FOR THE RECORD

Crystallization and preliminary X-ray diffraction analysis of gingipain R2 from *Porphyromonas gingivalis* in complex with H-D-Phe-Phe-Arg-chloromethylketone

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(RECEIVED November 24, 1997; ACCEPTED January 20, 1998)

Abstract: Gingipain R2 is a 50 kDa proteinase from the oral pathogenic bacterium *Porphyromonas gingivalis*. This proteinase, which displays no significant sequence homology to any protein previously analyzed by X-ray crystallography, has been crystallized using the vapor diffusion method. Two different crystal forms were obtained from a solution containing polyethylene glycol (MW 8,000) (space group $P2_12_12_1$) or magnesium sulfate (space group R3) as precipitating agent. Complete diffraction data sets have been collected up to 2.0 and 2.9 Å resolution, respectively. Cell dimensions are $a = 51.9$ Å, $b = 79.9$ Å, and $c = 99.6$ Å ($P2_12_12_1$), and $a = b = 176.6$ Å, and $c = 143.4$ Å (R3). Considerations of the possible values of V_m accounts for the presence of one monomer per asymmetric unit in the case of the orthorhombic crystal form, whereas the rhombohedral crystal form, together with the analysis of the self-rotation function, could accommodate a tetramer in the asymmetric unit.

Keywords: cysteine proteinase; gingipain R2; gingipains; *Porphyromonas gingivalis*; protein crystallization; X-ray diffraction

The gram-negative microorganism *Porphyromonas gingivalis*, an oral anaerobic bacterium, is recognized as an important agent in the etiology of chronic adult periodontitis (White & Mayrand, 1981; Slots et al., 1986; Holt et al., 1988). Proteolytic enzymes, which are produced in large quantities by this bacterium are considered as important virulence factors (Sundqvist, 1993; Marsh et al., 1994; Bhogal et al., 1997). They are capable of degrading and/or inactivating a broad range of host proteins including both structural proteins and those involved in host defense. This latter effect results in the dysregulation of the cascade systems, which normally regulate coagulation, bradykinin release, and fibrinolysis

(for review see Potempa et al., 1996; Potempa & Travis, 1996). The majority of proteolytic activity from *P. gingivalis* is due to the concerted action of two cysteine proteinases, referred to as gingipains, with either Arg-Xaa peptide bond specificity (gingipains R, RGP) or Lys-Xaa specificity (gingipain K, KGP) (Potempa et al., 1995b; Potempa et al., 1997). Both proteinases appear to play central roles in the physiological and pathological functions of *P. gingivalis* (Smalley et al., 1989; Nakayama et al., 1995, 1996; Kesavalu et al., 1996; Onoe et al., 1996).

Gingipains R are products of two closely related genes (Pavloff et al., 1995; Nakayama, 1997). One gene, referred to as either *rgp-1* (Pavloff et al., 1995), *prpR1* (Aduse-Opoku et al., 1995) or *prtR* (Slakeski et al., 1996), encodes a polypeptide consisting of a pre-profragment, a 492 residue catalytic domain and a C-terminal hemagglutinin/adhesin domain (Pavloff et al., 1995). Because of both, proteolytic processing and post-translational modification, several forms of RGP-1 can originate from the initial gene product including a free catalytic domain, a lipopolysaccharide-modified catalytic domain, and a noncovalent complex of the catalytic domain with the hemagglutinin domain (Pike et al., 1994; Pavloff et al., 1995; Rangarajan et al., 1997a, 1997b). In contrast to *rgp-1*, the *rgp-2* gene (also known as *rgpB* and *prR2*) lacks a fragment encoding the hemagglutinin/adhesin domain and is expressed as a proteinase of 45–50 kDa, with the primary structure being 93% identical with the gingipain R1 (RGP-1) catalytic domain (Nakayama, 1997; Rangarajan et al., 1997a). In cultures of *P. gingivalis* strains W50, W83, Pg100 and ATCC 33277, gingipain R2 (RGP-2) occurs either as a heavily lipopolysaccharide-modified membrane protein or as a nonmodified monomer released into the medium (Potempa et al., 1995b; Rangarajan et al., 1997a, 1997b). In strain HG66, however, a monomeric RGP-2 is the predominant form of this gingipain (Pike et al., 1994; Potempa et al., 1995b). In order to further characterize the properties of members of the gingipain family, we have crystallized RGP-2 as part of our effort to determine its three-dimensional structure. Here, we report the X-ray diffraction analysis of two crystal forms. Because no phase

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information of any related protein is available, complete structural analysis will require use of the multiple isomorphous replacement method.

Results and discussion: Initial crystallization experiments were carried out using the screening protocols described by Jancarik & Kim (1991). Irregular crystals were obtained in one week, when polyethylene glycol was used as precipitating agent. Attempts to improve the quality of these crystals were performed using different molecular weight polyethylene glycols, and varying the pH of the solutions. Best results were obtained when using 0.1 M sodium cacodylate, pH 6.0, 15% polyethylene glycol (MW 8,000) and 0.1 M zinc acetate (Fig. 1A). These crystals, with a mean size of $0.7 \text{ mm} \times 0.7 \text{ mm} \times 0.2 \text{ mm}$, belong to the orthorhombic space group $P2_12_12_1$, have unit cell dimensions $a = 51.9 \text{ \AA}$, $b = 79.9 \text{ \AA}$, and $c = 99.6 \text{ \AA}$, and diffract to $2.0 \text{ \AA}$ resolution.

Another crystalline form with a rhombohedral habit diffracting to $2.9 \text{ \AA}$ resolution was obtained when using 0.1 M MES pH 6.3

and 1.6 M magnesium sulfate as precipitating agent. Crystals grew in approximately 1 month with a mean size of $0.3 \text{ mm} \times 0.3 \text{ mm} \times 0.2 \text{ mm}$ (Fig. 1B). Their space group was determined to be rhombohedral R3, and their unit cell dimensions were $a = b = 176.6 \text{ \AA}$ and $c = 143.4 \text{ \AA}$.

Data collection and statistics for both data sets are summarized in Table 1. The calculated packing parameter V_m (Matthews, 1968) is $2.13 \text{ \AA}^3/\text{Da}$ for the orthorhombic crystal form. This accounts for the presence of one monomer per asymmetric unit, with a solvent content of 42%. In the case of the rhombohedral crystal form, analysis of the self-rotation function together with the most probable calculated packing parameter V_m , $2.22 \text{ \AA}^3/\text{Da}$, accounts for the presence of a tetramer in the asymmetric unit, with a solvent content of 45%. Data for several heavy-atom derivatives are being collected and their difference Patterson maps analyzed.

The successful crystallization and initial data collection is the first step in determining the tertiary structure of RGP-2. This enzyme is an archetype of the gingipain family of cysteine proteinases comprising three members, RGP-1, RGP-2, and KGP (Potempa et al., 1995a). At the primary structure level gingipains show no significant similarity to any other protein in data banks and, therefore, may have unique fold. A recently discovered strong positive correlation between periodontitis and cardiovascular diseases (Editorial, 1997) makes this study even more urgent since gingipains R can activate the coagulation cascade and in this way contribute to the increased incidence of cardiovascular diseases in chronic periodontitis patients (Imamura et al., 1997).

Materials and methods: *Protein purification and complex formation:* RGP-2 was obtained from culture media of *P. gingivalis* HG66 grown to early stationary phase as described previously (Chen et al., 1992). Initial steps of gingipain fractionation, acetone precipitation, and Sephadex G-150 chromatography were performed according to the method designed for HRGP and KGP (Pike et al., 1994). After size exclusion chromatography, RGP-2 was further purified from the low molecular mass peak of activity

Fig. 1. Crystals of gingipain R2 in complex with H-D-Phe-Phe-Arg-chloromethylketone. **A:** Orthorhombic crystal (space group $P2_12_12_1$) grown in 0.1 M sodium cacodylate, pH 6.0, 13% PEG 8,000 and 0.1 M zinc acetate. **B:** Rhombohedral crystal (space group R3) grown in 0.1 M MES, pH 6.3 and 1.6 M magnesium sulfate. Crystal size is $0.3 \text{ mm} \times 0.3 \text{ mm} \times 0.2 \text{ mm}$.

Table 1. Data collection and processing statistics

	$P2_12_12_1$ crystal form	R3 crystal form
Cell constants	$a = 51.9 \text{ \AA}$ $b = 79.9 \text{ \AA}$ $c = 99.6 \text{ \AA}$	$a = 176.6 \text{ \AA}$ $c = 143.4 \text{ \AA}$
Crystal to detector distance	150 mm	210 mm
Maximum resolution	$2.0 \text{ \AA}$	$2.9 \text{ \AA}$
Rotation for each exposure	1°	1°
Time for each image	1,200 s	1,200 s
Total rotation for a data set	90°	60°
Number of measured reflections	174,312	75,426
Number of unique reflections	26,373	35,888
R_{merge}^a	8.6%	17.0%
Completeness	98.7% (20.0– $2.0 \text{ \AA}$)	97.4% (20.0– $2.9 \text{ \AA}$)
Last resolution shell	99.2% (2.12 – $2.0 \text{ \AA}$)	98.3% (3.00 – $2.9 \text{ \AA}$)

^a $R_{\text{merge}} = \sum \sum |I(h)_i - \langle I(h) \rangle| / \sum \langle I(h) \rangle$; $I(h)$ is the observed intensity of the i th measurement of reflection h , and $\langle I(h) \rangle$ the mean intensity of reflection h ; calculated after loading, and scaling.

(50 kDa) by sequential chromatography steps on DE-52 cellulose and Arginine-Sepharose, and by chromatofocusing on a mono-P column using an FPLC system (Pharmacia). The last step allows separation of four major isoforms of RGP-2 differing in pI (Potempa et al., unpubl. obs.).

For crystallization purposes the form of enzyme with pI 5.09 was selected. The purified isoform of RGP-2 (6.7 mg) was activated by incubation for 30 min in a total volume of 4 mL of 0.1 M HEPES, 2.5 mM CaCl₂, pH 8.0, 37 °C, containing 10 mM L-cysteine. The active gingipain was treated with the fast acting, irreversible inhibitor H-D-Phe-Phe-Arg-chloromethylketone (Bachem) (Potempa et al., 1997) (final concentration 20 µM) to allow alkylation of the active-site cysteine residue. The sample was then dialyzed against 3 mM MOPS, 0.02% Na₂S₂O₃, pH 7.2 and concentrated.

Crystallization: Crystallization experiments were carried out at a constant temperature of 20 °C using the sitting-drop vapor diffusion method. All reservoir solutions contained 0.02% (w/v) sodium azide. The initial protein concentration was 10 mg/mL in 3 mM MOPS buffer, pH 7.2. Drops were prepared by mixing 2 µL of the protein solution with 2 µL of the reservoir solution. The mixture was allowed to equilibrate against 0.4 mL reservoir solution.

X-ray diffraction analysis: Crystals were harvested using the reservoir solution and mounted in thin-walled glass capillary tubes. X-ray diffraction data were collected on a 300 mm MAR-Research image plate detector attached to a Rigaku RU200 rotating anode generator providing graphite monochromatized CuKα radiation operated at 100 mA and 50 kV. The data were indexed with DENZO, scaled and reduced with SCALEPACK (Otwinowski & Minor, 1997), and truncated with programs from the CCP4 suite (Collaborative Computational Project, No. 4, 1994).

Acknowledgments: We thank Prof. Robert Huber for the possibility of performing this work in his department. This work was supported by National Institute of Health grant DE 09761 to JT, by grant 6 P204A 019 11 from the State Committee of Scientific Research (KBN, Poland) to JP, and by EU project ERBB104CT960464 and the SFB 469 to WB.

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