

Remarkable enhancement of the hydrolyses of phosphoesters by dinuclear centers: *Streptomyces* aminopeptidase as a 'natural model system'†

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The transition-state analogues bis(*p*-nitrophenyl)phosphate and *p*-nitrophenyl phenylphosphonate for peptide hydrolysis are shown to be very effectively hydrolyzed by *Streptomyces* dinuclear aminopeptidase and its Co²⁺, Ni²⁺, Mn²⁺ and Cd²⁺ derivatives with high catalytic proficiencies and specific activities comparable to those of some phosphoesterases.

Enzymes are able to specifically recognize a certain type of substrates and stabilize their transition states during reactions to afford enormous rate enhancement.^{1,2} Consequently, transition-state analogues can often serve as inhibitors.² For peptide, ester, and amide hydrolysis, the transition state is a *gem*-diolate that can be well mimicked by the tetrahedral phospho-center in phosphoesters, phosphonates and phosphoamidates, which are known to inhibit the corresponding enzymes.^{3–5} Thus, it would be unusual that peptides and the transition state-like phosphoesters can both be effectively hydrolyzed by the same hydrolytic enzyme because the recognition of the substrates at the ground- and the transition-states and the hydrolytic mechanisms of these two substrate families are quite different.

Proteins from the same origin with structural and sequence similarities may have evolved to exhibit completely different functions that are not related to each other, such as α -lactalbumin and lysozyme,⁶ alkaline phosphatase and arylsulfatase,⁷ and among the many β -barrel proteins.⁸ Some of these enzymes indeed exhibit 'alternative catalysis',^{6,9} which demonstrates their evolutionary relatedness and would not by all means be completely unexpected. On the other hand, it would be quite unusual to observe an 'alternative catalysis' of an enzyme that is attributable to an evolutionarily and structurally unrelated enzyme.

Recently, we have observed that the di-Zn aminopeptidase from *Streptomyces griseus* (sAP) exhibits a remarkable hydro-

lytic activity towards bis(*p*-nitrophenyl)phosphate (BNPP) with a specific activity comparable to several native phosphoesterases.¹⁰ However, it was not clear whether or not this catalysis was unique toward only that particular one phosphoester substrate. Here, we describe an extensive analysis of the hydrolyses of a few different kinds of phosphoesters, including phosphomonoester, phosphodiester, phosphotriester and phosphonate monoester, by metal-substituted derivatives of sAP. The effectiveness toward the hydrolyses of both peptide and phosphoester substrates by sAP offers a rare opportunity to investigate different hydrolytic mechanisms in a single enzymatic system.

The hydrolysis of BNPP by a few homonuclear derivatives of sAP (M₂-sAP; M = Mn²⁺, Co²⁺, Ni²⁺, Zn²⁺ and Cd²⁺)^{11,12} has been analyzed with Michaelis–Menten kinetics (Table 1; Fig. S1A in ESI†). The rate constants ($k_{\text{cat}}/K_{\text{m}} = 0.94\text{--}100\text{ M}^{-1}\text{ s}^{-1}$) are much higher than the second-order constants of many synthetic chemical models, e.g. $(1.3\text{--}43) \times 10^{-5}\text{ M}^{-1}\text{ s}^{-1}$ at 35 °C and pH 7.3–10.5 for several Zn²⁺ complexes¹³ (also see footnote 14). The specific activity values (1.6–158 nmol min⁻¹ mg⁻¹; Table 1) are in the range for several phosphodiesterases and phosphatases (0.3–2450 and ca. 2–40 nmol min⁻¹ mg⁻¹, respectively),¹⁵ and comparable to ethyl(*p*-nitrophenyl)phosphate hydrolysis by metal-substituted derivatives of *Pseudomonas* phosphotriesterase (1.7–11.4 nmol min⁻¹ mg⁻¹ calculated from ref. 16 with $k_{\text{cat}}/K_{\text{m}}$ in the range 1.1–7.2 M⁻¹ s⁻¹).

In order to demonstrate that BNPP hydrolysis by M₂-sAP is not just an incidental catalysis, the hydrolyses of structurally distinct phosphoesters were investigated. The phosphonate ester *p*-nitrophenylphenylphosphonate (NPPP) contains a P–C bond and resembles the primary hydrolytic products of some chemical warfare agents.¹⁷ Thus, the study of its hydrolysis has practical value. The second order rate constants of NPPP hydrolysis (ca. 1–7 M⁻¹ s⁻¹; Table 1 and Fig. S1B in ESI†) by M₂-sAP are much greater than those of some metal ions,¹⁸ and are approaching those of a few highly active tetra-valent

† Electronic supplementary information (ESI) available: Michaelis–Menten kinetics plots. See <http://www.rsc.org/suppdata/cc/b0/b004544f/>

Table 1 The hydrolyses of BNPP and NPPP by several di-metal substituted derivatives of sAP in 0.1 M HEPES buffer at pH 8.0 in the presence of 2 mM Ca²⁺ at 30 °C (BNPP) and 50 °C (NPPP)^a

M ₂ -sAP ^b	Bis(<i>p</i> -nitrophenyl)phosphate (BNPP) ^c					<i>p</i> -Nitrophenyl phenylphosphonate (NPPP) ^c				
	$k_{\text{cat}}/\text{s}^{-1}$	K_{m}/mM	$(k_{\text{cat}}/K_{\text{m}})/\text{M}^{-1}\text{ s}^{-1}$	CP ^d	SA ^e	$k_{\text{cat}}/\text{s}^{-1}$	K_{m}/mM	$(k_{\text{cat}}/K_{\text{m}})/\text{M}^{-1}\text{ s}^{-1}$	CP ^d	SA ^e
ZnZn ¹⁰	0.45	4.5	100	4.1×10^{10}	158	0.014	14.9	0.94	1.8×10^5	1.7
MnMn	0.21	12.0	18	1.9×10^{10}	31	0.010	4.9	2.0	1.3×10^5	3.3
CoCo	0.74	9.5	78	6.7×10^{10}	136	0.022	7.9	2.8	2.9×10^5	4.8
NiNi	0.010	10.6	0.94	0.91×10^9	1.6	0.0033	3.0	1.1	0.43×10^5	1.6
CdCd	0.043	9.7	4.4	3.9×10^9	7.8	0.017	2.4	7.1	2.2×10^5	9.7

^a The hydrolyses of the phosphotriesters parathione and tris(*p*-nitrophenyl)phosphate and the phosphomonoester *p*-nitrophenylphosphate by the enzyme are not detectable on the spectrophotometer after several hours at pH 8.0 and 50 °C. ^b Enzyme concentrations were in the range 0.1–0.7 μM . The di-Cu derivative did not show observable activity with a concentration of 2.0 μM under the experimental conditions, which indicates that the activity of CuCu-sAP would be at least ca. $10^4 \times$ smaller than the native di-Zn sAP. ^c The substrate concentrations were in the range 1.0–20 mM. The initial rates were obtained from the change of absorbance at 405 nm ($\epsilon = 17\,500\text{ M}^{-1}\text{ cm}^{-1}$), plotted against substrate concentration, and then fitted directly to the hyperbolic Michaelis–Menten equation, $\text{rate} = k_{\text{cat}}[\text{E}_0][\text{S}]/(K_{\text{m}} + [\text{S}])$ to give the kinetic parameters k_{cat} and K_{m} . ^d CP: catalytic proficiency.²² ^e The specific activity (SA) is for the hydrolysis of 1 mM substrate in 1 min by 1 mg enzyme (nmol min⁻¹ mg⁻¹) or extrapolated by the use of the Michaelis–Menten equation.

lanthanide micelles ($13.1\text{--}76.8\text{ M}^{-1}\text{ s}^{-1}$)¹⁹ which have Lewis acidities several orders of magnitude higher.²⁰ The specific activities of $1.6\text{--}9.7\text{ nmol min}^{-1}\text{ mg}^{-1}$ against 1 mM NPPP (Table 1) are comparable to those of a few phosphatases and phosphodiesterases at $30\text{ }^{\circ}\text{C}$ ($12\text{--}355$ and *ca.* $1\text{--}38\text{ 900 nmol min}^{-1}\text{ mg}^{-1}$, respectively).¹⁵ Conversely, the hydrolyses of the phosphomonoester *p*-nitrophenylphosphate and the phosphotriesters parathion and tris(*p*-nitrophenyl)phosphate are beyond the spectrophotometric detection limit, indicating the presence of specificity toward different phosphoesters.

Although the *p*-nitrophenol in both BNPP and NPPP is a very good leaving group, the auto-hydrolytic rates of BNPP and NPPP are still *extremely slow* with a rate constant $k_1 = 1.1 \times 10^{-11}\text{ s}^{-1}$ for BNPP at pH 7.0 and $25\text{ }^{\circ}\text{C}$ ²¹ and $7.65 \times 10^{-8}\text{ s}^{-1}$ for NPPP at pH 8.0 and $50\text{ }^{\circ}\text{C}$ (comparable to $1.7 \times 10^{-7}\text{ s}^{-1}$ at $60\text{ }^{\circ}\text{C}$ ¹⁸). Tremendous catalytic proficiencies²² are obtained for M₂-sAP toward BNPP and NPPP hydrolyses *i.e.* $(0.94\text{--}67) \times 10^9$ and $(0.43\text{--}2.9) \times 10^5$, respectively (Table 1). Co₂-sAP virtually decreases the half-life of BNPP hydrolysis from *ca.* 2000 years to *ca.* 1 second! These rate enhancements are remarkable when it is taken into account that the phospho-substrates are transition-state analogues of peptides during hydrolysis.^{1,3-5} In this case their corresponding trigonal bipyramidal transition states requires significantly more stabilization in support of their hydrolysis. For instance, an association constant of 108 M^{-1} (approximated from the average K_m of 9.3 mM for BNPP hydrolysis) would contribute 11.6 kJ mol^{-1} energy in ground-state stabilization at 298 K , which would increase the activation energy by the same amount and would reduce the reaction rate by *ca.* $100\times$. In the mean time, a 67×10^9 fold rate enhancement requires a decrease of 61.7 kJ mol^{-1} in activation energy at 298 K .

Finally, it is interesting that the Mn²⁺, Ni²⁺ and Cd²⁺ derivatives of sAP also exhibit potent hydrolytic power [*i.e.* $(ca. 1\text{--}4) \times 10^9$ and $(ca. 40\text{--}200) \times 10^3$ fold rate enhancements toward BNPP and NPPP hydrolyses, respectively, Table 1] suggesting that these metal ions should be included in future design of chemical models for more extensive structural and mechanistic studies of metal-centered hydrolysis.²³

The results have provided some mechanistic insight. The K_m values for BNPP and NPPP are similar, suggesting that they are recognized by sAP in a similar fashion. In both substrates, a hydrophobic *p*-nitrophenyl/phenyl group (as a hydrophobic anchor to bind to the active site) and a $-\text{PO}_2^-$ group (as a gem-diolate transition-state analogue) are essential. On the other hand, the non-competitive property of phosphate²⁴ may be attributable to the lack of a hydrophobic anchor; the competitive inhibitor *p*-nitrophenylphosphate²⁵ contains both recognition moieties, yet is not hydrolyzed owing to the lack of an additional hydrolyzable group; and the two phosphotriesters are not hydrolyzed owing to the lack of a $-\text{PO}_2^-$ group.

Many synthetic metal complexes have been utilized as models²⁶ to provide insight into the mechanistic roles of the metal ion(s) and the nucleophilic water in metallohydrolases. The specificity and tremendous effectiveness of enzymes offer a very challenging task for chemical modeling studies to achieve. We describe here that the transition-state analogues BNPP and NPPP are indeed substrates for M₂-sAP, and are hydrolyzed with remarkable rate accelerations. Although the rates of catalysis are not comparable to those of 'perfect enzymes' and much slower than those of the specific substrates of sAP,^{11a,b} M₂-sAP can serve as unique 'natural model systems' (as opposed to 'synthetic model systems') for further studies of phosphoester hydrolysis. The results from these studies should lead us to a better understanding of dinuclear hydrolysis in chemical and biological systems.

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