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

Altan Ercan, Hyun Ik Park and Li-June Ming*

Department of Chemistry and Institute for Biomolecular Science, University of South Florida, Tampa, FL 33620-5250, USA. E-mail: ming@chuma.cas.usf.edu

Received (in Irvine, CA, USA) 2nd June 2000, Accepted 27th October 2000
First published as an Advance Article on the web

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 Co^{2+}, Ni^{2+}, Mn^{2+} and Cd^{2+} 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 hydrolytic 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 (M_2-sAP; M = Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+})[11,12] has been analyzed with Michaelis–Menten kinetics (Table 1; Fig. S1A in ESI†). The rate constants (k_{cat}/K_m = 0.94–100 M^{-1} s^{-1}) are much higher than the second-order constants of many synthetic chemical models, *e.g.* (1.3–43) × 10^{-5} M^{-1} s^{-1} at 35 °C and pH 7.3–10.5 for several Zn^{2+} complexes[13] (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_{cat}/K_m in the range 1.1–7.2 M^{-1} s^{-1}).

In order to demonstrate that BNPP hydrolysis by M_2-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 M_2-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 Ca^{2+} at 30 °C (BNPP) and 50 °C (NPPP)[a]

	Bis(*p*-nitrophenyl)phosphate (BNPP)[c]					*p*-Nitrophenyl phenylphosphonate (NPPP)[c]				
M,M-sAP[b]	k_{cat}/s^{-1}	K_m/mM	$(k_{cat}/K_m)/M^{-1}$ s^{-1}	CP[d]	SA[e]	k_{cat}/s^{-1}	K_m/mM	$(k_{cat}/K_m)/M^{-1}$ 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 × 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 (ε = 17 500 M^{-1} cm^{-1}), plotted against substrate concentration, and then fitted directly to the hyperbolic Michaelis–Menten equation, rate = $k_{cat}[E_o][S]/(K_m + [S])$ 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.

DOI: 10.1039/b004544f

lanthanide micelles (13.1–76.8 M^{-1} s^{-1})[19] which have Lewis acidities several orders of magnitude higher.[20] The specific activities of 1.6–9.7 nmol min^{-1} mg^{-1} against 1 mM NPPP (Table 1) are comparable to those of a few phosphatases and phosphodiesterases at 30 °C (12–355 and *ca*. 1–38 900 nmol min^{-1} 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 $k_1 = 1.1 \times 10^{-11}$ s^{-1} for BNPP at pH 7.0 and 25 °C[21] and 7.65×10^{-8} s^{-1} for NPPP at pH 8.0 and 50 °C (comparable to 1.7×10^{-7} s^{-1} at 60 °C[18]). Tremendous catalytic proficiencies[22] are obtained for M_2-sAP toward BNPP and NPPP hydrolyses *i.e.* (0.94–67) $\times 10^9$ and (0.43–2.9) $\times 10^5$, respectively (Table 1). Co_2−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×. 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 Mn^{2+}, Ni^{2+} and Cd^{2+} derivatives of sAP also exhibit potent hydrolytic power [*i.e.* (*ca*. 1–4) $\times 10^9$ and (*ca*. 40–200) $\times 10^3$ 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 $–PO_2^-–$ group (as a *gem*-diolate transition-state analogue) are essential. On the other hand, the non-competitive property of phosphate[24] may be attributable to the lack of a hydrophobic anchor; the competitive inhibitor *p*-nitrophenylphosphate[25] 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 $–PO_2^-–$ group.

Many synthetic metal complexes have been utilized as models[26] 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 M_2-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] M_2-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.

This research was partially supported by the Petroleum Research Funds administrated by the American Chemical Society (ACS-PRF #35313-AC3).

Notes and references

1 L. Pauling, *Am. Sci.*, 1948, **36**, 51.
2 C. Walsh, *Enzymatic Reaction Mechanisms*, Freeman, New York, 1979.
3 K. Nishida, Y. Ohta, M. Ito, Y. Nagamura, S. Kitahara, K. Fujii and I. Ishiguro, *Biochim. Biophys. Acta.*, 1996, **1313**, 47; C. V. Preuss and C. K. Svensson, *Biochem. Pharmacol.*, 1996, **51**, 1661; L. Luan, T. Sugiyama, S. Takai, Y. Usami, T. Adachi, Y. Katagiri and K. Hirano, *Biol. Pharmacol. Bull.*, 1997, **20**, 71.
4 N. Sträter and W. N. Lipscomb, *Biochemistry*, 1995, **34**, 9200; B. Lejczak, P. Kafarski and J. Zygmunt, *Biochemistry*, 1989, **28**, 3549.
5 D. E. Tronrud, H. M. Holden and B. W. Matthews, *Eur. J. Biochem.*, 1986, **157**, 261.
6 E. M. Prager and A. C. Wilson, *J. Mol. Evol.*, 1988, **27**, 326; K. Nitta and S. Sugai, *Eur. J. Biochem.*, 1989, **182**, 111; H. A. McKenzie and F. H. White, Jr., *Adv. Protein Chem.*, 1991, **41**, 173; P. K. Qasba and S. Kumar, *Crit. Rev. Biochem. Mol. Biol.*, 1997, **32**, 255.
7 C. S. Bond, P. R. Clements, S. J. Ashby, C. A. Collyer, S. J. Harrop, J. J. Hopwood and J. M. Guss, *Structure*, 1997, **5**, 277; G. Lukatela, N. Krauss, K. Theis, T. Selmer, V. Gieselmann, K. von Figura and W. Saenger, *Biochemistry*, 1998, **37**, 3654.
8 R. Pickersgill, G. Harris, L. Lo Leggio, O. Mayans and J. Jenkins, *Biochem. Soc. Trans.*, 1998, **26**, 190.
9 P. J. O'Brien and D. Herschlag, *J. Am. Chem. Soc.*, 1998, **120**, 12 369.
10 H. I. Park and L.-J. Ming, *Angew. Chem., Intl. Ed.*, 1999, **38**, 2914.
11 The purification of sAP (*ca*. 30 kDa) and preparation of its apo form followed the literature procedures.[11a,b,12] The kinetic measurements by the metal-substituted derivatives were conducted in the presence of excess amount of the corresponding metal ions to ensure the complete formation of the derivatives. The background hydrolysis of BNPP by the excess metal ion is negligible and that of NPPP is considerably small and has been corrected. (*a*) A. Spungin and S. Blumberg, *Eur J. Biochem.*, 1989, **183**, 471; (*b*) D. Ben-Meir, A. Spungin, R. Ashkenazi and S. Blumberg, *Eur J. Biochem.*, 1993, **212**, 107.
12 L.-Y. Lin, H. I. Park and L.-J. Ming, *J. Biol. Inorg. Chem.*, 1997, **2**, 744.
13 T. Koike and E. Kimura, *J. Am. Chem. Soc.*, 1991, **113**, 8935; E. Kimura, H. Hashimito and T. Koike, *J. Am. Chem. Soc.*, 1996, **118**, 10 963.
14 Second-order rate constants in the range of (0.18–2.8) $\times 10^{-5}$ M^{-1} s^{-1} are calculated from corresponding pseudo-first order rate constants at pH 8.36 and 55 °C[14a] and (5.4–11.5) $\times 10^{-5}$ M^{-1} s^{-1} at pH 10.9–11.5 and 35 °C[14b] for several mono- and di-nuclear Zn^{2+} complexes. (*a*) W. H. Hapman and R. Breslow, *J. Am. Chem. Soc.*, 1995, **117**, 5462; (*b*) A. Bencini, E. Berni, A. Bianchi, V. Fedi, C. Giorgi, P. Paoletti and B. Valtancoli, *Inorg. Chem.*, 1999, **38**, 6323.
15 J. S. Kelly, D. E. Dardinger and L. G. Butler, *Biochemistry*, 1975, **14**, 4983; J. S. Kelly and L. G. Burtler, *Biochem. Biophys. Res. Commun.*, 1975, **66**, 316.
16 H. Shim, S.-B. Hong and F. M. Raushel, *J. Biol. Chem.*, 1998, **273**, 17 445.
17 Y. C. Yang, J. A. Baker and J. R. Ward, *Chem. Rev.*, 1992, **92**, 1729.
18 NPPP hydrolysis by La^{3+} is enhanced only by 100-fold at 60 °C which is much smaller than the catalytic proficiency of sAP (Table 1), whereas Cu^{2+}, Ni^{2+} and Zn^{2+} are ineffective; J. S. Loran, R. A. Naylor and A. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1977, 418.
19 R. Moss and K. G. Ragunathan, *Langmuir*, 1999, **15**, 107.
20 J. Burgess, *Metal Ions in Solution*, Halstead, New York, 1978.
21 B. K. Takasaki and J. Chin, *J. Am. Chem. Soc.*, 1995, **117**, 8582.
22 The catalytic proficiency is expressed as k_{cat}/k_1[22a] instead of $(k_{cat}/K_m)/(k_1/55.5)$,[9] which is not appropriate here since H_2O is not the nucleophile in the hydrolysis. (*a*) A. Radzicka and R. Wolfenden, *Science*, 1995, **26**, 90.
23 Recent references: F. Hampl, F. Liska, F. Mancin, P. Tecilla and U. Tonellato, *Langmuir*, 1999, **15**, 405; J. Suh and W. J. Kwon, *Bioorg. Chem.*, 1998, **26**, 103; C. He, V. Gomez, B. Spingler and S. J. Lippard, *Inorg. Chem.*, 2000, **39**, 4188.
24 M. N. Harris and L.-J. Ming, *FEBS Lett.*, 1999, **455**, 321.
25 H. I. Park, Ph.D. Dissertation 1999, University of South Florida, FL, USA.
26 Some recent reviews: E. Kimura and T. Koike, *Adv. Inorg. Chem.*, 1997, **44**, 229; E. L. Hegg and J. N. Burstyn, *Coord. Chem. Rev.*, 1998, **173**, 133; N. H. Williams, B. Takasaki, M. Wall and J. Chin, *Acc. Chem. Res.*, 1999, **32**, 485; A. Blasko and T. C. Bruice, *Acc. Chem. Res.*, 1999, **32**, 475; H. Vahrenkamp, *Acc. Chem. Res.*, 1999, **32**, 589.