

Catalytic properties of the PepQ prolidase from *Escherichia coli* [☆]

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Abstract

The PepQ prolidase from *Escherichia coli* catalyzes the hydrolysis of dipeptide substrates with a proline residue at the C-terminus. The *pepQ* gene has been cloned, overexpressed, and the enzyme purified to homogeneity. The k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ values for the hydrolysis of Met-Pro are 109 s^{-1} and $8.4 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$, respectively. The enzyme also catalyzes the stereoselective hydrolysis of organophosphate triesters and organophosphonate diesters. A series of 16 organophosphate triesters with a *p*-nitrophenyl leaving group were assessed as substrates for PepQ. The *S*_P-enantiomer of methyl phenyl *p*-nitrophenyl phosphate was hydrolyzed with a k_{cat} of 36 min^{-1} and a $k_{\text{cat}}/K_{\text{m}}$ of $710\text{ M}^{-1}\text{ s}^{-1}$. The corresponding *R*_P-enantiomer was hydrolyzed more slowly with a k_{cat} of 0.4 min^{-1} and a $k_{\text{cat}}/K_{\text{m}}$ of $11\text{ M}^{-1}\text{ s}^{-1}$. The PepQ prolidase can be utilized for the kinetic resolution of racemic phosphate esters. The PepQ prolidase was shown to hydrolyze the *p*-nitrophenyl analogs of the nerve agents GB (sarin), GD (soman), GF, and VX.
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Two distinct groups of proline-specific peptidases have been identified in a wide range of organisms. The first group contains enzymes which hydrolyze the Pro-Xaa bond while the second group consists of enzymes which specifically hydrolyze the Xaa-Pro bond. The former group, including lipases and esterases, belongs to a family of proteins with an α/β -hydrolase structural fold [1,2]. The second group belongs to a family of metallo-proteases that have a pita-bread structural fold [3,4]. The N-terminal and C-terminal domains of the pita-bread family of enzymes contain two α -helices and two antiparallel β -strands. This family of enzymes is further subdivided into type I and type II classes where the type II enzymes have an extra helical subdomain of approximately 60 residues inserted within the C-termi-

nal domain [4]. The pita-bread family of enzymes includes methionine aminopeptidase (Map),¹ proline aminopeptidase (PepP), and prolidase (PepQ). Methionine aminopeptidase removes the N-terminal methionine residue from proteins (Met-/Xaa-), whereas proline aminopeptidase catalyzes the cleavage of the peptide bond between any N-terminal amino acid and a penultimate proline residue (NH₂-Xaa-/Pro-Xaa-) in both short and long peptides. Prolidase is a ubiquitous enzyme, which hydrolyzes dipeptides with proline at the C-terminus (NH₂-Xaa-/Pro-CO₂H). Although these three enzymes have different activities and substrate specificities, the amino acid residues that are used to coordinate the binuclear metal center are conserved [3,5,6]. Two metal ions are coordinated in the active site

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¹ Abbreviations used: PTE, phosphotriesterase; Map, methionine aminopeptidase; PepP, proline aminopeptidase; PepQ, prolidase; OpaA, organophosphorus acid anhydrolase; DFP, diisopropyl fluorophosphate.

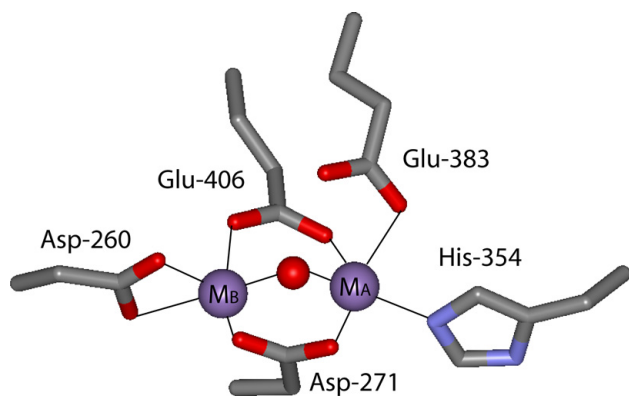
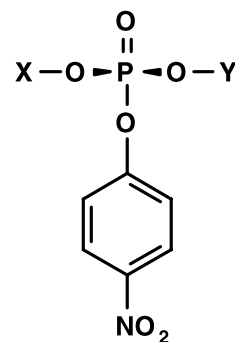


Fig. 1. Structure of the binuclear metal center from PepP prolidase from *E. coli*. The coordinates are taken from PDB file 1az9.

by ligation to two aspartates, two glutamates, and a histidine residue. A representation of the binuclear metal center found in proline amino peptidase is shown in Fig. 1 [7,8].

The amino acid sequences of Map, PepP, and PepQ have a high level of amino acid sequence similarity to organophosphorus acid anhydrolase (OpaA) from *Altermonas* sp. JD6.5. OpaA is capable of hydrolyzing organophosphorus compounds with P–O and P–F bonds. The amino acid sequence of OpaA is approximately 47% identical to that of the *Escherichia coli* prolidase [5]. Organophosphorus acid anhydrolases are found in a wide variety of prokaryotes and eukaryotes [9,10]. Although the natural function of these enzymes is not clear, these proteins may play an important role in the cellular metabolism of dipeptides. The organophosphorus acid anhydrolase, possessing a high level hydrolytic activity toward diisopropyl fluorophosphate (DFP), was purified from *Altermonas* sp. strain JD6.5 [5,11]. The catalytic activity of OpaA toward a series of organophosphate (see Scheme 1) and organophosphonate analogs of toxic chemical warfare agents has been reported [12,13].

Organophosphate triesters are acetyl cholinesterase (AChE) inhibitors that exhibit high toxicity because of their ability to promote the accumulation of acetyl choline and the disruption of nerve transmission [14]. Nevertheless, organophosphates have been distributed extensively for the control of insects and other agricultural pests. The hazardous nature of these compounds and their broad usage have led to efforts for the development of improved methods for the destruction of these toxic substances. The catalytic detoxification of organophosphates was first reported by Mazur [15], who identified an enzyme from rabbit tissue with the ability to hydrolyze DFP, a structural analog of the nerve agents, sarin and soman. In an effort to find safer and more effective alternatives to chemical degradation, attention has turned to the discovery of more proficient enzymes for the recognition and catalytic detoxification of orga-



Scheme 1.

nophosphate nerve agents. We report the cloning, expression, purification, and characterization of the PepQ prolidase from *E. coli* toward the hydrolysis of dipeptides and a wide range of organophosphate nerve agents.

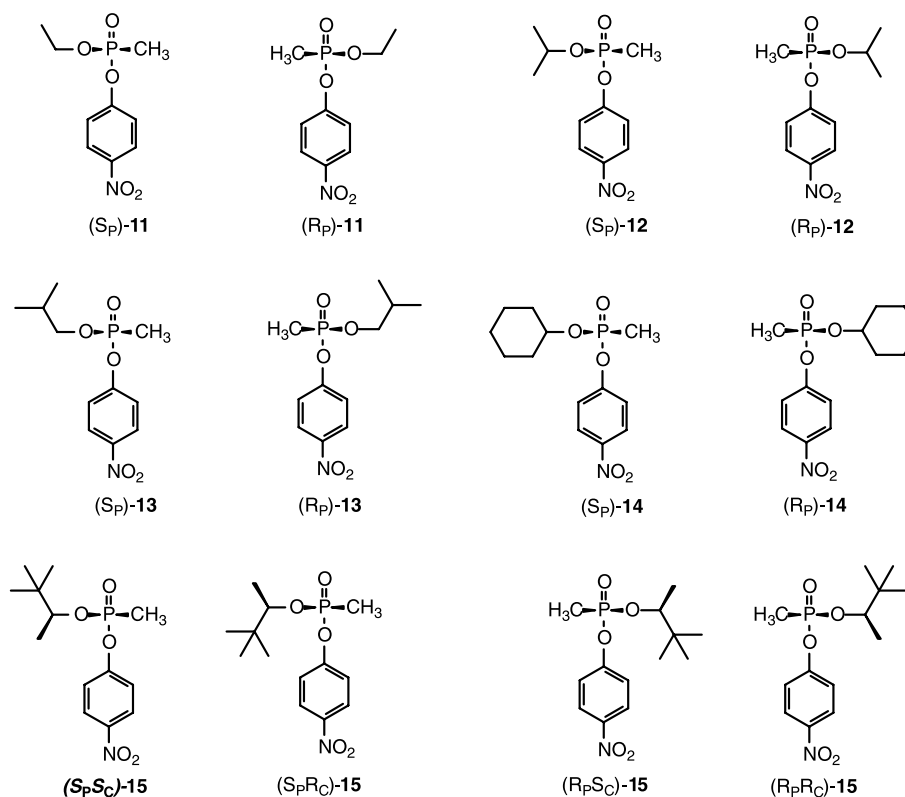
Materials and methods

Materials

The library of chiral organophosphate compounds (1–10) was synthesized as previously described [16–18]. The structures, based upon the template shown in Scheme 1, are presented in Table 2. The chiral analogs of VX (11), sarin (12), rVX (13), GF (14), and soman (15) were prepared according to established protocols [19]. The structures of these compounds are presented in Scheme 2. These organophosphonates are extremely toxic and should be used with the appropriate safeguards. DFP was purchased from Sigma. Wizard Genomic DNA purification kits were purchased from Promega. The gel extraction kit was obtained from Qiagen. Restriction enzymes and T4 DNA ligase were acquired from New England BioLabs. The Xaa-Pro dipeptides listed in Table 1 were purchased from TCI America.

Cloning of pepQ gene

Chromosomal DNA from *E. coli* BL21 (DE3) was purified with a commercial kit (Promega) and used as a template for the amplification of the pepQ gene using the polymerase chain reaction (PCR). PCR was carried out using two sets of primers, designed on the basis of the sequence deposited in GenBank (X54687). The sequence for the first primer was 5'-GGAATTAAGCTTAAGGA GATACATATGGAATCACTGGCCTCG-3' while the sequence for the second primer was 5'-CCGGAATT CTTATTCTTCAATCGCTAACA-3'. The first primer included a *Hind*III restriction site and a ribosomal binding site. The second primer included an *Eco*RI site. PCR fragments were digested with *Hind*III and *Eco*RI and



Scheme 2.

then subcloned into a pBluescript SK+ vector. The recombinant pBluescript vector was transferred to the host *E. coli* BL21 strain. The DNA sequence of the cloned *pepQ* gene was confirmed by the Gene Technology Laboratory at Texas A&M University.

Purification of *PepQ*

The *E. coli* culture was grown at 37°C in 2 liters of LB media in the presence of ampicillin (50 mg/L). Protein expression was induced by the addition of 0.5 mM IPTG after 6 h. Incubation was continued at 37°C for another

Table 1
Kinetic constants for the hydrolysis of Xaa-Pro dipeptides at pH 8.0 and 25°C

Substrate	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (mM)
Ala-Pro	$6.5 (0.5) \times 10^4$	120 ± 30	1.9 ± 0.5
Arg-Pro	$1.4 (0.1) \times 10^6$	142 ± 5	0.10 ± 0.01
Gly-Pro	$2.5 (0.1) \times 10^4$	34 ± 1	1.4 ± 0.2
His-Pro	$8.2 (0.3) \times 10^5$	123 ± 5	0.15 ± 0.01
Ile-Pro	$5.7 (0.4) \times 10^5$	57 ± 3	0.10 ± 0.01
Leu-Pro	$2.1 (0.1) \times 10^5$	215 ± 25	1.0 ± 0.1
Lys-Pro	$8.8 (0.5) \times 10^5$	240 ± 11	0.27 ± 0.02
Met-Pro	$8.4 (0.8) \times 10^5$	109 ± 7	0.13 ± 0.01
Phe-Pro	$4.6 (0.3) \times 10^5$	200 ± 20	0.43 ± 0.06
Pro-Pro	$4.1 (0.3) \times 10^5$	129 ± 15	0.31 ± 0.05
Ser-Pro	$6.0 (0.3) \times 10^5$	281 ± 25	0.46 ± 0.06
Tyr-Pro	$7.1 (0.3) \times 10^5$	116 ± 6	0.16 ± 0.01
Val-Pro	$1.6 (0.1) \times 10^5$	64 ± 7	0.40 ± 0.07

10 h. The cells were harvested by centrifugation and suspended in 50 mM Hepes buffer (pH 8.5). After disrupting the cells by ultra-sonication for 40 min, the cell debris was removed by centrifugation and the supernatant solution was fractionated with $(\text{NH}_4)_2\text{SO}_4$ at 70% saturation. After centrifugation, the pellet was dissolved in 50 mM Hepes buffer (pH 8.5) containing 0.1 mM MnCl_2 and loaded onto a Superdex-200 column. The catalytically active fractions were applied to a Q-Sepharose column and eluted from the column with a linear gradient of 50 mM Hepes buffer (pH 8.5) containing 1.0 M NaCl. Fractions containing the enzyme were concentrated and loaded onto the Superdex-200 column to remove NaCl. SDS gel electrophoresis indicated that the purity of the prolidase was >95%. The purified protein was subjected to N-terminal amino acid sequence analysis. The sequence obtained for the first five residues, **MESLA**, agreed with the published gene sequence. The elution profile from a calibrated gel-filtration column was consistent with the formation of a dimer in solution.

Enzyme assays

The activity of prolidase with a series of Xaa-Pro dipeptides was monitored by following the change in absorbance at 222 nm for the hydrolysis of the peptide bond using an extinction coefficient of $904 \text{M}^{-1}\text{cm}^{-1}$. The assays were conducted in a volume of 1.0 mL in

10mM Tris–HCl buffer, pH 8.0, using a Gilford 260 UV–Vis spectrophotometer at 25°C. The initial velocities for the enzymatic hydrolysis of the series of organophosphate triesters (**1–10**) and organophosphonate diesters (**11–15**) were conducted by measuring the change in absorbance at 400 nm for the release of *p*-nitrophenol. Each 3.0 mL assay contained 50 mM Hepes buffer, pH 8.0, 0.1 mM MnCl₂, 10% (v/v) methanol, substrate, and various amounts of PepQ. The full time courses for the enzymatic hydrolysis of racemic mixtures of organophosphates and organophosphonates were monitored by following the absorbance change for the complete hydrolysis of both enantiomers using variable enzyme concentrations. The total substrate concentration was determined by hydrolysis of all enantiomers with KOH. The hydrolysis of DFP was monitored by following the release of fluoride.

Data analysis

The steady state initial velocities for the hydrolysis of dipeptides, organophosphates (**1–10**), and organophosphonates (**11–15**) were fit to Eq. (1). In this equation k_{cat} is the turnover number, K_{m} is the Michaelis constant, A is the substrate concentration, and E_{t} is the total enzyme concentration. The kinetic constants and the associated errors were obtained by fitting the data to the appropriate equation using SigmaPlot.

$$v/E_{\text{t}} = k_{\text{cat}}A/(K_{\text{m}} + A). \quad (1)$$

Results

Cloning and sequencing of *pepQ* gene

The *pepQ* gene from the BL21 strain of *E. coli* was sequenced and found to contain a stop codon after 1329

nucleotides. Therefore, the prolidase is expected to contain 443 amino acids. The DNA sequence from BL21 matched well with that of the *pepQ* gene from *E. coli* K12 (GenBank Accession No. P21165) except for 24 differences at the third codon position. However, the derived amino acid sequence was exactly the same as for *E. coli* K12. The purified recombinant PepQ, cloned from *E. coli* BL21, has a molecular weight of ~50 kDa as determined from a calibrated SDS electrophoresis gel. The molecular weight of PepQ, based on the DNA sequence, is calculated to be 50,176.

Hydrolysis of dipeptides

The substrate specificity of the recombinant *E. coli* prolidase was evaluated using 13 proline-containing dipeptides (Table 1). Significant catalytic activity occurred with dipeptides containing polar and nonpolar amino acids at the N-terminal position. Dipeptides containing large hydrophobic side chains at the N-terminus have low K_{m} values and high values of $k_{\text{cat}}/K_{\text{m}}$. For example, Met-Pro has a K_{m} value of 0.13 mM and the highest value for $k_{\text{cat}}/K_{\text{m}}$ of $8.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. High values of $k_{\text{cat}}/K_{\text{m}}$ were also obtained with dipeptides containing charged residues. For example, Arg-Pro and Lys-Pro have $k_{\text{cat}}/K_{\text{m}}$ values of 1.4×10^6 and $8.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively.

Hydrolysis of organophosphates

The prolidase from *E. coli* has a broad substrate specificity toward the hydrolysis of organophosphate triesters (Table 2). The active site within the PepQ *E. coli* prolidase can accommodate a variety of bulky side chains attached to the phosphorus center. In addition, the enzyme exhibits a marked stereoselectivity toward the hydrolysis of chiral *p*-nitrophenyl phosphotriester substrates with a clear preference for the S_{P} -enantiomer

Table 2
Kinetic constants for the hydrolysis of organophosphates at pH 8.0, 25°C

Substrate	X	Y	k_{cat} (min ⁻¹)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	K_{m} (mM)
1	CH ₃	CH ₃	0.56 ± 0.06	7.2 ± 0.5	1.3 ± 0.2
(<i>R</i> _P)- 2	CH ₂ CH ₃	CH ₃	0.36 ± 0.04	2.6 ± 0.2	2.3 ± 0.4
(<i>S</i> _P)- 2	CH ₃	CH ₂ CH ₃	39 ± 1	105 ± 3	6.2 ± 0.8
(<i>R</i> _P)- 3	CH(CH ₃) ₂	CH ₃	0.15 ± 0.01	4.8 ± 0.3	0.51 ± 0.07
(<i>S</i> _P)- 3	CH ₃	CH(CH ₃) ₂	12 ± 1.3	100 ± 8	2.0 ± 0.3
(<i>R</i> _P)- 4	C ₆ H ₅	CH ₃	0.40 ± 0.03	11 ± 1	0.58 ± 0.07
(<i>S</i> _P)- 4	CH ₃	C ₆ H ₅	36 ± 4	707 ± 42	0.9 ± 0.1
5	CH ₂ CH ₃	CH ₂ CH ₃	1.2 ± 0.1	3.2 ± 0.1	6.2 ± 0.5
(<i>R</i> _P)- 6	CH(CH ₃) ₂	CH ₂ CH ₃	0.12 ± 0.01	1.1 ± 0.06	1.8 ± 0.3
(<i>S</i> _P)- 6	CH ₂ CH ₃	CH(CH ₃) ₂	0.59 ± 0.06	8.3 ± 0.6	1.2 ± 0.2
(<i>R</i> _P)- 7	C ₆ H ₅	CH ₂ CH ₃	0.22 ± 0.02	4.6 ± 0.2	0.8 ± 0.1
(<i>S</i> _P)- 7	CH ₂ CH ₃	C ₆ H ₅	0.6 ± 0.1	28 ± 2	0.37 ± 0.06
8	CH(CH ₃) ₂	CH(CH ₃) ₂	1.0 ± 0.1	1.7 ± 0.1	10 ± 1
(<i>R</i> _P)- 9	C ₆ H ₅	CH(CH ₃) ₂	0.05 ± 0.01	9.5 ± 0.3	0.09 ± 0.01
(<i>S</i> _P)- 9	CH(CH ₃) ₂	C ₆ H ₅	0.17 ± 0.02	8.1 ± 0.4	0.30 ± 0.05
10	C ₆ H ₅	C ₆ H ₅	0.05 ± 0.01	6.9 ± 0.5	0.13 ± 0.02

over the R_P -enantiomer. The physically smaller substituent is preferentially found at the *proR* position (denoted by X in Scheme 1) while the physically larger substituent is preferentially attached to the *proS* position (denoted by Y in Scheme 1). The stereoselectivity ranges from ~ 60 for the discrimination between phenyl and methyl substituents to ~ 1 for the discrimination between isopropyl and phenyl substituents.

The catalytic constants are influenced by the specific substituents attached to the phosphorus center. Those substrates with a methyl substituent at the *proR* position (X in Scheme 1) have the highest values of k_{cat} and k_{cat}/K_m . The values of k_{cat} are reduced when the methyl group is replaced by other substituents. For example, (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**4**) has a higher k_{cat} value, compared to the values measured for either (S_P)-ethyl phenyl *p*-nitrophenyl phosphate (**7**) or (S_P)-isopropyl phenyl *p*-nitrophenyl phosphate (**9**), which are reduced by 60- and 257-fold, respectively. The enzyme exhibits the highest value of k_{cat}/K_m for (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**4**) and the lowest for (R_P)-ethyl isopropyl *p*-nitrophenyl phosphate (**6**). The enzyme exhibited a clear stereoselective preference for the S_P -enantiomer over the R_P -enantiomer when the substrate has a methyl substituent at the phosphorus center. However, the chiral selectivity is essentially eliminated upon replacement of the methyl substituent with other groups, and the loss of stereoselectivity is also accompanied by poor overall kinetic parameters.

Hydrolysis of organophosphonates

The catalytic activity of the prolidase from *E. coli* was also tested with a variety of methyl phosphonates that are analogs of the nerve agents, sarin (**12**), soman (**15**), GF (**14**), VX (**11**), and rVX (**13**). The kinetic constants are presented in Table 3. For this set of substrates, the R_P -configuration at the phosphorus center is clearly pre-

ferred over the S_P -configuration. However, the relative stereochemical preference (with regard to the orientation of the large and small substituents) is the same as it is for the corresponding series of organophosphate substrates. The best of the methyl organophosphonate substrates is the R_P -enantiomer of the rVX analog (**13**) and the worst is the S_P -enantiomer of the GF analog (**14**). The organophosphonate compounds containing a bulky carbon center within one substituent exhibited greater stereoselectivity. For example, the R_P -enantiomers of the VX (**11**) and rVX (**13**) analogs are 2 and 11 times faster than the corresponding S_P -enantiomers, whereas the R_P -enantiomers of the analogs for sarin (**12**) and GF (**14**) are hydrolyzed 94 and 121 times faster than the corresponding S_P -enantiomers.

The most extreme stereoselectivity is observed among the four analogs of soman (**15**) where there is an additional stereogenic carbon center within the *O*-pinacolyl substituent. The stereochemical preference for the four soman analogs is as follows: $R_P S_C > R_P R_C > S_P R_C > S_P S_C$. These results indicate that the R_P -configuration is preferred relative to the S_P -configuration whereas the preference for the configuration at the carbon center is dependent on configuration at the phosphorus center. The stereoselective preference for the phosphorus center is high, but the difference in the catalytic constants for the chiral carbon center within the *O*-pinacolyl group is relatively small. The $R_P S_C$ -diastereomer of **15** is preferred by a factor of 6100 over the $S_P S_C$ -diastereomer.

Hydrolysis of DFP

The hydrolysis of the P–F bond in DFP is faster than the P–O bond in the organophosphate triesters and organophosphonate diesters tested for this investigation. The values of k_{cat} , k_{cat}/K_m , and K_m for the hydrolysis of DFP are 590 min^{-1} , $250 \text{ M}^{-1} \text{ s}^{-1}$, and $38 \pm 17 \text{ mM}$, respectively. The value of k_{cat}/K_m for the hydrolysis of DFP is ~ 150 -fold higher than that for the hydrolysis of diisopropyl *p*-nitrophenyl phosphate (**8**).

Table 3

Kinetic constants for the hydrolysis of organophosphonates at pH 8.0, 25°C

Substrate	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{ s}^{-1}$)	K_m (mM)
(S_P)- 11	1.6 ± 0.1	8.1 ± 0.3	3.3 ± 0.4
(R_P)- 11	3.4 ± 0.3	17 ± 1.7	3.3 ± 0.5
(S_P)- 12	0.07 ± 0.01	0.32 ± 0.01	3.7 ± 0.5
(R_P)- 12	2.3 ± 0.2	30 ± 1.6	1.3 ± 0.2
(S_P)- 13	2.7 ± 0.2	20 ± 1.7	2.3 ± 0.3
(R_P)- 13	134 ± 27	218 ± 6	10 ± 2
(S_P)- 14	nd	0.6 ± 0.1	nd
(R_P)- 14	45 ± 7	73 ± 3	10 ± 2
($S_P S_C$)- 15	0.010 ± 0.001	0.07 ± 0.01	2.3 ± 0.5
($S_P R_C$)- 15	0.040 ± 0.01	0.11 ± 0.01	5.9 ± 0.6
($R_P S_C$)- 15	33 ± 2	427 ± 16	1.1 ± 0.1
($R_P R_C$)- 15	5.0 ± 0.04	30 ± 2	2.7 ± 0.3

Discussion

The PepQ prolidase from *E. coli* catalyzes the hydrolysis of dipeptides possessing a proline residue at the C-terminus. The enzyme also catalyzes the stereoselective hydrolysis of phosphate and phosphonate esters but the turnover numbers for the dipeptides are significantly greater than for the phosphorus containing esters. The hydrolysis of Met-Pro, for example, is hydrolyzed more than three orders of magnitude faster than (S_P)-methyl phenyl *p*-nitrophenyl phosphate (**4**), the organophosphate substrate with the largest k_{cat}/K_m . The proline amino peptidase (PepP) from *E. coli* has also been reported to cleave a limited number of dipeptides (Xaa-

Pro) [20]. However, the catalytic activity with dipeptides is considerably slower than the turnover numbers for longer peptides. Proline aminopeptidase preferentially hydrolyzes longer polypeptides and is able to distinguish among amino acids that occupy the third and fourth amino acids from the N-terminus of the substrate [21]. The PepP and PepQ prolidases from *E. coli* are from the same enzyme superfamily and thus the amino acid residues that are used to coordinate the binuclear metal centers are conserved but the specific residues that define the substrate binding pockets are different. Recently, the three-dimensional structure of the prolidase from *Pyrococcus furiosus*, that is analogous to PepQ, has been solved and the structural similarity within the active sites of this superfamily has been confirmed [21].

The PepQ prolidase from *E. coli* hydrolyzes the *p*-nitrophenyl analogs of the organophosphorus nerve agents GB, GD, GF, VX, and rVX. The enzyme stereoselectively hydrolyzes organophosphonate esters with a preference for the R_P -configuration of the substrates tested for this investigation (Table 3). This stereoselectivity is similar to that previously observed for OpaA [13]. However, the prolidase from *E. coli* exhibits a more restricted stereoselectivity and a reduced rate of hydrolysis for organophosphonate esters. The k_{cat}/K_m value for the $R_P S_C$ diastereomer of **15** with PepQ is two orders of magnitude slower than the value previously measured for OpaA. Likewise, the k_{cat}/K_m value for the hydrolysis of the R_P -enantiomer of **12** is approximately an order of magnitude slower than the value previously measured for OpaA.

Acetyl cholinesterase is inactivated by the chiral forms of GB, GD, GF, and VX at different rates. For example, the two most toxic diastereomers of GD are those with the S_P -configuration at the phosphorus center [22]. The PepQ prolidase characterized in this investigation preferentially hydrolyzes the analogs of these organophosphonates (**11–15**) with the R_P -configuration. The PepQ prolidase preferentially hydrolyzes the least toxic enantiomer since the stereochemical designations for the analog and the parent organophosphonate are the same. Therefore, if this enzyme is to be considered for the catalytic detoxification or organophosphate nerve agents the rate of hydrolysis of the most toxic enantiomers will have to be enhanced via mutagenesis.

The bacterial phosphotriesterase (PTE) is another enzyme with a demonstrated potential for utilization in the catalytic decomposition of chemical warfare agents. This enzyme has a similar stereoselectivity to that displayed by PepQ for the set of *p*-nitrophenyl analogs of the nerve agents **11–15** (Table 3) and the organophosphates **1–10** (Table 2) [23–25]. However, the overall kinetic parameters obtained with PTE for these substrates are significantly higher than those found in this study for PepQ. For example, the k_{cat}/K_m values for (S_P)- and (R_P)-methyl ethyl *p*-nitrophenyl phosphate

(**2**) with PTE are 10^4 - and 10^5 -fold higher than for prolidase, respectively [23]. Therefore, PTE is clearly superior for the catalytic hydrolysis of these organophosphate nerve agents. The stereochemical selectivity of prolidase for the organophosphates with a methyl substituent (e.g., **2**, **3**, and **4**) is higher than that of PTE. However, the remaining *p*-nitrophenyl organophosphates have a similar degree of stereoselectivity as does PTE.

The X-ray structure of the prolidase from *P. furiosus* has recently been solved in the presence of the inhibitor, (2*S*,3*R*)-3-amino-2-hydroxyl-5-methyl-hexanoyl-1-proline, which resembles Xaa-Pro, and provides some insight into the role of the binuclear metal center for substrate hydrolysis [21]. The carbonyl oxygen from the proline moiety is coordinated to M_A , the hydroxyl group at C-2 displaces the hydroxide that bridges the two divalent cations, and the amino group from C-3 coordinates to M_B . His-192 and His-291 form hydrogen bonds with the C-terminal carboxylic acid and the carbonyl oxygen of the bound inhibitor, respectively. This substrate analog is actually a pseudo-dipeptide substrate linking proline with a β -amino acid. Therefore, caution should be applied in using this structure as a guide for how true substrates actually bind to the active site. Nevertheless, the bridging hydroxide is nicely positioned to initiate nucleophilic attack on the carbonyl group that is polarized via ligation to M_A . The significance of the interaction of the amino group with M_B is not clear. The identity of the group that donates a proton to the leaving group amine is not obvious from the crystal structure obtained in the presence of the bound inhibitor although this function may be served by one of the glutamate residues that also coordinates to M_A (Glu-384 in the PepQ prolidase from *E. coli*). The PepP prolidase likely activates organophosphate esters in a similar fashion; M_A could polarize the phosphoryl oxygen bond prior to nucleophilic attack from the bridging hydroxide. Mechanistic experiments are in progress to test these proposals.

References

- [1] J.D. Schrag, Y. Li, S. Wu, M. Cygler, Nature 351 (1991) 761–764.
- [2] T. Kabashima, M. Fujii, Y. Hamasaki, K. Ito, T. Yoshimoto, Biochim. Biophys. Acta 1429 (1999) 516–520.
- [3] J.F. Bazan, L.H. Weaver, S.L. Roderick, B.W. Matthews, Proc. Natl. Acad. Sci. USA 91 (1994) 2473–2477.
- [4] W.T. Lowther, B.W. Matthews, Biochim. Biophys. Acta 1477 (2000) 157–167.
- [5] T.-C. Cheng, S.P. Harvey, G.L. Chen, Appl. Environ. Microbiol. 62 (1996) 1636–1641.
- [6] M. Ghosh, A.M. Grunden, D.M. Dunn, R. Weiss, M.W.W. Adams, J. Bacteriol. 180 (1998) 4781–4789.
- [7] M.C.J. Wilce, C.S. Bond, N.E. Dixon, H.C. Freeman, J.M. Guss, P.E. Lilley, J.A. Wilce, Proc. Natl. Acad. Sci. USA 95 (1998) 3472–3477.

- [8] S.C. Graham, M. Lee, H.C. Freeman, J.M. Guss, *Acta Crystallogr. D* 59 (2000) 897–902.
- [9] J.J. DeFrank, W.T. Beaudry, T.-C. Chang, S.P. Harvey, A.N. Stroup, L.L. Szafraniec, *Chem. Biol. Interact.* 87 (1993) 141–148.
- [10] W.G. Landis, J.J. DeFrank, *Adv. Appl. Biotechnol.* 4 (1990) 183–201.
- [11] T.-C. Cheng, L. Liu, J. Wang, J. Wu, J.J. DeFrank, D.M. Anderson, V.K. Rastogi, A.B. Hamilton, *J. Ind. Microbiol.* 18 (1997) 49–55.
- [12] C.M. Hill, F. Wu, T.-C. Cheng, J.J. DeFrank, F.M. Raushel, *Biochem. Med. Chem.* 10 (2000) 1285–1288.
- [13] C.M. Hill, W.-S. Li, T.-C. Cheng, J.J. DeFrank, F.M. Raushel, *Bioorg. Chem.* 29 (2001) 27–35.
- [14] D.J. Ecobichon, in: C.D. Klaassen (Ed.), *The Basic Science of Poisons*, McGraw-Hill, New York, 1995, pp. 655–666.
- [15] A. Mazur, *J. Biol. Chem.* 164 (1946) 271–289.
- [16] S.B. Hong, F.M. Raushel, *Biochemistry* 38 (1999) 1159–1165.
- [17] T. Koizumi, Y. Kobayashi, H. Amitani, E. Yoshii, *J. Org. Chem.* 42 (1977) 3459–3460.
- [18] W.J. Donarski, D.P. Dumas, D.P. Heitmeyer, V.E. Lewis, F.M. Raushel, *Biochemistry* 28 (1989) 4650–4655.
- [19] F. Wu, W.-S. Li, M. Chen-Goodspeed, M.A. Sogorb, F.M. Raushel, *J. Am. Chem. Soc.* 122 (2000) 10206–10207.
- [20] T. Yoshimoto, A.T. Orawski, W.H. Simmons, *Arch. Biochem. Biophys.* 1 (1994) 28–34.
- [21] M.J. Maher, M. Ghosh, A.M. Grunden, A.L. Menon, M.W. Adams, H.C. Freeman, J.M. Guss, *Biochemistry* 43 (2004) 2771–2783.
- [22] H.P. Benschop, L.P.A. De Jong, *Acc. Chem. Res.* 21 (1988) 368–374.
- [23] D.P. Dumas, H.D. Durst, W.G. Landis, F.M. Raushel, J.R. Wild, *Arch. Biochem. Biophys.* 164 (1989) 1137.
- [24] S.B. Hong, F.M. Raushel, *Chem. Biol. Int.* 119–120 (1999) 225–234.
- [25] W.S. Li, K.T. Lum, M. Chen-Goodspeed, M.A. Sogorb, F.M. Raushel, *Bioorg. Med. Chem.* 9 (2001) 2083–2091.