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 efficiencies 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. Consequently, transition-state analogues can also serve as inhibitors. For peptide, ester, and amide hydrolysis, the transition state is a tetrahedral that can be well mimicked by the tetrahedral phospho-center in the enzyme because the recognition of the substrates at the ground-state of the enzyme cannot 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-state 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, alkaline phosphatase and arylsulfatase, and among the many β-barrel proteins. Some of these enzymes indeed exhibit ‘alternative catalysis’, 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. 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⁺) has been analyzed with Michaelis–Menten kinetics (Table 1; Fig. S1A in ESI†). The rate constants (k₅/Kₘ, 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 Zn²⁺ complexes (also see footnote 1). The specific activity values (1.0–138 nmol min⁻¹ mg⁻¹) are 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 k₅/Kₘ 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-nitrophenyl phenylphosphonate (NPPP) contains a P–C bond and resembles the primary hydrolytic products of some chemical warfare agents. 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, and are approaching those of a few highly active tetra-valent phosphoesters.

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)

<table>
<thead>
<tr>
<th>M·M·sAP</th>
<th>k₅cat s⁻¹</th>
<th>Kₘ mM</th>
<th>(k₅/Kₘ)</th>
<th>CP</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnZn¹⁰</td>
<td>0.45</td>
<td>4.5</td>
<td>100</td>
<td>4.1 × 10⁻⁶</td>
<td>158</td>
</tr>
<tr>
<td>Mn Mn</td>
<td>0.21</td>
<td>12.0</td>
<td>18</td>
<td>1.9 × 10⁻³</td>
<td>31</td>
</tr>
<tr>
<td>Co Co</td>
<td>0.74</td>
<td>9.5</td>
<td>78</td>
<td>6.7 × 10⁻⁶</td>
<td>136</td>
</tr>
<tr>
<td>Ni Ni</td>
<td>0.010</td>
<td>10.6</td>
<td>0.94</td>
<td>0.91 × 10⁻²</td>
<td>1.6</td>
</tr>
<tr>
<td>Cu Cd</td>
<td>0.043</td>
<td>9.7</td>
<td>4.4</td>
<td>3.9 × 10⁻⁶</td>
<td>7.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M·M·sAP</th>
<th>k₅cat s⁻¹</th>
<th>Kₘ mM</th>
<th>(k₅/Kₘ)</th>
<th>CP</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnZn¹⁰</td>
<td>0.014</td>
<td>14.9</td>
<td>0.94</td>
<td>1.8 × 10⁶</td>
<td>1.7</td>
</tr>
<tr>
<td>Mn Mn</td>
<td>0.010</td>
<td>4.9</td>
<td>2.0</td>
<td>1.3 × 10⁶</td>
<td>3.3</td>
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<tr>
<td>Co Co</td>
<td>0.022</td>
<td>7.9</td>
<td>2.8</td>
<td>2.9 × 10⁸</td>
<td>4.8</td>
</tr>
<tr>
<td>Ni Ni</td>
<td>0.0033</td>
<td>3.0</td>
<td>1.1</td>
<td>0.43 × 10⁶</td>
<td>1.6</td>
</tr>
<tr>
<td>Cu Cd</td>
<td>0.017</td>
<td>2.4</td>
<td>7.1</td>
<td>2.2 × 10⁸</td>
<td>9.7</td>
</tr>
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</table>

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

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lanthanide micelles (13.1–76.8 M⁻¹ s⁻¹) which have Lewis acidities several orders of magnitude higher. 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 phosphohydrolases and phosphodiesterases at 30 °C (12–355 and ca. 1–38 900 nmol min⁻¹ mg⁻¹, respectively). Conversely, the hydrolyses of the phosphomonoester p-nitrophenylphosphate and the phosphotriesters paranitrophenylphosphotriester and tris(p-nitrophenyl)phosphate are beyond the spectrophotometric detection limit, indicating the presence of specificity toward different phosphomonoesters.

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 k₁ = 1.1 × 10⁻¹¹ s⁻¹ for BNPP at pH 7.0 and 25 °C and 7.65 × 10⁻¹⁰ s⁻¹ for NPPP at pH 8.0 and 50 °C (comparable to 1.7 × 10⁻⁷ s⁻¹ at 60 °C). Tremendous catalytic properties are obtained for M₂-sAP toward BNPP and NPPP hydrolases i.e. (0.94–67) × 10⁵ and (0.43–2.9) × 10⁴, respectively (Table 1). Consistent with these observations, the catalytic efficiency of 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 phosphosubstrates are transition-state analogues of peptides during hydrolysis. 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 Kₐ of 9.3 mM for BNPP hydrolisis) would contribute 11.6 kJ mol⁻¹ in activation energy at 298 K.

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Notes and references
11. The purification of sAP (ca. 30 kDa) and preparation of its apo form followed the literature procedures.¹¹b⁻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. Spangin and B. Blumberg, Eur. J. Biochem., 1989, 183, 471; (b) D. Ben-Meir, A. Spangin, R. Ashkenazi and S. Blumberg, Eur. J. Biochem., 1993, 212, 107.
14. Second-order rate constants in the range of (1.8–2.8) × 10⁻³ M⁻¹ s⁻¹ are calculated from corresponding pseudo-first order rate constants at pH 8.36 and 55 °C and (5.4–11.5) × 10⁻³ M⁻¹ s⁻¹ at pH 10.9–11.5 and 35 °C for several mono- and di-nuclear Zn²⁺ complexes. (a) W. H. Kaplan and R. Breslow, J. Am. Chem. Soc., 1995, 117, 5462; (b) A. Bencini, E. Berni, A. Bianchi, V. Fedi, C. Giorgi, P. Paolotti and B. Valtancoli, Inorg. Chem., 1999, 38, 6323.
18. NPPP hydrolysis by La³⁺ is enhanced only by 100-fold at 60 °C which is much smaller than the catalytic proficiency of sAP (Table 1), whereas Cu²⁺, Ni²⁺ and Zn²⁺ are ineffective; J. S. Loran, R. A. Naylor and A. Williams, J. Chem. Soc., Perkin Trans. 2, 1977, 418.
22. The catalytic proficiency is expressed as kₜₐₖ/kᵦ (as opposed to sAP) instead of kₜₐₖ/kᵦ which is not appropriate here since H₂O is not the nucleophile in the hydrolysis. (a) A. Radzicka and R. Wolfenden, Science, 1995, 26, 90.
25. H. I. Park, Ph.D. Dissertation 1999, University of South Florida, Fl., USA.