The HA2 haemagglutinin domain of the lysine-specific gingipain (Kgp) of *Porphyromonas gingivalis* promotes μ -oxo bishaem formation from monomeric iron(III) protoporphyrin IX

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The lysine- and arginine-specific gingipains (Kgp, and RgpA and RgpB) are the major proteinases produced by the black-pigmented periodontopathogen *Porphyromonas gingivalis*. They play a role in degrading host proteins, including haemoglobin, from which is formed the μ -oxo bishaem complex of iron(III) protoporphyrin IX, [Fe(III)PPIX]₂O, the major haem component of the black pigment. Kgp and RgpA bind haem and haemoglobin via the haemagglutinin-adhesin 2 (HA2) domain, but the role of this domain in the formation of μ -oxo bishaem-containing pigment is not known. UV-visible spectroscopy was used to examine the interaction of iron(III) protoporphyrin IX monomers [Fe(III)PPIX.OH] with recombinant HA2 and purified HRgpA, Kgp and RgpB gingipains. The HA2 domain reacted with Fe(III)PPIX.OH to form μ -oxo bishaem, the presence of which was confirmed by Fourier transform infrared spectroscopy. Both HRgpA and Kgp, but not RgpB, also mediated μ -oxo bishaem formation and aggregation. It is concluded that the Arg- and Lys-gingipains with HA2 haemagglutinin domains may play a crucial role in haem-pigment formation by converting Fe(III)PPIX.OH monomers into [Fe(III)PPIX]₂O and promoting their aggregation.

INTRODUCTION

Porphyromonas gingivalis, a Gram-negative anaerobe, is strongly implicated in the pathogenesis of adult periodontitis (Holt *et al.*, 1988; Machtei *et al.*, 1997). One of its phenotypic characteristics is the production of a black haem-containing pigment composed of iron(III) protoporphyrin IX in the form of the μ -oxo bishaem complex [Fe(III)PPIX]₂O (Smalley *et al.*, 1998, 2002, 2004), also referred to as μ -oxo dimer. It is composed of two iron(III) protoporphyrin IX molecules covalently joined through an oxygen atom interbridge, and it is this component which imparts the green-black coloration to the pigment. The μ -oxo bishaem complex acts as a defensive molecule, since its formation from Fe(II) protoporphyrin IX monomers (derived from haemoglobin) ties up dioxygen and toxic oxygen intermediates (Smalley *et al.*, 1998, 2002, 2004). Cell-surface μ -oxo bishaem acts as a barrier against ingress of oxygen and reactive oxygen species and breaks down hydrogen

peroxide through inherent catalase activity (Smalley *et al.*, 2000).

Gingipains specific for Arg-Xaa (RgpA and RgpB) and Lys-Xaa (Kgp) peptide bonds are the major proteases produced by *P. gingivalis* (Potempa *et al.*, 1995). While Kgp is the product of a single gene (*kgp*), Rgps are encoded by two related but individual genes (*rgpA* and *rgpB*). In contrast to the single-chain enzyme RgpB, mature Kgp and RgpA (HRgpA) proteins are multidomain complexes generated by proteolytic processing of the nascent translated polypeptide chains. They are composed of divergent protease domains associated with virtually identical haemagglutinin-adhesin (HA) domains. In addition to playing an important role in pathogenicity, either by degrading or inactivating proteins essential for host immunity and connective tissue integrity (see Potempa *et al.*, 2000), gingipains are implicated in haem acquisition by proteolytic degradation of haemoglobin (Sroka *et al.*, 2001; Smalley *et al.*, 2004). However, it is not known whether these proteases play any other role in converting proteolytically freed haems into the μ -oxo bishaem complex. The HA2 domain of Kgp and HRgpA is a receptor for both haem and haemoglobin (Nakayama *et al.*,

Abbreviations: ATR FT-IR spectroscopy, attenuated total reflectance Fourier transform infrared spectroscopy; HA domain, haemagglutinin-adhesin domain.

1998; DeCarlo *et al.*, 1999; Paramaesvaran *et al.*, 2003), whilst RgpB, lacking this domain, shows little or no binding to either haem or haemoglobin (Olczak *et al.*, 2001). Mutants with *kgp* truncated with respect to the HA2 domain are attenuated in haemoglobin and haem binding (Sztukowska *et al.*, 2004). In view of these facts, we have raised the question whether the HA2 domain of Kgp and HRgpA plays an additional role in pigmentation by binding and converting haems into μ -oxo bishaem, and have thus examined the interactions of HA2, and Arg- and Lys-gingipains, with monomeric iron(III) protoporphyrin IX. We report here that HA2, and HRgpA and Kgp, but not RgpB, mediate the formation and aggregation of the μ -oxo bishaem complex.

METHODS

Purification of recombinant HA2. Forward and reverse primers (CGGGATCCGACAGCTTCACGGAACGTTTCG and CGGAATTC-CCTTAATTATCCGTTAGGCGTTTCTTC) bearing restriction sites for *Bam*HI and *Eco*RI were used to amplify the HA2 domain from the *kgp* gene. The digested PCR product was ligated into the pGEX-4T-2 vector (Amersham Biosciences).

The expression construct of the HA2 domain from the *kgp* gene in the pGEX-4T-2 vector was sequenced completely to ensure that no mutations were introduced during the cloning procedure, and was found to be 100% identical to the coding sequence. The construct was transformed into *Escherichia coli* BL21 and expression of the recombinant protein induced with 1 mM IPTG when the culture reached an OD₆₀₀ of 0.5. After 3 h, *E. coli* cells were collected, suspended in PBS, and disrupted by sonication. Insoluble material was harvested by centrifugation, resuspended in 20 mM Tris/HCl, pH 8.0, 2 M urea, 0.5 M NaCl, 2% Triton X-100, stirred for 1 h and subjected to centrifugation (18 000 g for 30 min). The pellet of inclusion bodies was dissolved in 50 mM Tris/HCl, pH 8.0, 8 M urea, 3 mM DTT, and the solution cleared by centrifugation. To refold the protein, the supernatant was rapidly diluted in PBS then loaded on glutathione-Sepharose 4 FF (Amersham Biosciences). The column was washed with PBS until the A₂₈₀ baseline was reached, and the GST-tagged HA2 domain retained on the matrix was subjected to overnight digestion with 200 units of thrombin. The released HA2 domain was eluted with 50 mM Tris/HCl, pH 8.5, and purified from thrombin using a FPLC Mono Q column, and the final product was dialysed against 50 mM Tris/HCl, pH 8.0.

Gingipain purification. Soluble HRgpA, RgpB and Kgp proteins were purified from the culture medium of *P. gingivalis* HG66 as described previously (Chen *et al.*, 1992; Pike *et al.*, 1994; Potempa *et al.*, 1998). Briefly, HRgpA and Kgp were purified using gel-filtration and arginine-Sepharose chromatography, while RgpB was separated using a combination of gel-filtration and anion-exchange chromatography on a Mono Q FPLC column (Potempa *et al.*, 1998). The protein content and concentration of active proteases in each batch were measured using the bicinchoninic acid (BCA) method with bovine albumin as the standard, and by active-site titration employing D-Phe-Phe-Arg-chloromethane, as described previously (Potempa *et al.*, 1997), respectively. The purity of enzymes in each batch was checked using SDS-PAGE. RgpB migrated as a single 48 kDa band, whilst both HRgpA and Kgp resolved into four major and one minor band on SDS-PAGE (Pike *et al.*, 1994), the identities of which were confirmed by N-terminal sequence analysis.

Spectroscopic methods. UV-visible spectroscopy has been widely used to study both the kinetics of dimerization (Inada & Shibata, 1962) and the aggregation of iron protoporphyrin IX (Brown *et al.*,

1970, 1976, 1980; Silver & Lukas, 1983; Miller *et al.*, 1987). In aqueous solution, iron(III) protoporphyrin IX exists as a binary system comprising the monomeric and dimeric species in dynamic equilibrium, dependent upon the pH and the total ferrihaem concentration (Brown *et al.*, 1976, 1980; Silver & Lukas, 1983). At acid pH, the dominant species is the monomer, which displays a Soret band $\lambda_{\max}$ at 365 nm and a β band at $\sim$ 630 nm, whilst at alkaline pH the dominant form is the μ -oxo dimer, with Soret $\lambda_{\max}$ at 385 nm and a 608 nm β band (Silver & Lukas, 1983; Miller *et al.*, 1987). Iron(III) protoporphyrin IX solutions were prepared from bovine haemin (Sigma; product no. H-2250) in 0.14 M NaCl, buffered at pH 6.5 with 0.2 M Na₂HPO₄/NaH₂PO₄, or at pH 8.5 with 0.1 M Tris/HCl, as previously described (Smalley *et al.*, 2003), to give the monomeric and dimeric ferrihaem species, respectively (Silver & Lukas, 1983). For calculation of the relative proportions of the haem species (see below), these solutions were taken to represent 100% monomer or μ -oxo dimer. The A₃₆₅ and A₃₈₅ values for 20 μ M solutions of the above were used to calculate the millimolar extinction coefficients, from which the concentrations of monomer ([mon]) and dimer ([dim]) were determined according to the following equations, where ϵ_{mon} and ϵ_{dim} are the millimolar extinction coefficients of monomer and dimer species at these wavelengths, respectively:

$$A_{365} = \epsilon_{\text{mon}365}[\text{mon}] + \epsilon_{\text{dim}365}[\text{dim}]$$

$$A_{385} = \epsilon_{\text{mon}385}[\text{mon}] + \epsilon_{\text{dim}385}[\text{dim}]$$

The values of $\epsilon_{\text{mon}365}$ and $\epsilon_{\text{mon}385}$ were determined as 43.9 and 41.5, and those for $\epsilon_{\text{dim}365}$ and $\epsilon_{\text{dim}385}$ as 87.8 and 92.8, respectively. Spectra were recorded in an LKB-Biochrom Ultraspec 2000 spectrophotometer, as previously described (Smalley *et al.*, 2002), using plastic or quartz semi-micro optical cuvettes with a 1 cm pathlength. The relative proportions of the monomeric and dimeric species were expressed on a haem monomer basis.

Attenuated total reflectance Fourier transform infrared (ATR FT-IR) measurements were performed on liquid samples on a Thermo Nicolet instrument using a Smart Omni-Sampler. Aliquots (80 μ l) were placed on the sampler and 128 spectra were collected at a resolution of 4 cm⁻¹. Haem spectra were obtained by subtraction of the background spectrum of the buffer.

Gingipain-haem interactions. HA2 and purified gingipains (0.2 or 2 μ M) were incubated at 37 °C with a fixed excess concentration of iron(III) protoporphyrin IX (20 μ M) in 0.14 M NaCl, pH 6.5, and the spectra recorded periodically. In some experiments, HA2 (4 μ M) was incubated with 400 μ M iron(III) protoporphyrin IX in 250 mM Tris/HCl, pH 7. The Q band region of the visible spectrum was monitored periodically, and the samples subjected to FT-IR.

RESULTS

During short-term incubation of recombinant HA2 with iron(III) protoporphyrin IX monomers there was a progressive drop in A₃₆₅, accompanied by a broadening of the Soret band and a reduction in the ratio of A₃₆₅ to A₃₈₅ (Fig. 1). These changes in the spectra are indicative of ferrihaem dimerization and aggregation (Inada & Shibata, 1962; Brown *et al.*, 1970, 1976, 1980). In the control haem there were slight reductions with time in both the Soret band intensity and the A₃₆₅ to A₃₈₅ ratio, indicative of a small amount of μ -oxo dimer formation (data not shown). The difference spectrum made by subtraction of the haem control from the HA2 test spectrum taken after 1 h incubation revealed a haem β band at 608 nm (Fig. 1; inset).

Fig. 1. UV-visible spectra of iron(III) protoporphyrin IX incubated with recombinant HA2 over a 6 h period. The molarities of the iron porphyrin and HA2 were 20 and 2 μ M, respectively. Incubation was carried out at 37 °C in 0.14 M NaCl, 0.2 M phosphate, pH 6.5. Inset: difference spectrum between HA2-haem and haem control after 1 h incubation, showing the β band at 608 nm attributable to the presence of the μ -oxo dimer; the concentrations of HA2 and iron(III) protoporphyrin IX were 4 and 400 μ M, respectively.

This is indicative of the presence of the μ -oxo-bridged species and not of the iron(III) monomer, which displays a β band in the region of 630 nm (Silver & Lukas, 1983; Miller *et al.*, 1987). Importantly, this demonstrated that the μ -oxo dimer was formed during the phase represented by the drop in A_{365} . Final confirmation of the production of μ -oxo bishaem was provided by ATR FT-IR spectroscopy. To improve the IR spectroscopic detection of the μ -oxo dimer, HA2 was incubated for 24 h with an increased concentration of iron(III) monomer (400 μ M) at a haem:protein molar ratio of 100:1 (Fig. 2). This revealed an absorbance band at ~ 900 cm^{-1} attributable to the asymmetric stretching frequency of the oxo-bridged Fe-O-Fe dimer (Brown *et al.*, 1969; Kapetanaki & Varotsis, 2000). As a negative control, Fe(III)PPIX.OH was incubated with bovine albumin, which resulted in a Soret band with a 403 nm λ_{max} (data not shown), indicating the formation of a haem monomer-albumin complex (Beaven *et al.*, 1974; Kamal & Behere, 2002) and not the μ -oxo bishaem. The amounts of the μ -oxo dimer formed and monomer depleted during incubation of HA2 with iron(III) protoporphyrin IX monomers were calculated from the A_{365} and A_{385} ratios and the millimolar extinction coefficients at these wavelengths (Fig. 3). As expected, we observed a low level of μ -oxo dimer formation from some of the ferrihaem monomer in solution in the control (Inada & Shibata, 1962). In contrast, incubation of Fe(III)PPIX.OH with HA2 resulted in an immediate increase in the amount of μ -oxo dimer and depletion of the monomer. Approximately 50% (10 μ M) of the monomer was converted into μ -oxo dimer after 1 h. This is in reasonable agreement with the value of 14 μ M calculated using a

Fig. 2. ATR FT-IR spectra of (a) iron(III) protoporphyrin IX (400 μ M) after incubation with HA2 (4 μ M), showing the 900 cm^{-1} band attributable to the asymmetric stretching frequency of the oxo-bridged Fe-O-Fe dimer (arrowed); (b) control iron(III) protoporphyrin IX. Incubations were carried out for 24 h at 37 °C in 250 mM Tris/HCl, pH 7.0.

millimolar extinction coefficient of 5 (Silver & Lukas, 1983) for the 608 nm band shown in Fig. 1. Incubation of iron(III) protoporphyrin IX monomers with purified Kgp also gave a series of spectra similar to those of HA2, characterized by a fall in A_{365} and gradual shift in the Soret λ_{max} to 385 nm (Fig. 4). As for the HA2 protein, there was an initial rapid increase in the concentration of the μ -oxo dimer formed in the presence of the Kgp polyprotein (Fig. 4, inset).

Taken together, these data show that the HA2 domain and the Kgp polyprotein can mediate dimerization of monomeric ferrihaems, accompanied by aggregation of the μ -oxo bishaem. In this context, aggregation should not be confused with the process of bacterial aggregation, but rather be understood as the process of stacking of μ -oxo dimers in solution through weak π -bonding interactions to give larger molecular aggregates (Brown *et al.*, 1976, 1980).

A comparison was made between the polyprotein gingipains HRgpA and Kgp, and the single-chain protease RgpB, for the ability to promote dimerization and aggregation. As seen in Fig. 5, the spectra of the RgpB-haem and control

Fig. 3. Time-course of the formation of μ -oxo dimer and depletion of monomer in the control and during incubation of HA2 with iron(III) protoporphyrin IX (20 μ M). The amounts of μ -oxo dimer and monomer were calculated from the absorbances at 365 and 385 nm, as derived from Fig. 1, and are expressed as a percentage of the total iron porphyrin concentration.

haem incubations were almost identical. In contrast, the HRgpA- and Kgp-haem incubations resulted in a broadening of the Soret band and greater reductions in intensity, indicative of a greater extent of μ -oxo dimer formation and aggregation compared to the control or the RgpB protease. Reduction of Soret band absorbance intensity and broadening are measures of the extent of dimerization (Inada & Shibata, 1962) and molecular aggregation of ferrihaems

Fig. 4. UV-visible spectra of iron(III) protoporphyrin IX (20 μ M) incubated with purified Kgp (2 μ M) at 37 °C in 0.14 M NaCl, 0.2 M phosphate, pH 6.5. The inset shows the μ -oxo dimer formed as a percentage of total iron porphyrin during incubation with Kgp (●) and in the control (■).

(Wood *et al.*, 2004), respectively. On this basis it was clearly demonstrated that both HRgpA and Kgp mediated greater dimer formation and aggregation than RgpB, which lacks the HA2 domain.

DISCUSSION

The covalent μ -oxo bishaem complex, the major component of the haem pigment, forms through the spontaneous reaction between dioxygen and Fe(II)PPIX monomers

Fig. 5. UV-visible spectra of the Soret band regions of 10 μ M iron(III) protoporphyrin IX incubated with purified gingipains. (a) Control haem; (b) RgpB; (c) Kgp; (d) HRgpA. The iron porphyrin:protein ratio in each case was 100:1, and incubations were carried out at 37 °C in 0.14 M NaCl, 0.2 M phosphate, pH 6.5.

proteolytically released by *P. gingivalis* from deoxyhaemoglobin [where the haems are in the iron(II) state] (Smalley *et al.*, 2002):

It also forms at a much slower rate through dimerization of iron(III) protoporphyrin IX monomers (Inada & Shibata, 1962; Brown *et al.*, 1970; Silver & Lukas, 1983; Miller *et al.*, 1987), according to the equation:

Because the μ -oxo dimer is formed from Fe(III)PPIX.OH released from methaemoglobin (the oxidized form of haemoglobin) (Smalley *et al.*, 2002), we examined interactions of Fe(III)PPIX.OH with purified gingipains and the HA2 haemagglutinin, which is known to mediate haem binding to the Kgp and HRgpA polyproteins.

Using UV-visible spectroscopy, we demonstrated that the isolated HA2 protein can convert the monomeric iron(III) species into the μ -oxo dimer. The formation of the covalent Fe-O-Fe bridged haem complex was confirmed using IR spectroscopy. The generation of the μ -oxo dimer was accompanied by aggregation. In addition to the HA2 protein, μ -oxo dimer formation was mediated by both Kgp and HRgpA polyproteins which possess this adhesin. The inability of RgpB to promote these effects is in keeping with its lack of HA2. Thus, in addition to acting as a multifunctional adhesin, the HA2 domain of Arg- and Lys-gingipains, which is identical in both gingipains (Pavloff *et al.*, 1997), may play an important role in haem-pigment formation by facilitating both dimerization and aggregation of the μ -oxo bishaem complex.

The mechanism of HA2-mediated dimer formation is not clear, but we speculate that this domain serves as a template to transiently bind Fe(III) monomers such that they may react with other Fe(III)PPIX.OH molecules, either free in solution or bound to the protein, to form $[\text{Fe(III)PPIX}]_2\text{O}$ according to reaction (2). Newly formed μ -oxo bishaem released from the protein would be free to aggregate through weak π -bonding interactions and porphyrin stacking to form micelles which would become segregated from solution (Brown *et al.*, 1980). Rendering μ -oxo dimer aggregates insoluble would result in greater monomer to dimer conversion so as to maintain the solution equilibrium between the monomeric and dimeric forms (Brown *et al.*, 1976; Silver & Lukas, 1983). This behaviour of the ferrihaems in aqueous solution would drive pigment production and may explain, in part, why *P. gingivalis* accumulates up to 50 % of its biomass dry weight as haem (Rizza *et al.*, 1968; Smalley *et al.*, 1998) in the form of aggregated μ -oxo dimer (Smalley *et al.*, 1998, 2004).

P. gingivalis displays a pH growth optimum of 7.5–8 (McDermid *et al.*, 1988), and its preferred habitats, the inflamed gingival sulcus and diseased periodontal pocket, have a slightly alkaline pH (Bickel & Cimasoni, 1985; Eggert

et al., 1991), which will promote μ -oxo bishaem formation from Fe(III)PPIX.OH (Silver & Lukas, 1983). Although acid pH ordinarily favours formation of Fe(III)PPIX.OH monomers from the $[\text{Fe(III)PPIX}]_2\text{O}$ complex, Silver & Lukas (1983) have shown that once formed at low pH, μ -oxo dimers remain stable. In this context, we demonstrated that μ -oxo dimer formation was mediated by HA2, HRgpA and Kgp under slightly acid conditions (pH 6.5). This is significant, as it demonstrates that *P. gingivalis* may be capable of promoting μ -oxo dimer pigment formation at below neutral pH, such as in supragingival plaque, as well as in the subgingival environment.

Several other proteins are expressed by *P. gingivalis* which are involved in the binding and/or uptake of haem. These include outer-membrane proteins expressed under haem limitation (Bramanti & Holt, 1993; Smalley *et al.*, 1993), the iron haem transport protein (IhtB) (Hendtlass *et al.*, 2000), the TonB-like proteins Tla and Tlr (Aduse-Opoku *et al.*, 1997; Slakeski *et al.*, 2000), the haem-regulated protein HemR (Karunakaran *et al.*, 1997) and a haem/haemoglobin-binding receptor (HmuR) (Simpson *et al.*, 2000, 2004). It should also be noted that the cell-surface haemagglutinin A (HagA) protein of *P. gingivalis* possesses four repeats of the HA2 domain sequence in its structure (Shi *et al.*, 1999), but its role, and that of the above proteins in μ -oxo bishaem formation, has not been investigated. Importantly, in addition to targeting the catalytic functions of the Arg- and Lys-gingipains to abrogate the aggressive proteolytic nature and pathogenic potential of *P. gingivalis*, consideration must now be given to perturbing production of the protective haem pigment by inhibiting protease-mediated μ -oxo bishaem formation from monomeric haem precursors.

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