

Expression, purification and preliminary crystallographic analysis of dipeptidyl peptidase IV from *Porphyromonas gingivalis*

Dean Rea,^a Anne-Marie Lambeir,^b Yumi Kumagai,^c Ingrid De Meester,^b Simon Scharpe^b and Vilmos Fülöp^{a*}

^aDepartment of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, England, ^bDepartment of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, Wilrijk, Belgium, and ^cDepartment of Microbiology, Nippon Dental University, Tokyo, Japan

Correspondence e-mail: vilmos@globin.bio.warwick.ac.uk

The asaccharolytic periodontopathogen *Porphyromonas gingivalis* produces membrane-anchored proteases such as dipeptidyl peptidase IV that are involved in the destruction of host periodontal tissue. The extracellular domain of this enzyme was overexpressed in *Escherichia coli* as an N-terminal His-tag fusion protein, purified using standard metal-affinity chromatography and crystallized using the hanging-drop vapour-diffusion technique in 40% 2-methyl-2,4-pentanediol and 100 mM Tris-HCl pH 8.0. Diffraction data to 2.7 Å resolution were collected using synchrotron radiation. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 117.0$, $b = 112.9$, $c = 310.0$ Å, $\beta = 95.0^\circ$. There are ten molecules per asymmetric unit, indicating a solvent content of 50%. Data were also collected from selenomethionine-derived crystals and structure solution by SAD or MAD is in progress.

Received 1 June 2004
Accepted 17 July 2004

1. Introduction

Porphyromonas gingivalis is a major pathogen associated with adult periodontitis (Holt *et al.*, 1988; Allaker & Hardie, 1998; Genco *et al.*, 1998). This organism is also implicated in systemic diseases such as aspiration pneumonia in the elderly and in endocarditis and pulmonary infections as a blood-borne pathogen (Allaker & Hardie, 1998; Allen & Duerden, 1998; Genco *et al.*, 1998; Read & Finch, 1998). It is not known at present how oral bacteria are able to penetrate the systemic circulation and cause infection at sites other than the oral cavity.

P. gingivalis is asaccharolytic and cannot take up free amino acids, utilizing only short oligopeptides as carbon and energy sources (Dashper *et al.*, 1998). A complex cell-surface-associated proteolytic system comprising several unique peptidases has evolved to meet this nutritional requirement. The best characterized of these enzymes are gingipains R and K, which are arginine and lysine-specific cysteine proteases, respectively (Curtis *et al.*, 1999). These and other cysteine proteases are involved in degrading the periodontal ligament and surrounding area (Potempa *et al.*, 1995; Travis *et al.*, 1997). The gingival connective tissue is comprised predominantly of collagen type I, which can be degraded by human and bacterial collagenases. The resulting fragments are then susceptible to further degradation by endopeptidases released by plaque bacteria. These collagen-derived peptides are rich in proline and hydroxyproline, making them resistant to hydrolysis by most proteases. For asaccharolytic organisms, the ability to process

these peptides is important for providing nutrients. A number of serine proteases from *P. gingivalis* have been identified that possess the ability to hydrolyse peptides at proline residues. A post-proline endopeptidase showing homology to human angiotensin-converting enzyme has been described (Awano *et al.*, 1999). A pair of post-proline exopeptidases belonging to the prolyl oligopeptidase family have been discussed, namely prolyl tripeptidyl peptidase (PtpA) and dipeptidyl peptidase IV (DPPIV; Banbula *et al.*, 1999, 2000; Kumagai *et al.*, 2000). Both enzymes contain a catalytic serine residue within the sequence GX X SGG, the consensus sequence for this enzyme family, where X is any amino acid (Rawlings *et al.*, 1991).

P. gingivalis has the ability to survive on dipeptides as the sole source of carbon, suggesting an important nutritional function for DPPIV (Takahashi & Sato, 2001, 2002). An animal study involving the use of a DPPIV-deficient knockout mutant of the bacterium has shown this enzyme to be a potential virulence factor (Kumagai *et al.*, 2000). Mice infected with the DPPIV-deficient strain became infected less severely and recovered more quickly from infection than those infected with the wild-type bacterium (Kumagai *et al.*, 2000; Yagashita *et al.*, 2001). Reintroduction of the cloned DPPIV gene into the null mutant using a shuttle vector restored virulence (Kumagai *et al.*, 2003). Expression of the catalytically deficient DPPIV enzyme in which the catalytic serine is mutated to alanine results in an intermediate virulence between the strain expressing wild-type DPPIV and the null mutant. This indicates that DPPIV cata-

lytic activity is important but not sufficient for virulence (Kumagai *et al.*, 2003).

DPPIV is also found in eukaryotes, where soluble and membrane-anchored forms have been characterized. Although the physiological and pathological roles of the bacterial protein are not well understood, a wealth of literature exists concerning the biological role of the mammalian enzyme. It has the ability to degrade a range of signalling peptides, including chemokines and neuropeptides (Augustyns *et al.*, 1999; Lambeir, Durinx *et al.*, 2001; Lambeir, Proost *et al.*, 2001). In addition to enzymatic activity, eukaryotic DPPIV is involved in protein-protein interactions with collagen and/or fibronectin (Kikkawa *et al.*, 2003) and adenosine deaminase (Abbott *et al.*, 1999). The enzyme has been implicated in a number of disease states, including AIDS, breast cancer and diabetes (Gutheil *et al.*, 1994; Wrenger *et al.*, 1997; Cheng *et al.*, 1998; Ohtsuki *et al.*, 1998; Augustyns *et al.*, 1999). It is possible that *P. gingivalis* DPPIV (PgDPPIV) not only helps to fulfil a nutritional requirement, but is also capable of hydrolysing chemokines and other host peptides, helping to evade the host's response to infection.

Kinetic studies revealed similarities and differences between the enzymatic properties of eukaryotic and bacterial DPPIV (Lambeir *et al.*, 2003). Differences in inactivation kinetics using dipeptide-derived diaryl-phosphonate esters suggest there are significant structural differences in the active sites. A different residue is preferred at the P2 position of the substrate (Lambeir *et al.*, 2003). The crystal structures of both human and porcine DPPIV have been solved (Engel *et al.*, 2003; Hiramatsu *et al.*, 2003; Oefner *et al.*, 2003; Rasmussen *et al.*, 2003; Thoma *et al.*, 2003). The overall structure is reminiscent of the closely related prolyl oligopeptidase (Fülöp *et al.*, 1998). The catalytic domain has α/β -hydrolase topology and is structurally homologous to lipases and esterases. There is a further open-topology β -propeller domain that is eight-bladed in DPPIV but seven-bladed in prolyl oligopeptidase (Fülöp *et al.*, 1998). Whilst prolyl oligopeptidase is monomeric, both eukaryotic and bacterial DPPIV enzymes exist as dimers. PgDPPIV exhibits around 28% sequence identity to its eukaryotic counterparts, so a similar overall three-dimensional structure would be anticipated. The crystal structure of the bacterial enzyme may help to explain the observed enzymatic differences and assist in the development of selective inhibitors for the treatment of periodontitis.

2. Materials and methods

2.1. Cloning, overexpression and purification

A plasmid containing the PgDPPIV gene was used as published previously (Kumagai *et al.*, 2000). The region of the gene corresponding to the extracellular part of the protein (starting at glutamine 20) was amplified by PCR with primers incorporating *Nco*I and *Eco*RI sites at the 5' and 3' ends, respectively. The resulting PCR fragment was digested with these enzymes and ligated into the pProEx-Hta expression vector. Positive recombinants were selected and the expression plasmid was used to transform the *Escherichia coli* BL21(DE3) expression strain, which also contained the pRARE plasmid for supplementation of rare tRNAs. Bacteria were grown at 310 K in 2 l LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 35 $\mu\text{g ml}^{-1}$ chloramphenicol. Protein expression was induced by the addition of IPTG to 1 mM and growth continued at 293 K for 18 h. The cells were harvested by centrifugation at 6000g for 15 min and resuspended in 50 mM HEPES pH 8.0, 1 M NaCl, 10 mM imidazole (buffer A) prior to sonication and clarification of the extract by centrifugation at 20 000g for 30 min. The N-terminal histidine-tagged recombinant enzyme was purified in one step using nickel-affinity chromatography. A nickel Sepharose column was equilibrated in buffer A. The cell extract was applied, the column was washed with buffer A, buffer B (50 mM HEPES pH 8.0, 1 M NaCl, 100 mM imidazole) and buffer C (50 mM HEPES pH 8.0, 0.1 M NaCl, 100 mM imidazole) and the enzyme was eluted with buffer D (50 mM HEPES pH 8.0, 0.1 M NaCl, 500 mM imidazole). Fractions containing protein were combined, diluted twofold with elution buffer and glycerol was added to 20% (v/v) to prevent aggregation. The sample was concentrated using centrifugal concentrators and applied onto a Hi-trap desalting column equilibrated with 20 mM Tris-HCl pH 8.5,

20% glycerol. DPPIV was judged by Coomassie-stained SDS-PAGE to be greater than 99% pure and was concentrated to 9 mg ml⁻¹ prior to crystallization.

2.2. Expression of selenomethionine-substituted PgDPPIV

For the production of selenomethionine (SeMet) substituted PgDPPIV, the expression construct was used to transform the methionine auxotrophic *E. coli* strain B834(DE3) and a 5 ml culture was grown overnight at 303 K in LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin. 100 ml LB was inoculated with 1 ml overnight culture and grown at 310 K to an OD₆₀₀ of 0.2; the cells were pelleted using centrifugation, resuspended in M9 minimal media, pelleted and resuspended twice more to remove SeMet. The cell suspension was used to inoculate 1 l pre-warmed (310 K) M9 minimal media supplemented with 0.4% glucose, 40 mg l⁻¹ of 19 amino acids (all except Met), 40 mg l⁻¹ SeMet, 1 mg l⁻¹ of the vitamins thiamine, thymine, niacinamide and pyridoxine monohydrochloride, 25 mg l⁻¹ iron (II) sulfate and 2 mM magnesium sulfate. The culture was grown to an OD₆₀₀ of 0.6, protein expression was induced by addition of IPTG to 1 mM and growth continued at 293 K for 16 h. SeMet-substituted PgDPPIV was purified as described above, except that 2-mercaptoethanol was used at 2 mM in all buffers.

2.3. Crystallization

Initial crystallization screens were performed using the hanging-drop vapour-diffusion technique with 0.5 ml reservoir solutions taken from Hampton Research Crystal Screens I and II (Jancarik & Kim, 1991) or the Clear Strategy Screen buffered at 0.1 M MES pH 6.5 (Brzozowski & Walton, 2001). Clusters of plate-like crystals appeared after several months in a solution containing 40% (v/v) MPD, 100 mM Tris pH 7.5. Using the microseeding technique, single crystals suitable for X-ray diffraction studies were grown in a solution containing between 35 and 40% (v/v) MPD, 100 mM Tris pH 8.0 and had dimensions of 0.1 × 0.2 × 0.3 mm (Fig. 1). SeMet-substituted PgDPPIV crystals grew under identical conditions.

2.4. X-ray diffraction analysis

Crystals were picked up from the crystallization drop using a nylon loop, transferred directly into a cryostream, cooled to 100 K and then stored in liquid nitrogen



Figure 1
Photograph of a PgDPPIV crystal; the largest crystal dimension is 0.3 mm.

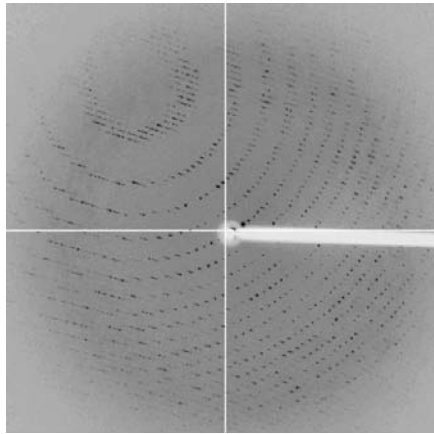


Figure 2
A typical diffraction image of a selenomethionine-substituted crystal collected at beamline ID14-4 at the ESRF using an ADSC Q4 CCD detector. The oscillation range was 0.5° and the resolution at the edge is 2.4 \AA .

until needed for data collection. A partial data set was collected from native crystals to a resolution of 2.8 \AA with an oscillation range of 1° per frame on beamline 14.1 at the SRS, Daresbury using an ADSC Q4 CCD detector. All data were indexed, integrated and scaled using the *HKL* suite of programs (Otwinowski & Minor, 1997). The crystals belong to space group $P2_1$, with unit-cell parameters $a = 117.0$, $b = 112.9$, $c = 310.0 \text{ \AA}$, $\beta = 95.0^\circ$, resulting in a unit-cell volume of $4.1 \times 10^6 \text{ \AA}^3$. The Matthews probability calculation suggests the presence of ten molecules in the asymmetric unit with a V_M value of $2.46 \text{ \AA}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 50%. The structures of both human and porcine DPPIV have been solved. However, the *P. gingivalis* enzyme shares only 28% sequence identity with both the porcine and human proteins. Low sequence homology coupled with the large number of molecules in the asymmetric unit resulted in the unsurprising failure of molecular replacement using either monomeric or dimeric eukaryotic DPPIV coordinates as search models. Selenomethionine-derived crystals have been prepared and data were collected to 2.7 \AA at three wavelengths on beamline ID14-4 at the ESRF, Grenoble. A typical diffraction image is shown in Fig. 2. All data were indexed, integrated and scaled with the *HKL* package (Otwinowski & Minor, 1997). Data-collection and processing statistics for highly redundant (11.5-fold) data collected at the selenium absorption-peak wavelength are shown in Table 1. Structure solution by SAD or MAD is in progress and heavy-atom soaks are under way to provide additional phasing options.

Table 1
Data-collection and processing statistics.

Data were collected at the wavelength of the selenium absorption peak. Values in parentheses refer to the highest resolution shell.

Synchrotron radiation	ESRF ID14-14
Detector	ADSC Q4 CCD
Wavelength (\AA)	0.9794
Space group	$P2_1$
Unit-cell parameters	
a (\AA)	117.0
b (\AA)	112.9
c (\AA)	311.0
β ($^\circ$)	95.0
Molecules per AU	10
Matthews coefficient ($\text{\AA}^3 \text{ Da}^{-1}$)	2.46
Solvent content (%)	50
Resolution range (\AA)	80–2.7 (2.8–2.7)
Total observations	9885450
Unique reflections	425879
Average $I/\sigma(I)$	14.7 (2.4)
R_{merge} (%)	0.062 (0.231)
Completeness (%)	97.8 (99.9)

We are grateful for access and user support at the synchrotron facilities of ESRF, Grenoble and SRS, Daresbury. DR thanks the BBSRC for the award of a studentship. VF is a Royal Society University Research Fellow.

References

- Abbott, C. A., McCaughan, G. W., Levy, M. T., Church, W. B. & Gorrell, M. D. (1999). *Eur. J. Biochem.* **266**, 798–810.
- Allaker, R. P. & Hardie, J. M. (1998). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, 9th ed., edited by W. J. Hausler & M. Sussman, Vol. 3, pp. 373–390. London: Arnold.
- Allen, S. D. & Duerden, B. I. (1998). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, 9th ed., edited by W. J. Hausler & M. Sussman, Vol. 3, pp. 743–776. London: Arnold.
- Augustyns, K., Bal, G., Thonus, A., Belyaev, X. M., Zhang, W., Bollaert, W., Lambeir, A. M., Durinx, C., Goosens, F. & Haemers, A. (1999). *Curr. Med. Chem.* **6**, 311–327.
- Awano, S., Ansai, T., Mochizuki, H., Yu, W., Tanzanawa, K., Turner, A. J. & Takehara, T. (1999). *FEBS Lett.* **460**, 139–144.
- Banbula, A., Bugno, M., Goldstein, J., Yen, J., Nelson, D., Travis, J. & Potempa, J. (2000). *Infect. Immun.* **68**, 1176–1182.
- Banbula, A., Mak, P., Bugno, M., Silberring, J., Dubin, A., Nelson, D., Travis, J. & Potempa, J. (1999). *J. Biol. Chem.* **274**, 9246–9252.
- Brzozowski, A. M. & Walton, J. (2001). *J. Appl. Cryst.* **34**, 97–101.
- Cheng, H. C., Abdel-Ghany, M., Elble, R. C. & Pauli, B. U. (1998). *J. Biol. Chem.* **273**, 24207–24215.
- Curtis, M. A., Kuramitsu, H. K., Lantz, M., Macrina, F. L., Nakayama, K., Potempa, J., Reynolds, E. C. & Aduse-Opoku, J. (1999). *J. Periodontol. Res.* **34**, 464–472.
- Dashper, S. G., Kandasamy, S., O'Brien-Simpson, N. & Reynolds, E. C. (1998). *J. Dent. Res.* **77**, 1133.
- Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H. U. & Brandstetter, H. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 5063–5068.
- Fülöp, V., Böcskei, Z. & Polgár, L. (1998). *Cell*, **94**, 161–170.
- Genco, C. A., Van Dyke, T. & Amar, S. (1998). *Trends Microbiol.* **6**, 444–449.
- Gutheil, W. G., Subramanyam, G. R., Flentke, G. R., Sanford, D. G., Munoz, E., Huber, B. T. & Bachovin, W. W. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 6594–6598.
- Hiramatsu, H., Kyono, K., Higashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A. & Shimizu, R. (2003). *Biochem. Biophys. Res. Commun.* **302**, 849–854.
- Holt, S. C., Ebersole, J., Felton, M., Brunsvold, M. & Kornman, K. S. (1988). *Science*, **239**, 55–57.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Kikkawa, F., Kajiyama, H., Ino, K., Shibata, K. & Mizutani, S. (2003). *Int. J. Cancer*, **105**, 779–783.
- Kumagai, Y., Konishi, K., Gomi, T., Yagashita, H., Yajima, A. & Yoshikawa, M. (2000). *Infect. Immun.* **68**, 716–724.
- Kumagai, Y., Yajima, A. & Konishi, K. (2003). *Microbiol. Immunol.* **47**, 735–743.
- Lambeir, A. M., Durinx, C., Proost, P., Van Damme, J., Scharpé, S. & de Meester, I. (2001). *FEBS Lett.* **507**, 327–330.
- Lambeir, A. M., Proost, P., Durinx, C., Bal, G., Senten, K., Augustyns, K., Scharpé, S., Van Damme, J. & de Meester, I. (2001). *J. Biol. Chem.* **276**, 29839–29845.
- Lambeir, A. M., Rea, D., Fülöp, V., Kumagai, Y., Augustyns, K., de Meester, I., Haemers, A. & Scharpé, S. (2003). *Adv. Exp. Med. Biol.* **524**, 29–35.
- Oefner, C., D'Arcy, A., Mac Sweeney, A., Pierau, S., Gardiner, R. & Dale, G. E. (2003). *Acta Cryst.* **D59**, 1206–1212.
- Ohtsuki, T., Hosono, O., Kobayashi, H., Munakata, A., Souta, A., Shinoda, T. & Morimoto, C. (1998). *FEBS Lett.* **431**, 236–240.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Potempa, J., Pike, R. & Travis, J. (1995). *Prosp. Drug Discov. Des.* **2**, 445–458.
- Rasmussen, H. B., Branner, S., Wiberg, F. C. & Wagtman, N. (2003). *Nature Struct. Biol.* **10**, 19–25.
- Rawlings, N. D., Polgár, L. & Barrett, A. J. (1991). *Biochem. J.* **279**, 907–908.
- Read, R. C. & Finch, G. (1998). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, 9th ed., edited by W. J. Hausler & M. Sussman, Vol. 3, pp. 319–345. London: Arnold.
- Takahashi, N. & Sato, T. (2001). *J. Dent. Res.* **80**, 1425–1429.
- Takahashi, N. & Sato, T. (2002). *Oral Microbiol. Immunol.* **17**, 50–54.
- Thoma, R., Löffler, B., Stihle, M., Huber, W., Ruf, A. & Hennig, M. (2003). *Structure*, **8**, 947–959.
- Travis, J., Pike, R., Imamura, T. & Potempa, J. (1997). *J. Periodontol. Res.* **32**, 120–125.
- Wrenger, S., Hoffmann, T., Faust, J., Mrestani-Klaus, C., Brandt, W., Neubert, K., Kraft, M., Olek, S., Frank, R., Ansoerg, S. & Reinhold, D. (1997). *J. Biol. Chem.* **272**, 30283–30288.
- Yagashita, H., Kumagai, Y., Konishi, K., Takahashi, Y., Aoba, T. & Yoshikawa, M. (2001). *Infect. Immun.* **69**, 7159–7161.