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Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from *Alteromonas haloplanktis*

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Organophosphorus acid anhydrolases (OPAA) catalyzing the hydrolysis of a variety of toxic organophosphorus cholinesterase inhibitors offer potential for decontamination of G-type nerve agents and pesticides. The gene (*opa*) encoding an OPAA was cloned from the chromosomal DNA of *Alteromonas haloplanktis* ATCC 23821. The nucleotide sequence of the 1.7-kb DNA fragment contained the *opa* gene (1.3 kb) and its flanking region. We report structural and functional similarity of OPAAs from *A. haloplanktis* and *Alteromonas* sp JD6.5 with the enzyme prolidase that hydrolyzes dipeptides with a prolyl residue in the carboxyl-terminal position. These results corroborate the earlier conclusion that the OPAA is a type of X-Pro dipeptidase, and that X-Pro could be the native substrate for such an enzyme in *Alteromonas* cells.

Keywords: *Alteromonas* organophosphorus acid anhydrolase gene; X-Pro dipeptidase; sequencing and functional homology to prolidase

Introduction

Organophosphates (OPs) are hydrolyzed by a group of enzymes generally classified as organophosphorus acid anhydrolases (OPAA: EC 3.1.8.2). Fifty years ago, Mazur [23] first described the presence of an enzyme in crude preparations of rabbit tissue that hydrolyzed the acetylcholinesterase inhibitor diisopropyl fluorophosphate (DFP). Since then the DFP-hydrolyzing enzymes have been reported in diverse organisms such as squid [17], protozoa [19], mammals [20], clams [1], *Escherichia coli* [29] and soil bacteria [2]. The physiological significance of these enzymes in the cell has not been elucidated. DFP, a serine protease inhibitor, is commonly used as a substrate to screen for the presence of OPAA enzymes.

An interest in the use of microbial enzymes for the degradation of OPs began with the purification of a parathion-degrading enzyme from *Pseudomonas diminuta* MG and *Flavobacterium* species ATCC 27551 [10,16]. The enzyme, designated as organophosphorus acid hydrolase (OPH) (EC 3.1.8.1), is encoded by the *opd* gene which encodes a polypeptide of 325 amino acids with a molecular weight of 35 kDa [25]. Recently, we reported purification of an OPAA enzyme with high levels of DFP-hydrolyzing activity from a halophilic isolate, *Alteromonas* sp JD6.5 [7]. Subsequent screening established the presence of high levels of DFP-hydrolyzing activity in other *Alteromonas* species [8] and from *A. undina* [4]. OPAAs that catalyze the hydrolysis of a variety of toxic OP cholinesterase inhibitors offer potential for decontamination of G-type nerve agents and pesticides. The OPAAs from *Alteromonas* sp JD6.5 and *A. undina* are similar in their catalytic properties [4,7]. OPAAs from both the *Alteromonas* species and OPH have functional similarities in that they exhibit varying degrees of hydrolytic activity against different G-type nerve agents such as GB (Sarin; O-isopropyl methylphosphonofluoridate), GD (soman; O-pinacolyl methylphosphonofluoridate), GF (O-cyclohexyl methylphosphonofluoridate), and DFP [4,7,8]. Figure 1 shows the structure of these G-type nerve agents and DFP. In general, OPAAs from *Alteromonas* have a significantly higher soman-hydrolyzing activity. In contrast, OPH displays higher activity against the pesticide derivative paraoxon [4,7,8,11]. Recently, we reported the nucleotide sequence of the gene encoding OPAA (OPAA-2) from *Alteromonas* sp JD6.5 [5]. No sequence homology was found between this enzyme and OPH [5].

The amino acid sequence of OPAA-2 exhibited structural similarity to human prolidase (E.C. 3.4.13.9), an X-Pro dipeptidase [5]. In this paper, we report the molecular cloning and nucleotide sequence of an OPAA encoding gene (*opa*) from *A. haloplanktis* (ATCC 23821). Amino acid sequences deduced from the nucleotide sequence of the two OPAA-encoding genes were found to be over 80% identical. In addition, OPAAs from *A. haloplanktis* and *A. undina* exhibit X-Pro dipeptidase activity. Taken together, these observations are consistent with the idea that *Alteromonas* OPAAs are indeed X-Pro dipeptidases, and the native function of such enzymes may be involved in catabolism of X-Pro dipeptides.

Methods

Purification of OPAA and protein sequencing

The purification of OPAAs from either native or recombinant cells was performed by procedures similar to those described earlier [4,5,7].

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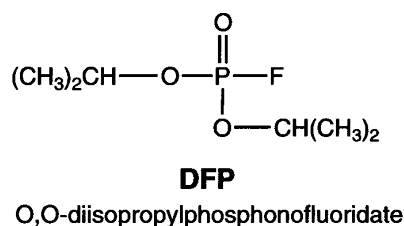

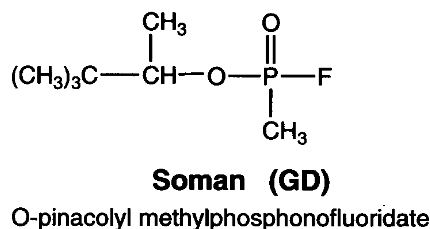

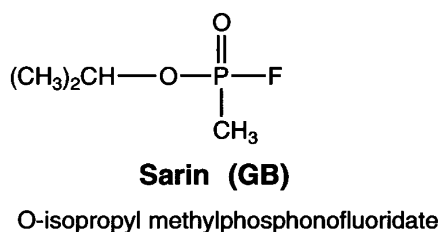

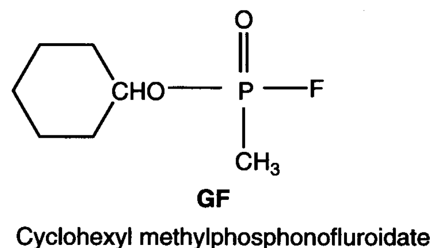

Figure 1 The chemical structure of DFP and organophosphorus G-agents.

For internal amino acid sequencing, about 15 mg of purified OPAA protein was dialyzed to remove salts and air-dried. A 70% formic acid and 0.15 M CNBr solution was added to the protein sample, and incubated overnight at room temperature. The formic acid was evaporated by blowing air over the sample to dryness. The CNBr cleavage products were separated by 15% SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (PVDF: Millipore, Bedford, MA, USA) [18,22]. Two peptide fragments (OPAA-C1 and OPAA-C2) were obtained. The purified enzyme and peptide fragments were sent to Jan Pohl, Emory University School of Medicine, Atlanta, GA, USA, for amino acid sequence determination. The sequences obtained were: OPAA-C1, PYGNIVALNENXAILHYTHFEPKA; and OPAA-C2, RDETGAHEX (X, unidentified residue).

Preparation of oligonucleotide probes

Based on the amino acid sequence of AA-1 (No. 16 to No. 23 of OPAA-C1) and AA-2 (No. 2 to No. 8 of OPAA-C2), two oligonucleotide pools oligo-3 (5′-CAYTAYACICAYTTYGARCCIAA-3′) (Y = T or C; R = A or G; I = inosine) and oligo-5 (5′-GAYGARACIGGIGGICAYCA-3′) were prepared. The oligonucleotide pools were labeled with digoxigenin-11-ddUTP (DIG) using the Genius system of Boehringer Mannheim (Indianapolis, IN, USA) according to the manufacturer's protocol.

Cloning of A. haloplanktis OPAA gene

A. haloplanktis genomic DNA was isolated following procedures described by Bagdasarian and Bagdasarian [3]. The total DNA was partially digested with *Sau*3AI and fragments from 8 to 12 kb in size were isolated from a preparative agarose gel. Pre-digested ZAP Express *Bam*HI/CIP vector cloning kit and Gigapack II packing extracts (Stratagene, La Jolla, CA, USA) were used according to the manufacturer's instructions to construct genomic libraries. About 5×10^5 plaques obtained in the initial construction of library were amplified by plating to prepare a high-titer phage stock.

The genomic library of *A. haloplanktis* was screened with DIG-labeled oligonucleotides according to the manufacturer's instruction. Briefly, in the screening procedure, the hybridization temperature for both labeled oligonucleotide pools was 40°C, whereas the washing temperatures for oligo-3 and oligo-5 were 45°C and 42°C, respectively.

Mutagenesis with transposon Tn1000 (γδ)

The plasmid pCG411 was mutagenized by Tn*1000* by selecting for F factor-mediated conjugative transfer of the plasmid from XL1 blue cell to the F recipient strain DH10β according to the procedures described [15]. Before mating, both parental cells were grown at 37°C without shaking to a concentration between 5×10^7 to 1×10^8 cells ml^{-1} in LB medium. One milliliter of donor cells and 0.5 ml of recipient cells were then mixed in a small flask and incubated for 30 min at 37°C. Ten milliliters of pre-warmed LB were added and incubated for an additional 2 h. Streptomycin (50 μg ml^{-1}) was then added to kill the donor cells. The surviving cells were collected and plated on LB agar containing both ampicillin (100 μg ml^{-1}) and streptomycin (50 μg ml^{-1}) to select for DH10β(pCG411::Tn*1000*) exconjugants. The plasmid DNA isolated from these exconjugants carried Tn*1000* insertions in pCG411. The insertion sites were determined by restriction endonuclease mapping. OPAA enzyme assays were performed to determine which insertions inactivated the gene.

DNA sequencing

Tn*1000* insertions were used to determine the nucleotide sequence of OPAA gene [21]. Both strands of the DNA fragments were sequenced. The nucleotide sequence of *A. haloplanktis* OPAA was determined by the Silver Sequence System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The system uses thermal cycle sequencing and a silver staining protocol to detect the ladders in the DNA sequencing gel. The sequence of the γ primer is 5′-ATATAAACAACGAATTATCTCC-3′, corresponding to the sequence 59 to 38 bases from the γ end, while the δ primer is 5′-GTATTATAATCAATAAGTTAT-

ACC-3′, corresponding to the 62 to 39 bases from the δ end. Plasmid DNA (pCG411::Tn*1000*), isolated by an alkaline method [28] and linearized by digestion with *Bam*HI, was purified by gel electrophoresis. The annealing temperature was determined by PCR using γ or δ primer in conjunction with M13 forward primer (5′-CGCCAGGG-TTTTCCCAGTCACGAC-3′). Sequencing cycles were as follows: 95°C for 2 min (pretreatment), and 60 cycles of 95°C for 30 s, 55°C for 30 s, and 70°C for 1 min followed by ending at 4°C.

Assay of OPA anhydrolase

Enzyme activity against DFP and G-agents was assayed by monitoring F^- release with an ion-specific electrode following a modified method as described previously [6]. Briefly, the reaction medium contained 50 mM $(NH_4)_2CO_3$, pH 8.7, 0.1 mM $MnCl_2$, 3 mM DFP or G-agents, and 5–25 μl of enzyme sample in a total volume of 2.5 ml. Assays were run at 25°C in a temperature-controlled vessel with stirring. One unit (U) of activity is defined as the release of 1.0 μmole of F^- min^{-1}. Specific activity is expressed as U mg^{-1} protein.

Dipeptidase and aminopeptidase activities

The relative activity of OPAA enzymes against several dipeptides and tripeptides was determined by measuring the release of amino acids from corresponding dipeptide or tripeptide using the following modified Cd-ninhydrin method (method-D) [9]. The ninhydrin reagent was prepared by dissolving 0.8 g of ninhydrin in a mixture of 80 ml of 99.9% ethanol and 10 ml of acetic acid, followed by addition of 1 g $CdCl_2$ dissolved in 1 ml of water. The reaction with a dipeptide or tripeptide was carried out in a 200-μl volume of 50 mM $(NH_4)_2$ CO_3, pH 8.7, 0.1 mM $MnCl_2$, 2 mM dipeptide at 25°C, and started by addition of 0.2 U of enzyme. Twenty microliters of reaction mixture were immediately transferred to a 96-well polystyrene micro-titer plate. The reaction at various times was stopped by addition of 150 μl Cd-ninhydrin reagent. The color was developed by heating the plate in an incubator for 15 min at 85°C. After cooling, the absorbance was read at 490 nm using a microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA). The amount of monomeric amino acids released was determined using a standard curve with amino acids from the test peptide. Specific activity was expressed as μmole of both amino acids released min^{-1} mg^{-1} protein.

Protein assay

The Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) was used for protein determination with bovine serum albumin (BSA) as a standard.

Protein sequence analysis

Homology searches of the protein sequences were carried out with MacVector version 4.0 sequence analysis software (Oxford Molecular, Campbell, CA, USA).

Nucleotide sequence accession number

The GenBank accession numbers for the *opa* gene of *A. haloplanktis* and *Alteromonas* sp JD6.5 are U56398 and U29240, respectively.

Results

Cloning of A. haloplanktis OPAA gene

The N-terminal amino acid sequence of purified *A. haloplanktis* OPAA was determined and yielded a very weak signal even though a large quantity of protein was used. The result suggested that the enzyme may be blocked at the N-terminus. The amino termini of two cyanogen bromide-derived internal peptides, OPAA-C1 (PYGNIVALNEN-X′AILHYTHFEPKA) and OPAA-C2 (RDETGAHEX′), were sequenced. The oligonucleotides derived from the two peptides, oligo-3 (5′-CAYTAYACICAYTTYGARCCIAA-3′) and oligo-5 (5′-GAYGARACIGGIGGICAYCA-3′) were used for screening the genomic library.

Four putative OPAA clones, pCG407, pCG408, pCG409 and pCG410 were identified from about 10^5 plaques after sequential screening with oligo-3 and oligo-5. Cell lysates obtained from cells harboring pCG407 or pCG408 clones exhibited the DFP-hydrolyzing activity. The specific activity of cell lysates from pCG408-harboring cells was higher than that from pCG407 (27.0 *vs* 6.0 U mg^{-1} protein), and was approximatley 300 times that of the parent *A. haloplanktis* strain C. After isolation and restriction endonuclease digestion of the inserts from recombinant plasmids with a number of restriction enzymes (Figure 2), both clones were found to contain the complete OPAA gene in the opposite orientation with respect to the vector. The other two clones, pCG409 and pCG410, contained DNA inserts with a partial overlap region with the sequence in pCG407 and pCG408. To confirm the transcription orientation, the insert DNA fragments from pCG407 and pCG408 were sub-cloned into pUC18 and pUC19 using restriction endonucleases *Eco*RI and *Sal*I. Two resultant plasmids, pCG413 and pCG416, derived from pCG408 and pCG407, respectively, produced high specific OPAA activity (40.0 U mg^{-1}) with DFP after IPTG induction. The data strongly suggested the OPAA gene is expressed and it has the same transcription orientation as the *lac* promoter in the vector plasmid. The locations of the oligo-3 and oligo-5 oligomers used for probing were mapped within 500 bp from the right-end of the coding fragment (Figure 3).

Gene mapping and DNA sequencing of A. haloplanktis OPAA gene

Tn*1000* is a transposon (also known as γ δ) on the *E. coli* F factor. Tn*1000* insertion mutagenesis [15] was used to map the location of the *A. haloplanktis* OPAA gene on the inserted DNA fragment in pCG411 to a 1.3-kb fragment (heavy line) by 13 insertions that resulted in loss of OPAA activity (Figure 2).

The ends of Tn*1000* insertions were used as primer binding sites for determining the nucleotide sequence of 1700 bp encompassing OPAA gene (Figure 3). Within this sequenced region, only one large open reading frame (ORF) was found. The deduced molecular weight of the protein encoded by this ORF is 50 kDa, smaller than that of OPAA-2 (59 kDa) from *Alteromonas* sp JD6.5. The start ATG codon is at position 277, and the termination codon TAA is at position 1596. Preceding the start codon is a putative 5′-GATGGG-3′ ribosome-binding site at position 265, in which four of the six nucleotides are identical to the ribo-

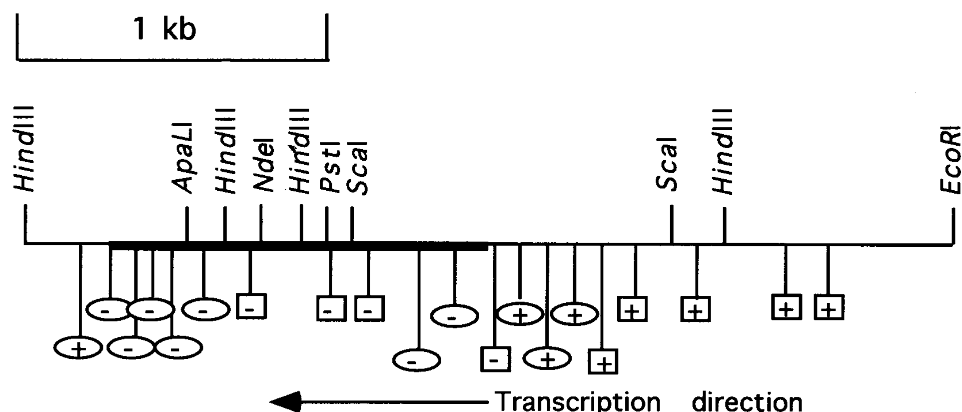

Figure 2 Restriction endonuclease site map and sequence strategy for the *A. haloplanktis* OPAA gene. *A. haloplanktis* OPAA gene (GenBank accession number U56398) was mapped and sequenced by Tn*1000* insertion mutagenesis in pCG411. The location of the OPAA gene was mapped to a 1.3-kb fragment that encodes enzyme activity against DFP. ○, γ to δ; □, δ to γ; + and −, with or without OPAA activity, respectively.

some-binding site of the OPAA-2 gene (5′-AGTGGG-3′) [5].

Structural similarity of OPAA from A. haloplanktis and Alteromonas sp JD6.5

The deduced primary structure of OPAA from *A. haloplanktis* is composed of 440 amino acids, whereas OPAA-2 of *Alteromonas* sp JD6.5 contains 517 amino acids [4]. As reported earlier [5], a significant structural identity was found between OPAA-2 and a protein (PepQ) encoded by the *E. coli pepQ* gene [26] and a human prolidase [14]. The amino acid sequences of *A. haloplanktis* and *Alteromonas* sp JD6.5 OPAAs, and *E. coli* PepQ are highly conserved throughout their aligned sequences. There is 81% amino acid sequence identity between the *A. haloplanktis* and *Alteromonas* sp JD6.5 enzymes over the full lengths of the *A. haloplanktis* sequence without any gap or insertion. When comparisons included checking for amino acids of similar functional groups, 91% similarity was observed. The sequence of the *E. coli* PepQ sequence (residues 1–442), with the introduction of several breaks and gaps, shows a 51% and 49% identity to that of enzymes from *A. haloplanktis* and *Alteromonas* sp JD6.5, respectively. Based on the structural similarities, it appears that *E. coli* PepQ is an enzyme similar to that of the *Alteromonas* enzymes. In addition, a segment of amino acid sequence from both *A. haloplanktis* and *Alteromonas* sp JD6.5 enzyme sequences (residues 56–440) have 28% identity to the human prolidase sequence (residues 96–466). Overall, these similarities suggest that OPAAs from *A. haloplanktis* and *Alteromonas* sp JD6.5, human prolidase and *E. coli* PepQ are evolutionarily and functionally related.

Functional similarities among OPAAs

The *A. haloplanktis* OPAA has optimum activity at pH 7.5 and 40°C (data not shown). Comparison of the recombinant OPAAs from *A. haloplanktis* and *Alteromonas* sp JD6.5 [7], as well as an OPAA purified from *A. undina* [4], revealed a wide spectrum of shared activities and biological properties: a) they all consist of a single polypeptide with a molecular weight ranging between 50 and 60 kDa; b) the pH optima is between 7.5 and 8.5 and temperature optima between 40 and 55°C; c) the three enzymes showed increased activity and stability in the presence of Mn^{2+}, and were inhibited by DFP analog Mipafox (N,N′-diisopropyl phosphorodiamidofluoridate), the sulfhydryl inhibitor *p*-chloromercuribenzoate (PCMB), and N-ethylmaleimide (NEM). The OPAA from the three *Alteromonas* species exhibit various degrees of enzyme activity against DFP, and G-type nerve agents GB, GD, and GF (Table 1). The OPAAs from *Alteromonas* sp JD6.5 and *A. undina* exhibited comparable high specific activity against GD with low specific activity against GB. The OPAA from *A. haloplanktis* had lower specific activity against all the substrates tested. However, with $(NH_4)_2CO_3$ adjusted to pH 7.5, the enzyme from *A. haloplanktis* exhibited similar specific activity (1760 ± 82 U mg^{-1}) against DFP as that from *Alteromonas* sp JD6.5. Overall, assays with *A. haloplanktis* enzyme and replacing 50 mM Bis-tris propane (pH 7.2) with $(NH_4)_2CO_3$ resulted in at least a six-fold increase of the specific activity.

Functional relationship with prolidase

Prolidase is ubiquitous in nature and hydrolyzes dipeptides with a prolyl residue in the carboxyl-terminal position. To determine whether the OPAAs from *A. haloplanktis*, *Alteromonas* sp JD6.5, and *A. undina* are functionally related to prolidase, the relative activities against various dipeptides and tripeptides were examined (Table 2). All these enzymes displayed high activity against dipeptides with proline at the C-terminus (X-Pro), but no activity against dipeptides with proline at the N-terminus (Pro-X). Leu-Pro was hydrolyzed at a rate higher than that of Ala-Pro. The *A. haloplanktis* enzyme exhibited a higher specific activity against the Leu-Pro and Ala-Pro than those from *Alteromonas* sp JD6.5 and *A. undina*, but lower for non-Pro dipeptides. The *A. undina* enzyme displayed a broad range of substrate hydrolysis with highest specific activity against Gly-Glu. All three *Alteromonas* enzymes also exhibited some activity against Leu-Ala, but displayed no catalytic activity against tested tripeptides including Gly-Pro-Ala and Ala-Pro-Phe, which are substrates for aminopeptidase P. On the basis of results summarized above, the OPAA from three *Alteromonas* species functionally appear to be a type of X-Pro dipeptidase, with a broad range of substrate specificity.

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AAGAAGCAAT GCCGGCTGAG CTATGGGTTG AACTTATTCC TTACAGAGAA ACCCGAGATT ATGTAAAAAA TGTATTTGCT TATCGGCAGG TTTATCATAC   100

TCGCCTTGGT CGGGAGGGAA ACCTTCTAGC CCCCATATTA GAAATGACGA TGGGCGGATA GTAAAAATAA ATCTACAACC ACAGAGGGAT ATTTACACAG   200

TAGTATCCCT CTTTTTTGAG CGCTTGTGTA TAAGTGGTAG ACTCAAAGCA AGTTAAATTT AACAGATGGG TTTATT ATG GAA AAA TTA GCC GTT   294
                                                                                     M   E   K   L   A   V>

TTA TAC GCC GAA CAT ATT GCA ACA TTG CAG CAG CGT ACA CGT ACT ATT TGT GAG CAA GAA GGG TTA GAA GGA TTA GTC ATT   375
 L   Y   A   E   H   I   A   T   L   Q   Q   R   T   R   T   I   C   E   Q   E   G   L   E   G   L   V   I>

CAT TCA GGC CAA GCT AAG CGC CAA TTT TTA GAT GAT ATG TAT TAC CCG TTT AAA GTT AAC CCT CAT TTT AAA GCG TGG CTA   459
 H   S   G   Q   A   K   R   Q   F   L   D   D   M   Y   Y   P   F   K   V   N   P   H   F   K   A   W   L>

CCG GTT ATT CAT AAT CCA CAT TGC TGG ATT GTG GTA AAT GGT AGC GAT AAG CCA AAA CTT ATT TTT TAT CGC CCA ATT GAT   537
 P   V   I   H   N   P   H   C   W   I   V   V   N   G   S   D   K   P   K   L   I   F   Y   R   P   I   D>

TTT TGG CAT AAA GTA CCT GAT GAG CCA AGA GAT TTT TGG GCA GAA TAC TTC GAT ATT GAA TTG TTA TTA CAA CCC GAT CAG   618
 F   W   H   K   V   P   D   E   P   R   D   F   W   A   E   Y   F   D   I   E   L   L   L   Q   P   D   Q>

GTT GAA AAG CTA CTA CCT TAC GAT AAA GCT AAA TTT GCC TAC ATT GGT GAA TAC CTC GAA GTA GCA CAA GCA CTT GGC TTT   699
 V   E   K   L   L   P   Y   D   K   A   K   F   A   Y   I   G   E   Y   L   E   V   A   Q   A   L   G   F>

AGT ATT ATG AAC CCT GAG CCA GTA CTT AAC TAT ATT CAT TAC CAC CGT GCT TAT AAA ACG CAA TAT GAA CTT GAA TGT TTA   780
 S   I   M   N   P   E   P   V   L   N   Y   I   H   Y   H   R   A   Y   K   T   Q   Y   E   L   E   C   L>

CGT AAT GCG AAT CGT ATT GCG GTT GAT GGC CAT AAA GCA GCG CGT GAT GCG TTT TTT AAT GGT GGT AGC GAG TTT GAT ATT   861
 R   N   A   N   R   I   A   V   D   G   H   K   A   A   R   D   A   F   F   N   G   G   S   E   F   D   I>

CAG CAA GCT TAC TTA ATG GCA ACG CGC CAA AGT GAA AAC GAA ATG CCA TAT GGC AAT ATT GTG GCA CTT AAC GAA AAC TGC   942
 Q   Q   A   Y   L   M   A   T   R   Q   S   E   N   E   M   P   Y   G   N   I   V   A   L   N   E   N   C>

GCT ATT TTG CAC TAC ACC CAT TTT GAG CCA AAA GCA CCA CAA ACG CAT AAT TCA TTT TTA ATT GAT GCG GGC GCT AAT TTT  1023
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 N   G   Y   A   A   D   I   T   R   T   Y   D   F   K   K   Q   G   E   F   A   D   L   V   N   A   M   T>

GCG CAT CAA ATT GAG TTA GGA AAA AGC TTA AAG CCA GGT TTA CTG TAT GGC GAT CTG CAT ATT GAT TGT CAT AAC CGT ATT  1185
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CCG CAT GGC TTA GGG CAT CAT TTA GGT GCA CAG GTT CAC GAT GTG GGT GGT TTT ATG CGT GAT GAA ACA GGG GCA CAT CAA  1347
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 Y   F   I   D   S   L   L   G   D   L   A   Q   T   D   N   K   Q   F   I   N   W   E   K   V   E   A   F>

AAA CCT TTT GGC GGT ATT CGT ATC GAG GAC AAT ATT ATT GTT CAC GAA GAT AGC CTA GAA AAT ATG ACG CGT AAT TTA TTA  1590
 K   P   F   G   G   I   R   I   E   D   N   I   I   V   H   E   D   S   L   E   N   M   T   R   N   L   L>

CTC GAC TAAATCCTTA TTAAAGACGA GTTAGTCAAT ACGCGCTAAA ATAAACGGAT ACAAAGGGGC CTTTTGGCCC CATTTTTTTT AATTAAAGCC   1686
 L   D>  *

TAAGTAAGTG CTAT (1700)
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Figure 3 Nucleotide sequence of the *opa* gene and the deduced amino acid sequence of OPAA from *A. haloplanktis* (NCBI accession number U56398). The putative ribosome-binding sequence GATGGG is indicated by a broken underline. The stop codon is indicated by (*). The locations of OPAA-C1 (PYGNIVALNENXAILHYTHFEPKA) and OPAA-C2 (RDETGAHEX) are solidly underlined (X, unidentified residue).

To see whether OPAA and prolidase are functionally related, we also tested partially purified prolidase from porcine liver (Sigma, St Louis, MO, USA) and human peripheral erythrocytes (prepared in our laboratory). Both preparations were tested for their hydrolytic capability against DFP and G-type nerve agents. Our results (data not shown) indicated that the human and porcine crude enzyme preparations possessed measurable activity but proportionally much less specific activity against DFP and G-agents (1/200 to 1/500th) than X-Pro dipeptides (1/6 to 1/10th) compared to OPAAs. Though these findings strongly suggest that prolidase and *Alteromonas* OPAAs are func-

Table 1 Specific activity of purified OPAAs with DFP and various G-agents

Substrate	Specific activity (U mg^{-1})[a]		
	A. undina	*A. haloplanktis*	*Alteromonas* sp JD6.5
DFP	1403 ± 49	691 ± 11	1820 ± 74
GB	426 ± 36	308 ± 24	611 ± 39
GD	2826 ± 127	1667 ± 74	3145 ± 95
GF	1775 ± 115	323 ± 22	1654 ± 125

[a]OPAA specific activity was determined by monitoring fluoride release with a fluoride-specific electrode as described in Methods. One unit (U) of OPAA activity is defined as catalyzing the release of 1.0 μmole of F$^-$ min^{-1}. Specific activity is expressed as U mg^{-1} protein (± standard deviation).

Table 2 Specific activity of purified OPAAs with different dipeptides and DFP

Substrate[b]	Specific activity (U mg^{-1})		
	A. undina	*A. haloplanktis*	*Alteromonas* sp JD6.5
Leu-Pro[a]	810	988	636
Ala-Pro[a]	658	725	510
His-Phe[a]	24	<1	<1
Phe-Leu[a]	19	<1	<1
Try-Gly[a]	86	<1	<1
Ile-Asn[a]	22	<1	<1
Met-Asn[a]	410	<1	<1
Ala-Ala[a]	105	<1	<1
Gly-Glu[a]	1391	<1	<1
Leu-Ala[a]	220	63	82

[a]The amount of monomeric amino acids released from individual dipeptide or tripeptide were determined from a standard curve. Specific activity is expressed as μmole of amino acids released min^{-1} mg^{-1} protein (U mg^{-1}).
[b]Other tested substrates with no detectable activity (<1 U mg^{-1}) include Pro-Gly, Pro-Leu, Glu-Gly, Gly-Pro-Ala, Ala-Pro-Phe, Gly-Gly-Val, Glu-Gly-Phe, and Gly-Gly-Ala.

tionally related, further studies on the structure and substrate specificity by purified prolidase from other sources should provide more insights into structure-function relationships.

Discussion

OPAA is an enzyme with broad substrate specificity, ie it catalyzes hydrolysis of P-F, P-O, and P-CN bonds of several OPs. Deduced amino acid sequence analysis of the OPAA gene from *A. haloplanktis* revealed a high similarity (81% identity) with OPAA-2 from *Alteromonas* sp JD6.5. The two recombinant OPAAs and one purified from *A. undina* also exhibit X-Pro dipeptidase activity. All three OPAAs have a significant structural and functional similarity to PepQ and prolidase (which also catalyzes hydrolysis of X-Pro dipeptides). In addition, despite the functional similarities between OPAAs [4,7] and OPH [2,11], no dipeptidase activity was observed for OPH. Furthermore, a purified dipeptidase from *Lactobacillus sake*, which does not possess activity against either X-Pro or Pro-X dipeptide [24], cannot hydrolyze DFP or the chemical G-agents (Montel *et al*, Institut National de la Recherche Agronomique, France, personal communication). Overall, the structural homology and functional similarities suggest that *Alteromonas* OPAAs and prolidase may have evolved from the same ancestral gene.

Prolidase catalyzes cleavage of a C-N bond. It is interesting that only prolidase has OP hydrolysis activity while other peptidases and dipeptidases do not. Although the biological role of native bacterial prolidase is not known, a deficiency of human prolidase results in a syndrome with abnormalities of the skin and other collagen tissue [27]. The affected patients excrete massive amounts of iminopeptides in the urine that are normally substrates for prolidase [12,13]. The significant activity of *Alteromonas* OPAAs against the X-Pro dipeptides suggests that these enzymes are indeed a type of prolidase, and may play a role in cellular dipeptide metabolism. The functional relationship between OPAA and prolidase was further established by observed hydrolysis of DFP and G-type nerve agents with human and porcine lysates. Further studies with purified enzymes from human and porcine sources are needed to provide conclusive evidence on catalytic capability of eukaryotic prolidase against DFP and G-agents.

In conclusion, the results summarized here clearly establish that *Alteromonas* OPAA enzymes function as X-Pro dipeptidases. Further studies are required to elucidate the structural features determining the specificity and the biological activity of the enzyme. This information is crucial for engineering and designing of the novel enzymes with improved catalytic efficiency for nerve agent decontamination. For large scale decontamination, the use of $(NH_4)_2CO_3$ in enzymatic reaction offers obvious advantages. It is effective, simple, inexpensive and nonhazardous to users, equipment, and the environment in general.

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