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Journal of Molecular Catalysis B: Enzymatic 27 (2004) 7–12

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Hydrolysis of organophosphate triesters by *Escherichia coli* aminopeptidase P

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Received 7 June 2003; received in revised form 28 July 2003; accepted 2 September 2003

Abstract

Aminopeptidase P (AMPP, EC 3.4.11.9) from *Escherichia coli* catalyzes the cleavage of amino-terminal X-Pro peptide bonds. In this study, AMPP was found to catalyze the hydrolysis of a wide range of organophosphate triesters (**1**–**7**). The activity of AMPP was promoted in the presence of Mn^{2+}, and slight activation was achieved by treatment with Cd^{2+}. The enzymatic rates of hydrolysis for organophosphate triesters (**3** and **5**) with both isopropyl (or isobutyl) and methyl substituents attached to the phosphorus atom were from 5- to 36-fold higher compared to the corresponding analogs (**4** and **6**, **7**). Three mutants, R153W, R153L and R370L, have been characterized and exhibited from 1.2- to 1.5-fold increases in hydrolyzing **1**–**7** compared to the wild-type enzyme. These results demonstrate that there is a flanking hydrophobic interaction between the substituent group of substrates and position 153 or 370 in AMPP to facilitate the hydrolysis of organophosphate triesters.
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Keywords: Aminopeptidase P; Mutants; Hydrolysis; Enzymatic catalysis; Organophosphate triesters

1. Introduction

Aminopeptidase P (AMPP, EC 3.4.11.9) from *Escherichia coli* is one member of proline-specific peptidases that is capable of catalytically hydrolyzing N-terminal peptide bonds containing a proline residue [1–3]. Two divalent Mn^{2+} ions are critical for maximal catalytic activity of the enzyme [1,2,4–6]. Replacement of the native Mn^{2+} with Co^{2+}, Zn^{2+}, Ca^{2+}, or Mg^{2+} results in an enzyme that only Co^{2+}-AMPP remains catalytically competent when Gly-Pro was used as the substrate [7]. The three-dimensional structure of AMPP by X-ray crystallography reveals that the enzyme is a tetramer with each subunit composed of the "pita-bread" fold of the C-terminal domain [8]. Interestingly, amino acid sequence alignments between AMPP, methionine aminopeptidase (EC 3.4.11.18) [9], creatinase (EC 3.5.3.3) [10,11] and prolidase (EC 3.4.13.9) [12,13] have clearly implicated that the catalytic domains of four enzymes all display similar "pita-bread" folds [5]. The active site of AMPP is located at the C-terminal portion of the β-sheet with the manganese ions separated by 3.3 Å. The two ions are coordinated by Asp 260, Asp 271, Glu 406, Asp 271, His 354, Glu 383, Glu 406 and two water molecules. It is believed that the water molecule or hydroxide ion bridging between the metal ions could be activated strongly enough to act as the nucleophile in the attack on the scissile peptide bond of Xaa-Pro. Furthermore, the X-ray structures suggested that some residues, like Arg 153, from an adjacent monomer in the tetramer are in proximity to the metal center of the other monomer. Thus, the active site is essentially extended across monomers.

Due to the inactivation of the enzyme acetylcholinesterase (AChE) and the subsequent loss of nerve function, the toxicological properties of organophosphate compounds still attract interest in view of environmental protection. The safety of the detoxification process is of great concern to the general public. Thus, an enzyme capable of hydrolyzing organophosphate compounds is of biological as well as environmental value. One of the examples, organophosphorus hydrolase [14], which belongs to a proline dipeptidase family (EC 3.4.13.9), not only catalyzes the cleavage of the peptide bond within a dipeptide containing a proline residue at the carboxy terminus (Xaa-Pro) but also hydrolyzes a wide variety of organophosphorus compounds.

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doi:10.1016/j.molcatb.2003.09.002

In general, AMPP catalyzes the hydrolysis of proline containing polypeptides. It has never been reported to cleave P–O bonds of organophosphate triesters. In this study, we cloned and overexpressed the *E. coli* gene of AMPP (*pepP*) and identified the activity of the enzyme to catalyze the hydrolysis of a wide range of organophosphorus compounds. In addition, an examination of the putative active site of AMPP in the holo- and apo-enzyme structures suggests at least two residues (Arg 153 and Arg 370), other than the above-mentioned, which may be of some significance to the catalytic activity of this protein toward organophosphorus compounds.

2. Experimental

2.1. Materials and methods

All buffers and chemicals were purchased from Sigma, Aldrich, or Acros Organics. Restriction endonucleases and DNA polymerase were from New England Biolabs. pET-15b and *E. coli* strains were from Novagen. Promega Wizard Genomic DNA purification kit was purchased from Promega. Primers were synthesized by MDBio Inc. HiTrap Chelating column was from Amersham Biosciences.

2.2. Chemical synthesis

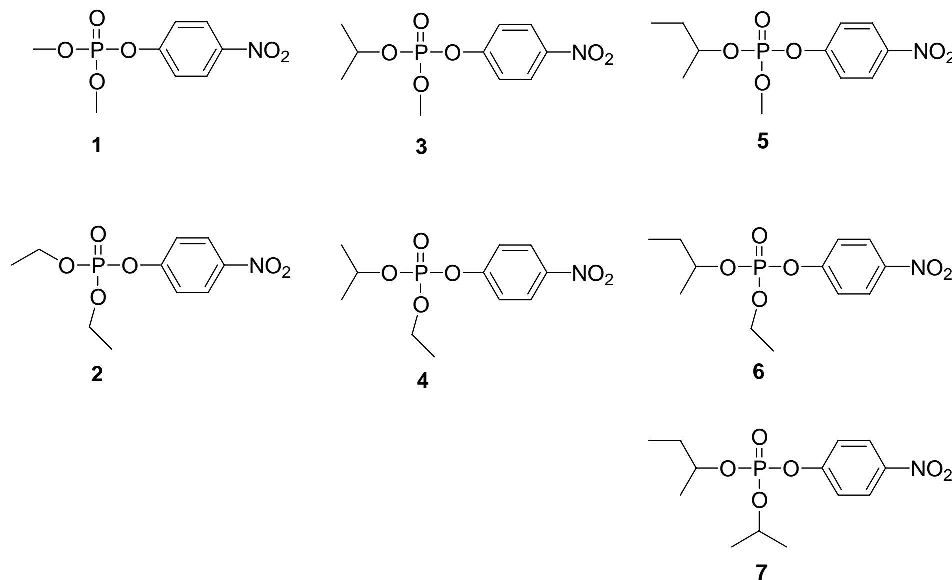
Dimethyl phosphotriester (**1**) and diethyl phosphotriester (**2**) were prepared from *p*-nitrophenol phosphorodichloridate and the corresponding alcohol according to the procedure reported by Steurbaut et al. [15]. The synthesis of racemic phosphotriesters (**3–7**) was conducted in two steps using a modification of the methods of Green and coworkers [16] and Raushel and coworkers [17]. Tris(4-nitrophenyl) phosphate was reacted with 10 equivalents of *i*-propyl alcohol in the presence of one equivalent of *N*,*N*-diisopropyl ethylamine to obtain bis(*p*-nitrophenyl)isopropyl phosphate with a yield of 88%. This material was then reacted with 10 equivalents of the corresponding alcohol (MeOH or EtOH) in the presence of one equivalent of 1,8-diazabicyclo[5.4.0]-undec-7-ene to yield the racemic **3–4** (78–74% yield). The racemic isobutyl phosphates **5–7** (68–59% overall yields) were obtained via a bis(*p*-nitrophenyl)isobutyl phosphate intermediate as described above for the preparation of the racemic isopropyl phosphates **3–4**. All of the racemic organophosphate triesters **1–7** used for this investigation are listed in Scheme 1.

2.2.1. *O-isobutyl-O-methyl-p-nitrophenyl phosphate (**5**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (m, 2H), 7.34 (m, 2H), 4.57 (m, 1H), 3.83 (d, J = 14.4 Hz, 3H), 1.66 (m, 2H), 1.30 (dd, J = 6.3, 20.9 Hz, 3H), 0.90 (dt, J = 7.5, 19.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.4 (d, $J[^{31}P, ^{13}C]$ = 6.1 Hz), 155.3 (d, $J[^{31}P, ^{13}C]$ = 6.4 Hz), 144.3, 125.4, 120.3 (d, $J[^{31}P, ^{13}C]$ = 5.2 Hz), 120.2 (d, $J[^{31}P, ^{13}C]$ = 5.3 Hz), 79.2 (d, $J[^{31}P, ^{13}C]$ = 6.9 Hz), 54.7 (d, $J[^{31}P, ^{13}C]$ = 6.4 Hz), 54.6 (d, $J[^{31}P, ^{13}C]$ = 6.3 Hz), 29.9 (d, $J[^{31}P, ^{13}C]$ = 5.6 Hz), 29.8 (d, $J[^{31}P, ^{13}C]$ = 5.7 Hz), 20.7 (d, $J[^{31}P, ^{13}C]$ = 3.4 Hz), 20.6 (d, $J[^{31}P, ^{13}C]$ = 2.2 Hz), 9.1 (d, $J[^{31}P, ^{13}C]$ = 2.2 Hz); ^{31}P NMR ($CDCl_3$, H_3PO_4 reference): δ −6.71 and −6.63; LRMS (FAB) *m/z*: 290 $(M + H)^+$; HRMS calculated for $C_{11}H_{17}O_6NP$ $(M + H)^+$, 290.0794; found, 290.0799.

2.2.2. *O-isobutyl-O-ethyl-p-nitrophenyl phosphate (**6**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (m, 2H), 7.35 (m, 2H), 4.56 (m, 1H), 4.21 (m, 1H), 1.63 (m, 2H), 1.32



Scheme 1. Structures of the organophosphate triesters.

(m, 6H), 0.90 (dt, $J = 7.5$, 19.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.6, 155.5, 144.4, 125.4, 120.4, 120.3, 79.1 (d, $J[^{31}P, ^{12}C] = 6.4$ Hz), 64.8, 64.7, 30.1 (d, $J[^{31}P, ^{13}C] = 5.8$ Hz), 30.0 (d, $J[^{31}P, ^{13}C] = 5.7$ Hz), 20.8 (d, $J[^{31}P, ^{13}C] = 2.3$ Hz), 20.7 (d, $J[^{31}P, ^{13}C] = 2.4$ Hz), 15.9 (d, $J[^{31}P, ^{13}C] = 6.4$ Hz), 9.2 (d, $J[^{31}P, ^{13}C] = 3.5$ Hz); ^{31}P NMR ($CDCl_3$, H_3PO_4 reference): δ −7.79 and −7.69; LRMS (FAB) *m/z*: 304 $(M+H)^+$; HRMS calculated for $C_{12}H_{19}O_6NP$ $(M + H)^+$, 304.0950; found, 304.0956.

2.2.3. *O-isobutyl-O-isopropyl-p-nitrophenyl phosphate* (**7**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (m, 2H), 7.31 (m, 2H), 4.71 (m, 1H), 4.51 (m, 1H), 1.61 (m, 2H), 1.30 (m, 9H), 0.85 (dt, $J = 7.5$, 19.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.0, 155.9, 144.5, 125.6, 120.6 (d, $J[^{31}P, ^{13}C] = 4.4$ Hz), 120.5 (d, $J[^{31}P, ^{13}C] = 4.3$ Hz), 79.1 (d, $J[^{31}P, ^{13}C] = 5.8$ Hz), 79.0 (d, $J[^{31}P, ^{13}C] = 5.9$ Hz), 74.3 (d, $J[^{31}P, ^{13}C] = 6.2$ Hz), 30.3 (d, $J[^{31}P, ^{13}C] = 6.2$ Hz), 30.2 (d, $J[^{31}P, ^{13}C] = 6.0$ Hz), 23.7 (d, $J[^{31}P, ^{13}C] = 5.0$ Hz), 23.6 (d, $J[^{31}P, ^{13}C] = 4.8$ Hz), 21.0 (d, $J[^{31}P, ^{13}C] = 3.1$ Hz), 20.9 (d, $J[^{31}P, ^{13}C] = 3.0$ Hz), 9.4 (d, $J[^{31}P, ^{13}C] = 6.3$ Hz); ^{31}P NMR ($CDCl_3$, H_3PO_4 reference): δ −8.60 and −8.48; LRMS (FAB) *m/z*: 318 $(M + H)^+$; HRMS calculated for $C_{13}H_{21}O_6NP$ $(M + H)^+$, 318.1107; found, 318.1106.

2.3. *Cloning, expression and purification of E. coli aminopeptidase P (AMPP)*

E. coli genomic DNA was first isolated according to the protocol in Promega Wizard Genomic DNA purification kit. Two primers used to amplify *pepP* were designed according to the DNA sequence deposited in GenBank D00398, with *Eco*RI, *Nde*I and *Bam*HI restriction sites (Table 1, pepP-S and pepP-A). The PCR product was ligated into *Ecor*V restriction site of pBluescript II SK(+) vector to yield pCJ01. The Quick-Change kit® from Stratagene was utilized according to manufacturer's instructions to get rid of the *Nde*I site with in *pepP* sequence. Two complementary oligonucleotides, dNdeI-S and dNdeI-A, were used as the primers (Table 1). The resulting plasmid pCJ02 was verified by sequence analysis. pCJ02 was then cut with *Nde*I and *Bam*HI to get *pepP* DNA insert, which was then ligated into the pET-15b expression vector between *Nde*I and *Bam*HI restriction sites to give pCJ03. This construct also allowed expression of AMPP in N-terminal fusion with a hexahistidine tag in *E. coli* under control of IPTG inducible T7 promoter. This expression construct was verified by sequence analysis and was transformed into *E. coli* strain BL21(DE3). A portion (15 ml) of overnight cell culture was inoculated for each 1 l of culture medium at 37 °C. IPTG was added to a final concentration of 1 mM when the OD_{600} reached between 0.9 and 1.0. The cells were harvested after 4 h by centrifugation at 6000 rpm and stored at −80 °C. The cell pellet was suspended in 25 ml, 20 mM Tris–HCl, pH 7.4, 0.2 mM DTT on ice and passed twice through a French Press with pressure <11,000 psi. The resulting cell lysate was centrifuged at 12,000 rpm for 20 min. To the supernatant added 5 M NaCl and 5 M imidazole solutions so the final concentration of NaCl and imidazole was 500 and 10 mM, respectively. The solution was again centrifuged at 12,000 rpm for 20 min. Finally, the supernatant was applied to a fully equilibrated Ni^{2+} chelating column, which was then washed with 25 ml bound solution (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 10 mM imidazole) to avoid non-specific binding. The bound AMPP was eluted by applying a gradient of imidazole from 10 to 300 mM and 1 ml fractions were collected. Fractions containing AMPP were pooled and dialyzed against 20 mM Tris–HCl, pH 8.2, 0.1 mM EDTA and 0.1 mM DTT overnight. After the first dialysis, 20 mM Tris–HCl without EDTA or DTT was used for the next two buffer changes. The resulting apoenzyme was finally stored in 20% glycerol at −80 °C.

Table 1
Primers used for cloning and site-directed mutagenesis (from **5′** to **3′**)

Primer	Sequence
pepP-S	AGAATTCCATATGAGTGAGATATCCCG
pepP-A	CGGATCCTTACGCTCATTGCTTTC
dNdeI-S	CAGGGCGAATATGCTTATGCTGATGTAATC
dNdeI-A	GATTACATCAGCATAAGCATATTCGCCCTG
R153W-S	GCGTAAAGGTTCGTGGCAAAATCTCACC
R153W-A	GGTGAGATTTTGCCACGAACCTTTACGC
R153L-S	GCGTAAAGGTTCGCTGCAAAATCTCACC
R153L-A	GGTGAGATTTTGCAGCGAACCTTTACGC
R370L-S	GTTTATGGTCAGGATCTCTCGCGCATTCTGG
R370L-A	CCAGAATGCGCGAGAGATCCTGACCATAAAC

2.4. *Site-directed mutagenesis and purification of mutant enzymes*

pCJ03 was used as template and the Quick-Change kit® from Stratagene was utilized according to manufacturer's instructions to give plasmids containing R153W, R153L and R370L mutants, designated as pCJ04, pCJ05 and pCJ06, respectively. Primers used to make the mutants are listed in Table 1. The plasmids containing desired mutations were confirmed by sequence analysis. AMPP mutants were purified using the same method for wild-type AMPP.

2.5. *Enzymatic hydrolysis of organophosphotriesters*

AMPP was first incubated with 1 mM $MnCl_2$ at 37 °C for 1 h. Activated AMPP (5 μL of 1 mg ml^{-1}) was added in 200 μl 50 mM CHES buffer (pH 8.5, 30 °C). The reaction was initiated by adding 2 μl of 50 mM organophosphotriester, which was prepared in 100% methanol. The enzymatic rate was measured by monitoring the difference of accumulation of *p*-nitrophenol at 400 nm ($\varepsilon = 17{,}000\,(M\,cm)^{-1}$) with or without AMPP at 30 °C. One unit (U) of AMPP was defined as the amount of enzyme required to produce 1 μmol *p*-nitrophenol per minute.

2.6. Enzyme activation and metal substitution experiments

AMPP was activated by incubating the apoenzyme ($1\,mg\,ml^{-1}$) at 37 °C for up to 1 h with various amounts of different divalent cations, i.e. Mn^{2+} ($MnCl_2$), Cd^{2+} ($CdSO_4$), Co^{2+} ($CoCl_2$), Mg^{2+} ($MgCl_2$), Ni^{2+} ($NiCl_2$) and Zn^{2+} ($ZnCl_2$). The activity was measured as described above.

2.7. Chemical hydrolysis of organophosphotriesters

The chemical hydrolysis was conducted by adding a large excess of KOH (a final concentration of 1.0 M) over organophosphotriester (50 μM) at 30 °C. The reactions were followed by monitoring the release of *p*-nitrophenolate at 400 nm for at least three half-lives. The pseudo-first-order rate constants were obtained by fitting the absorption (A) to the equation: $A = A_f - A_f[e^{-k(t+t_0)}]$, where A_f is final absorption of the product, k the pseudo-first-order rate constant, and t_0 the time from the start of the reaction to the reading of the first absorption. Data are expressed as means of duplicate assays.

3. Results and discussion

3.1. Mutagenesis and protein purification

The X-ray structure of *E. coli* AMPP with its inhibitor Pro-Leu (access code 1A16) identified residues of interest for mutagenesis. The Arginine residues at amino acid positions 153 and 370 were singly replaced with a tryptophan or leucine residue by site-directed mutagenesis. The wild-type and the mutant proteins were purified to homogeneity as shown by SDS–PAGE in Fig. 1. The calculated molecular weight of wild-type AMPP with His-tag is 51,979 for a monomer. The yield of the proteins was about 30 mg for 1 l flask culture.

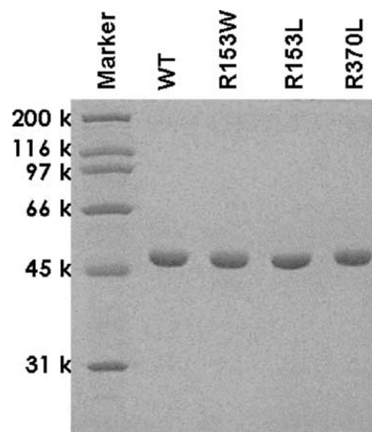


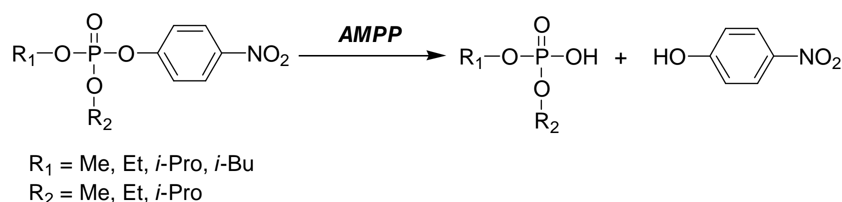
Fig. 1. SDS–PAGE of purified mutant and wild-type AMPP. Purified AMPP was run on a 10% SDS–PAGE gel and stained with coomassie R250. Each lane contains 2.5 μg of Ni chelating column-purified protein.

3.2. Potential roles of metal ions in catalysis

Recently, it was reported that the addition of divalent metal ions could substantially influence AMPP activity on the ability of hydrolysis of bradykinin and dipeptides [7,18]. The precise roles of the two metals are still unclear. To investigate whether the metal ions affect enzymatic hydrolysis of organophosphotriesters (Scheme 2), AMPP activity was assayed with various metal concentrations at pH 8.5. To date, we have tested AMPP with Mn^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} (Table 2). The enzyme was potentiated only by Mn^{2+} and Cd^{2+}. The latter stimulated AMPP activity at 2 μM but was inhibitory at higher concentrations (≥10 μM). Activity measurements performed under the same conditions showed that AMPP was negligibly activated by Co^{2+} and Mg^{2+} with isopropyl methyl *p*-nitrophenyl phosphate **3** as the substrate. Ni^{2+}- and Zn^{2+}-AMPP provided no detectable level of enzymatic activity.

3.3. Effect of various organic solvents on enzyme activity

The effect of organic solvent on AMPP activity was examined using *O*-isopropyl-*O*-methyl-*p*-nitrophenyl phosphate (**3**) as a substrate (Table 3). Enzyme activity was reduced when reaction solution contained organic solvents. Furthermore, the overall hydrolysis rate was decreased with increasing amounts of the same organic solvent (5–10%) in each case. Therefore, it was concluded that the stability of *E. coli* AMPP activity was eliminated by addition of organic solvents; the order of reduction of enzyme stability was methanol < acetonitrile < dimethyl sulfoxide < ethanol < *N*,*N*-dimethylformamide < 2-propanol.



Scheme 2. AMPP-catalyzed hydrolysis of organophosphate compounds.

Table 2
Effect of metal ions on the specific activity of AMPP from *E. coli*[a]

Metal ion (M^{2+})	[M^{2+}] (μM)	Relative specific activity (%)[b]
Mn^{2+}	2	92
	10	93
	25	100
	50	89
	100	75
Cd^{2+}	2	34
	≥10	0
Co^{2+}	2	5
	10	3
	25	2
	≥50	0
Mg^{2+}	2	1
	10	3
	25	2
	50	3
	100	1

[a] Metal activated AMPP was first incubated in 50 mM CHES buffer, pH 8.5 at 30 °C for 3 min. Organophosphotriester **3** (500 μM) was added next to initiate the reaction.
[b] Rates of hydrolysis are expressed relative to that for *O*-isopropyl-*O*-methyl-*p*-nitrophenyl phosphate **3** (100%), which is 80 mU mg^{-1}.

3.4. Chemical hydrolysis of organophosphotriesters (1–7)

In order to evaluate the relative chemical stability of organophosphotriesters (**1**–**7**), the irreversible reactions of these compounds with KOH were assessed by measuring the rate of the release of 4-nitrophenolate at 400 nm. The rate constants obtained under pseudo-first order reactions are listed in Table 4. It is apparent that the rate of hydrolysis decreases with an increase in the chain length and degree of branching for either of the two ligands added to the phosphorus atom.

Table 3
Effect of added organic solvent on organophosphotriester (compound **3**) hydrolysis by AMPP[a]

Organic solvent	Relative specific activity (%)[b]	
	[Organic solvent] = 5%	[Organic solvent] = 10%
1% Methanol[c]	100	100
Methanol	80	56
Acetonitrile	65	39
Dimethyl sulfoxide	55	33
Ethanol	53	24
N,*N*-Dimethylformamide	41	18
2-Propanol	35	19
Tetrahydrofuran	–[d]	–[d]

[a] $MnCl_2$ activated AMPP was first incubated in 50 mM CHES buffer, pH 8.5 at 30 °C for 3 min with 5 or 10% organic solvent. Organophosphotriester **3** (500 μM) was added next to initiate the reaction.
[b] Rates of hydrolysis are expressed relative to that for *O*-isopropyl-*O*-methyl-*p*-nitrophenyl phosphate **3** (100%), which is 80 mU mg^{-1}.
[c] 1% Methanol from substrate solution.
[d] Organophosphotriester **3** precipitated upon addition of the solvent.

Table 4
Chemical hydrolysis of organophosphotriesters by 1.0 M KOH

Organophosphotriesters	k_{obs}[a] (min^{-1})
1 (Me_2NP)	4.65
2 (Et_2NP)	1.23
3 (IspMe)	0.84
4 (IspEt)	0.39
5 (IsobMe)	0.52
6 (IsobEt)	0.26
7 (IsobIsp)	0.08

[a] Determined in 1% methanol solution.

3.5. Enzymatic hydrolysis of organophosphotriesters (1–7)

The specificity for substrate activity exhibited by AMPP was measured by systematically varying the substituents bonded to the phosphorus atom (Scheme 1). The results, shown in Table 5, indicate that AMPP is able to catalyze the cleavage of P–O bond in a variety of racemic organophosphate triesters **1**–**7** (Scheme 2).

O-Isopropyl-*O*-methyl-*p*-nitrophenyl phosphate (**3**) and *O*-isobutyl-*O*-methyl-*p*-nitrophenyl phosphate (**5**) were the preferred substrates for the wild-type enzyme under these conditions. Notably, substrate **3** is hydrolyzed five times faster than isopropyl ethyl *p*-nitrophenyl phosphate **4**. Furthermore, the wild-type AMPP hydrolyzes substrate **5** 12- and 33-folds faster than the analogs of *p*-nitrophenyl phosphate **6** and **7** (Table 5). This finding suggests that the lack of both isobutyl (or isopropyl) and methyl substituents attached to the phosphorus core results in the substrate being inappropriately positioned in the active site of the enzyme and preventing efficient catalysis. In comparison of the cleavage rate of the organophosphate triester **5** (500 μM **5**, 50 mM CHES buffer, 25 μM $MnCl_2$, 30 °C, pH 8.5) to that of a peptide substrate (20 mM Gly-Pro, 20 mM Tris–HCl buffer, 0.02–1.0 mM $MnCl_2$, 37 °C, pH 7.8), the former

Table 5
Hydrolysis[a] of organophosphotriesters by AMPP and selected mutants

Organophosphate compounds	Relative specific activity (%)			
	WT	R153W	R153L	R370L
1 (Me_2NP)	7	7	9	9
2 (Et_2NP)	7	9	9	8
3 (IspMe)	47	56	70	73
4 (IspEt)	9	11	13	13
5 (IsobMe)	100	115	135	144
6 (IsobEt)	8	10	11	10
7 (IsobIsp)	3	4	4	4

[a] The catalytic activity of wild-type and each mutant was assayed as described in Experimental procedures in the presence of CHES buffer (50 mM) and $MnCl_2$ (25 μM), with various organophosphotriesters (500 μM) at 30 °C and pH 8.5. Rates of hydrolysis are expressed relative to that for *O*-isobutyl-*O*-methyl-*p*-nitrophenyl phosphate **5** (100%), which is 170 mU mg^{-1}.

(0.17 U mg^{-1}) was hydrolyzed from 8- to 46-folds slower than the Gly-Pro (1.4–7.8 U mg^{-1}) [7] by wild-type enzyme.

Interestingly, the R153W, R153L and R370L single mutants had a similar effect as the wild-type AMPP on the substrate preference to hydrolyze **3** and **5** versus **1**, **2**, **4**, and **6**, **7**. As seen in Table 5, these mutants exhibited from 1.2- to 1.5-folds increases in the relative rate of hydrolysis of **1–7** as the wild-type enzyme. This observation provides concrete evidence that there is a contiguous hydrophobic interaction between the substituent group of substrates and arginine 153 or 370 in AMPP.

4. Conclusion

The present study demonstrates that aminopeptidase P from *E. coli* is capable of catalyzing the hydrolysis of a wide range of organophosphate triesters. The substitution of the positively-charged Arg residues at positions 153 and 370 with hydrophobic side chains results in the improvement of hydrolysis rates toward most of the substrates. These results suggest that further engineering of AMPP may significantly enhance the cleavage of P–O bonds in a variety of organophosphorus compounds.

Acknowledgements

We thank the National Science Council, Department of Health and Academia Sinica for financial support. We appreciate the preliminary effort of Kelvin Yang and C.-Y. Su in synthesizing organophosphorus compounds.

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