Phosphatase Model. As the Zn^{II} complex 1 earlier catalyzed the hydrolysis of DNP⁰,[8] the present 2 and 4 have promoted the hydrolyses of phosphotriester TNP⁰ as well as phosphodiester BNP⁻. Especially, the fact that an anionic phosphodiester hydrolysis is promoted by the Zn^{II} model complex in mild conditions (see Experimental Section) is the first observation. In the hydrolysis of anionic diester BNP⁻, the rates (see Table II) are generally 10⁵ times slower than those for neutral TNP⁰. Obviously this is due to the electrostatic repulsions between the attacking OH⁻ species and anionic substrates. When Na⁺ (added to keep ionic strength) is replaced by Mg²⁺ or Ca²⁺ ion, the BNP⁻ hydrolysis rate increases due to the partial neutralization of the anionic substrate (see Table II and structure I). The same effect

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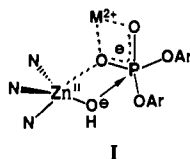
is well known in the enzymatic hydrolysis of anionic phosphate esters with Mg²⁺ ion.[1c] However, when Na⁺ is substituted by bulky tetraethylammonium (Me$_4$N⁺) that cannot so closely associate with P—O⁻ as the smaller M²⁺ ions, the rate enhancement does not occur. On the other hand, in the hydrolysis of neutral substrate TNP⁰, such an effect by the divalent metal ions is not observed, because M²⁺ will not have interaction with the neutral phosphate.

From the pH–rate profile in the TNP⁰ hydrolysis at 25 °C (Figure 2) and the BNP⁻ hydrolysis at 35 °C (Figure 3), respectively, the pK_a values are determined kinetically to be 7.3 and 7.2 with 4, and 7.9 and 7.8 with 2, which well agree with the pK_a(H$_2$O) values determined by the potentiometric pH titrations. Accordingly, it is concluded that L$_4$–Zn^{II}–OH⁻ and L$_2$–Zn^{II}–OH⁻ are kinetically active species. The same for phosphotriester hydrolysis was true with other Zn^{II} complex models, 1[8] and 4.[13] Thus, the reaction mechanisms of phosphate hydrolysis by 1–4 should

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(22) Massoud, S. S.; Sigel, H. *Inorg. Chem.* **1988**, *27*, 1447.

Table IV. Affinity Constants of Phosphates with 4 and Zn$^{II}_{aq}$, log $K(A^-)$ at 25 °C and I = 0.20 (NaClO$_4$)^a

| anion	4	Zn$^{II}_{aq}$	pK_a^e
PP²⁻	3.5 ± 0.1^c	2.1^d	5.80 ± 0.03^e
NP²⁻	3.1 ± 0.1c	1.7^d	5.09 ± 0.03^e
BPP⁻	<0.5^c		<2^e
BNP⁻	<0.5^c		<2^e
OH^{-b}	6.4	5.0	15.7
CH$_3$COO^{-b}	2.6	0.9	4.5

$^a K(A^-)$ = [L–Zn^{II}–A⁻]/[L–Zn^{II}][A⁻] (M⁻¹). ^{b} From ref 11. ^{c} Based on pK_a(H$_2$O) values of 4 in the presence of 5, 10, and 20 mM phosphate anion. ^{d} From ref 22 at 25 °C and I = 0.1 (KNO$_3$). ^{e} pK_a values of the conjugated acids at 25 °C and I = 0.20 (NaClO$_4$): K_a = [A²⁻][H⁺]/[HA⁻] for PP²⁻ and NP²⁻; K_a = [A⁻][H⁺]/[HA] for BPP⁻ and BNP⁻.

Table V. Comparison of the Second-Order Rate Constants (M⁻¹ s⁻¹) for Hydrolysis of Neutral Phosphotriesters

|	OH⁻	L$_1$–Zn^{II}–OH⁻	L$_2$–Zn^{II}–OH⁻
DNP⁰^a	2.8 × 10⁻²	2.8 × 10⁻¹	
DDP⁰^b		1.6 × 10⁻¹	2.7 × 10⁻¹
DEMP⁰^c	4.2 × 10	1.2	2.1

^{a} From ref 8 at 25 °C in 50% CH$_3$CN. ^{b} From ref 9a at 40 °C and I = 1.0 (KNO$_3$). ^{c} From ref 9b at 35 °C and I = 0.1 (KNO$_3$).

Chart II

(a)   (b)

(c) Hybrid of (a) and (b)
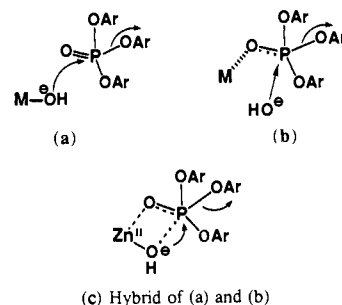
be all similar, which would permit a direct comparison of their hydrolysis efficiency and more detailed mechanistic pictures by the varying structures of 1–4 complexes.

Previously, Norman et al. reported that 2 (as the OH⁻ species) was ca. 2 times more powerful than 1 in hydrolyses of neutral substrates, 2,4-dinitrophenyl ethyl methyl phosphonate (DEMP⁰)

DEMP⁰
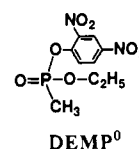
and phosphotriester DDP⁰ (see Table V).[9] The present results in the TNP⁰ hydrolysis have disclosed that 4 is a better promoter than the reference 2 (see Table I). Taken together with the data for 3[13] (Table I), a conclusion is drawn that among the reported Zn^{II} model complexes 1–4 the tetrahedral Zn^{II}–OH⁻ species with the macrocyclic N$_3$ ligand L$_4$ is the best reactant in neutral phosphate hydrolysis. Moreover, since 4 has the lowest pK_a(H$_2$O) value of 7.3, it also gives the fastest hydrolysis rate at neutral pH. Recently, Breslow et al. communicated that 4 is a better catalyst for an intramolecular phosphate cyclization than modified 1, although a detailed mechanism was unreported.[6a]
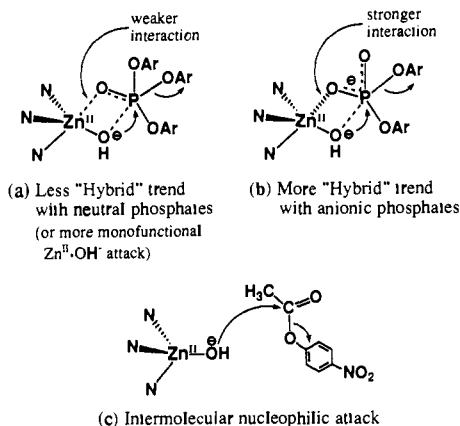
It is also to be noted that although monoanionic (from neutral phosphate) or dianionic phosphate products (from monoanionic phosphate) may have interactions with 4, they tend to be thermodynamically readily replaced by OH⁻ ion (see Table IV), which is quite advantageous in using Zn^{II} for phosphatases. With other metal ions such as Co^{III},[3d,31] the product anionic phosphates may strongly bind to the metal ion and then cause the product inhibition.

The Roles of Zn^{II} in Ester Hydrolysis. There are two limiting mechanistic possibilities for the phosphate hydrolysis with met-

Chart III

(a) Less "Hybrid" trend with neutral phosphates (or more monofunctional Zn^{II}·OH$^-$ attack)

(b) More "Hybrid" trend with anionic phosphates

(c) Intermolecular nucleophilic attack

Chart IV

(a)   (b)

al–OH$^-$ species, which are active reactants in the Zn^{II}-promoted reactions: (1) an intermolecular nucleophilic attack on the phosphorus atom (Chart II, a) and (2) an activation of the phosphoryl P=O bond by coordination to the electrophilic metal center to become susceptible to external nucleophilic attack (Chart II, b). For the neutral phosphotriester DNP^0 hydrolysis catalyzed by **1**, Breslow et al. postulated a "hybrid" mechanism (Chart II, c),[8] where the Zn^{II}–OH$^-$ acts as a nucleophile and at the same time the Zn^{II} offers an open site for P=O binding, on the basis of its 10 times greater second-order rate constant with respect to that for free OH$^-$ ion (see Table V). In contradiction, however, with another neutral phosphotriester (DEMP^0), free OH$^-$ ion is a more than 30 times better reactant than **1**[9b] (see Table V). Such a hybrid mechanism may be better tested by comparing the hydrolysis rates with **2** (with less open site) and **4** (with more open site) for a neutral (hence less nucleophilic) phosphate triester (TNP^0) (Chart III, a), an anionic (hence more nucleophilic) phosphate diester (BNP$^-$) (Chart III, b), and a neutral carboxylic ester, 4-nitrophenyl acetate.

In the carboxylic ester hydrolysis (Table III), free OH$^-$ ion has a much larger rate constant than those for the Zn^{II}–OH$^-$ species, where an approximately linear relationship exists between the nucleophilicities of the OH$^-$ species (i.e., rate constants) and the basicities. This fact indicates that a simple nucleophilic mechanism is predominant (Chart III, c) and the Zn^{II}-bound hydroxides (less basic than free OH$^-$ ion) act merely as nucleophiles (or general bases to generate OH$^-$) to the carbonyl group. Thus, **2** having a higher $pK_a(H_2O)$ of 7.9 reacts faster than **4** with a $pK_a(H_2O)$ of 7.2 (Table III). In the previous study on acetaldehyde hydration with **4** as a catalyst, we also concluded that the Zn^{II}–OH$^-$ species was acting as a simple nucleophile, just as so concluded by Woolley with **1** in the same reaction.[10]

Although the second-order rate constant for the reaction of free OH$^-$ ion with neutral substrate TNP^0 is larger than those for the reactions of L_2–Zn^{II}–OH$^-$ and L_4–Zn^{II}–OH$^-$ (Table I), its magnitude variation (within 3 times) is not as wide as those in the reactions of the carboxylic ester (Table III). Hence, the mere intermolecular Zn^{II}–OH$^-$ nucleophilic attack mechanism on the phosphate cannot account for this observation.

In the BNP$^-$ hydrolysis with Zn–OH$^-$ species, the rate constant for L_4–Zn^{II}–OH$^-$ is 4 times greater than that for L_2–Zn^{II}–OH$^-$, where a transient trigonal-bipyramidal phosphorus structure[23] might be envisaged (Chart III, b). The phosphate anion would be better accommodated on the more open coordination site of **4** than that of **2**. The phosphorus transient species may bear a dianionic character that has stronger affinity to the Zn^{II} like phosphomonoester dianions (see Table IV). Furthermore, the four-coordinate, tetrahedral conformation of **4** could easily be transformed to a five-coordinate, trigonal-bipyramidal conformation as previously shown by X-ray study of a phenolate–pendant
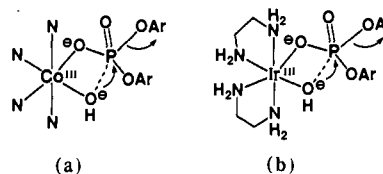
[12]aneN$_3$ Zn^{II} complex.[24] Hence, **4** would be a more powerful promoter in hydrolysis of BNP$^-$ than **2**. On the other hand, if a mere nucleophilic mechanism operates, **4** would be less reactive than **2**, as judged from the lower $pK_a(H_2O)$ value of **4**. Additional evidence in support of the bifunctional, "hybrid" mechanism is the rate constant for L_4–Zn^{II}–OH$^-$ which is greater than that for free OH$^-$ ion. It is now safely concluded that, in the TNP^0 hydrolysis, a less hybrid type of mechanism (Chart III, a) is operative, due to lesser interaction of TNP^0 with the Zn^{II}.

Comparison of Zn^{II} Complex and Other Metal Complex Promoted Hydrolyses of BNP$^-$. Phosphate hydrolysis promoted by cobalt(III) tetraamine complexes (N_4Co^{III}–OH$^-$) has been extensively studied, where N_4 is tris(2-aminoethyl)amine,[3d] tris(3-aminopropyl)amine,[3d] 1,8-diamino-3,6-diazoctane,[3c,n,o] (1,2-diaminoethane)$_2$,[3c,k,l] or L_2.[3d] For phosphodiester hydrolyses, the mechanism involves the initial interaction of the phosphodiester monoanion to the Co^{III} followed by an intramolecular Co^{III}–OH$^-$ attack (Chart IV, a). The rate-determining step involves binding of phosphate to Co^{III} (in the case of reactive esters) or phosphoester cleavage (in the case of less reactive esters). In the case of BNP$^-$ hydrolysis with N_4Co^{III}–OH$^-$,[3d] the conditional rates at pH ca. 7, 50 °C, and [N_4Co^{III} complex] = 10 mM are 10^5–10^8 times greater than that for noncatalytic BNP$^-$ hydrolysis. With Ir^{III} tetraamine complex (en)$_2$Ir^{III}–OH$^-$, where en is 1,2-diaminoethane, the rate increase is about 10^6 times at pH 8 and 25 °C, and the mechanism is almost the same (Chart IV, b).[7] In the BNP$^-$ hydrolysis with our Zn^{II} complex **4**, the conditional first-order rate constant k_{obs} is ca. 8×10^{-7} (s^{-1}) at pH 8.5 and [**4**] = 10 mM. In the absence of **4**, k_{obs} is ca. 8×10^{-11} (s^{-1}) at pH 8.5 and 35 °C. Therefore, **4** promotes the hydrolysis by 10^4 times. Taking into consideration the weaker phosphodiester interaction to the Zn^{II}, the distinct precoordinations to the M^{III} complexes would cause more promotion in the subsequent phosphate hydrolysis.

In the alkaline phosphatase **5** which has two Zn^{II} ions around different environments, it is conceivable that a role of one Zn^{II} ion is to capture phosphate esters, and another Zn^{II} is to generate Zn^{II}–OH$^-$ species to assist the hydrolysis to make a cooperative hybrid catalyst.

Interaction of Phosphate Anions with 4. It is significant that the phosphomonoester dianions PP^{2-} and NP^{2-} have strong interactions with **4** to form 1:1 complexes like **7**, whose log $K(A^-)$ values of 3.1 and 3.5 are greater than 2.6 of acetate anion (Table IV).[11] This is undoubtedly due to the -2 charge and bidentate

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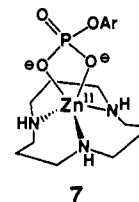
nature of phosphomonoesters. Monoanionic phosphodiesters showed only weak association with **4**. This trend is compatible with phosphate inhibition of alkaline phosphatases,[25] where highly anionic phosphates are strong inhibitors. We have observed that phosphomonoester dianion NP^{2-} was not hydrolyzed at all under the present conditions (reaction 3). We consider a lesser generation of the Zn^{II}–OH$^-$ species to attack the phosphate. On the other hand, with weakly associated monoanionic phosphate, the

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(25) (a) Morton, R. K. *Biochem. J.* **1955**, *61*, 232. (b) Fernley, H. N.; Walker, P. G. *Ibid.* **1967**, *104*, 1011.

Zn^{II}–OH$^-$ species coexists and remains reactive, as we saw above.

The more acidic Zn^{II} in L$_4$ (pK_a(H$_2$O) = 7.3) has ca. 25 times stronger affinity to the dianions than less acidic Zn$^{II}_{aq}$ ion (pK_a(H$_2$O) = 9.0) (see Table IV). The K(A$^-$) valuesu of the phosphodiester monoanions BPP$^-$ and BNP$^-$ with our model are much smaller than those with the phosphomonoester dianions PP^{2-} and NP^{2-}. This trend holds for the affinities with more basic PP^{2-} (pK_a = 5.8) vs less basic NP^{2-} (pK_a = 5.1). Previously, Sigel et al. reported a similar relationship between the phosphomonoester dianion affinity with Zn$^{II}_{aq}$ ion and phosphate ligand basicity.[22] In addition, the present results in phosphate anion complexation using our model have first disclosed that Zn^{II} acidity is also a significant factor determining the binding stability between Zn^{II} and phosphate anion.

Conclusion

Zinc(II) complex **4** of a 12-membered macrocyclic triamine ([12]aneN$_3$, L$_4$) has been shown to be the most suitable (structurally and kinetically) model for the zinc enzyme that catalyzes phosphate ester hydrolysis. We have found that the nucleophilic power of the Zn^{II}–OH$^-$ species from **4** effects phosphotriester and phosphodiester hydrolysis. The kinetic studies on the hydrolysis of phosphoesters and carboxyester by our model complex **4** have clearly disclosed that the Zn^{II} in the rigid triamine ligand acts as a hybrid-type catalyst. As shown in phosphate ester hydrolysis

promoted by other metal (e.g., Co^{III}, Ir^{III}, Mg^{II}) complexes, the Zn^{II} bound to the phosphorus oxygen atoms may also assist P–O bond rupture as a leaving group effect. The strongest affinity of **4** to OH$^-$ ion surpasses any other anions including the phosphodiester substrate or the hydrolysis products phosphomonoester dianion and phosphodiester monoanion. The former fact allows the hybrid mechanism, and the latter fact works favorably in the catalytic cycles. For the alkaline phosphatase **5** which has two Zn^{II} ions around different environments, it is conceivable that one Zn^{II} ion captures the phosphate ester or the phosphorylated (to serine) equivalent, and another one being Zn^{II}–OH$^-$ species to assist their hydrolysis to make a more efficient hybrid catalyst. Finally, further structural modification of the basic structure of **4** (e.g., attachment of an intramolecular hydroxy group for the phosphate binding site or a guanidyl group for substrate recognition) is likely to yield even closer alkaline phosphatase and other Zn^{II}-containing hydrolytic enzyme models, which is currently under investigation in our laboratory.

Communications to the Editor

Structured Self-Aggregates of 4-Methoxy-(*E*)-cinnamic Acid at the Air/Solution Interface As Detected by 2π + 2π Photodimerization and Their Role in the Control of Crystal Polymorphism

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Self-organization of molecules into structured clusters plays an important role in the early stages of crystal nucleation and growth. The different molecular arrangements within such clusters may lead, at the onset of crystallization, to precipitation of crystal polymorphs.[1–3] In the absence of analytical tools to probe the structure of such clusters in solution or in the melt, we have still to rely, for a while yet, on indirect methods. Here we describe the detection of structured self-aggregates of 4-methoxy-(*E*)-cinnamic acid (**1**) at the air/solution interface by topochemical photodimerization and the possible function of such aggregates for inducing the precipitation of a new crystalline polymorph.
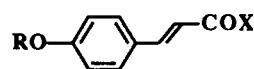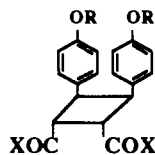

1	**R=CH$_3$**	**X=OH**
3	**R=CH$_3$**	**X=NH$_2$**
4	**R=C$_{18}$H$_{37}$**	**X=OH**
5	**R=C$_{18}$H$_{37}$**	**X=NH$_2$**

2	**R=CH$_3$**	**X=OH**
6	**R=C$_{18}$H$_{37}$**	**X=OH**

Surface tension and optical second harmonic generation measurements of aqueous solutions of **1** reveal that the solute molecules are sufficiently hydrophobic to accumulate at the solution surface,[4] with an estimated coverage of ~30%. These studies, however, do not provide information on the structure of the surface aggregates. Structural considerations, based on packing modes of molecules in crystals,[5] suggest that molecules of **1** should form, at the air/solution interface, close-packed stacks of "translationally" related molecules, separated by 4 Å, stabilized by aromatic ring interactions and Coulombic forces between the carboxyl groups. This organization should influence the photochemical behavior of these molecules as was established from photoreactivity in the crystalline state.[6] Thus while **1** undergoes trans/cis photoisomerization in bulk solution, as do all (*E*)-cinnamic acids, the self-organized molecules at the interface are expected to undergo a 2π + 2π photodimerization leading to the mirror-symmetric photodimer, 4,4'-dimethoxy-β-truxinic acid (**2**).

In order to differentiate between products formed in the bulk of the solution and at the interface, aqueous solutions of **1** (4.0–5.6 × 10^{-4} M) were irradiated (λ > 320 nm) in two different experimental setups: closed glass vessels completely filled with solution and open glass vessels with a large solution/air interface (Figure 1B,A). Irradiation of the solution in the closed vessel resulted[7] in a trans:cis ratio of about 1:3 (Figure 1b), whereas the open vessel yielded, in addition, formation of 5–20% of the photodimer **2**, depending upon the exposure time (Figure 1a). The molecular organization at the surface could be gradually removed by addition of EtOH (35%) to the solution, as made manifest by the formation of only traces of the photodimer **2**.

(1) Weissbuch, I.; Zbaida, D.; Addadi, L.; Lahav, M.; Leiserowitz, L. *J. Am. Chem. Soc.* **1987**, *109*, 1869–1870.

(2) Staab, E.; Addadi, L.; Leiserowitz, L.; Lahav, M. *Adv. Mater.* **1990**, *2*, 40–41.

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(4) From the SHG measurements we infer that the molecules are oriented with their long axis making an angle of ~25–30° with the normal to the water surface. It is obvious that the polar head group, as represented by the COX group, points into the water (SHG phase measurements); Berkovic, G., private communication.

(5) Leiserowitz, L. *Acta Crystallogr.* **1976**, *B32*, 775–802.

(6) (a) Cohen, M. D.; Schmidt, G. M. J.; Sonntag, F. I. *J. Chem. Soc.* **1964**, 2000–2013. (b) Schmidt, G. M. J. *J. Chem. Soc.* **1964**, 2014–2021.

(7) The photoproducts were assigned by reversed-phase HPLC analysis against synthetic standards.