Oral Microbiology Immunology 2004: 19: 50–56
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Oral Microbiology and Immunology

Hemoglobin hydrolysis and heme acquisition by *Porphyromonas gingivalis*

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Dashper SG, Cross KJ, Slakeski N, Lissel P, Aulakh P, Moore C, Reynolds EC. Hemoglobin hydrolysis and heme acquisition by Porphyromonas gingivalis. *Oral Microbiol Immunol 2004: 19: 50–56.* © Blackwell Munksgaard, 2004.

Porphyromonas gingivalis has been implicated in the progression of chronic periodontitis, an inflammatory disease of the supporting tissues of the teeth. This bacterium is a gram-negative, black-pigmented, asaccharolytic anaerobe that relies on the fermentation of amino acids for the production of metabolic energy. The Arg- and Lys-specific extracellular cysteine proteinases of *P. gingivalis*, RgpA, RgpB and Kgp have been implicated as major virulence factors. In this study we investigated the hydrolysis of human hemoglobin by whole cells of *P. gingivalis* W50 and the mutants W501 ($RgpA^-$), W50AB ($RgpA^-RgpB^-$) and W50ABK ($RgpA^-RgpB^-Kgp^-$) under strictly anaerobic conditions in a physiological buffer (pH 7.5) using mass spectrometric analysis. Incubation of *P. gingivalis* W50 with hemoglobin over a period of 30 min resulted in the detection of 20 hemoglobin peptides, all with C-terminal Arg or Lys residues. The majority of the hemoglobin α- and β-chain sequences were recovered as peptides except for two similar regions of the C-terminal half of each chain, α(92-127) and β(83-120). The residues of the unrecovered sequences form part of the interface between the α- and β-chains and an exposed surface area of the hemoglobin tetramer that may be involved in binding to *P. gingivalis*. *P. gingivalis* W501 ($RgpA^-$) produced similar peptides to those seen in the wild-type. All identified peptides from the hydrolysis of hemoglobin by the *P. gingivalis* W50AB ($RgpA^-RgpB^-$) mutant were the result of cleavage at Lys. The triple mutant W50ABK was unable to hydrolyze hemoglobin under the assay conditions used, suggesting that on whole cells the major cell surface activity responsible for hydrolysis of hemoglobin is from the RgpA/B and Kgp proteinases. However, the triple proteinase mutant W50ABK grew as well as the wild-type in a medium containing hemoglobin as the only iron source, indicating that the RgpA/B and Kgp proteinases are not essential for iron assimilation from hemoglobin by *P. gingivalis*.

Key words: growth; hemoglobin hydrolysis; mass spectrometry; mutants; *Porphyromonas gingivalis*; RgpA/B and Kgp proteinases

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Accepted for publication August 14, 2003

Periodontal diseases are complex, bacteria-associated inflammatory diseases of the supporting tissue of the teeth. The change from a periodontally healthy site to one undergoing destruction is accompanied by an increase in the relative abundance of a small number of opportunistic pathogens, in particular *Porphyromonas gingivalis* (10, 30, 32). To understand this shift in the species composition of subgingival plaque during disease initiation and progression, information on the ability of pathogenic bacteria to compete for, acquire and utilize nutrients is crucial. *P. gingivalis* is a black-pigmented, gram-negative, asaccharolytic anaerobe that relies on the fermentation of amino acids for the production of metabolic energy (23) and like the vast majority of bacteria it also requires iron.

The Arg- and Lys-specific extracellular cysteine proteinases of *P. gingivalis*, RgpA/B and Kgp, have been demonstrated to be essential for bacterial growth on bovine serum albumin (24) and are regarded as major virulence factors (12, 15, 18). RgpA is a calcium-stabilized, arginine-specific, cysteine proteinase with a C-terminal extension of adhesin domains. Kgp is a lysine-specific cysteine proteinase with C-terminal adhesin domains similar to those of RgpA. RgpA and Kgp exist as large complexes of the processed proteinase and adhesin domains located on the outer membrane (3) and one of the adhesin domains of RgpA and Kgp has recently been shown to bind to hemoglobin (17). A second Arg-specific

enzyme (RgpB), which is not associated with adhesins, is also cell-surface associated (27, 36). This enzyme is a calcium-stabilized, cysteine proteinase that has enzyme characteristics and an inhibitor/activator profile almost identical to the RgpA but does not contain the C-terminal adhesins of RgpA (27).

One of the characteristics of periodontitis is vascular disruption and bleeding. *P. gingivalis* produces a series of hemagglutinins, at least two hemolysins and a number of hemoglobin-binding proteins (12). This suggests that *in vivo* hemoglobin may be an important source of peptides and amino acids for growth of the bacterium as well as a source of heme, which provides both iron and protoporphyrin IX (PPIX) (31). Because *P. gingivalis* lacks the ability to synthesize PPIX *de novo*, it must be obtained from an exogenous source (21, 22). *P. gingivalis* preferentially utilizes hemoglobin-derived heme and this has been suggested to be acquired through the ability of the RgpA-Kgp proteinase-adhesin complexes of *P. gingivalis* to bind and hydrolyze hemoglobin, releasing heme at the cell surface (9, 13, 25). However, no studies have determined whether the extracellular RgpA/B and Kgp proteinases of *P. gingivalis* are essential for the extraction and utilization of heme from hemoglobin (33). Although partially purified Kgp has been demonstrated to hydrolyze hemoglobin *in vitro* (13) the degradation of hemoglobin by whole cells has not been studied, such that the actual proteinases involved in hemoglobin hydrolysis by *P. gingivalis* are as yet unknown.

The aim of this study was to investigate the hydrolysis of human hemoglobin by whole cells of *P. gingivalis* and isogenic mutants lacking the RgpA, RgpB and Kgp proteinases under strictly anaerobic conditions using mass spectrometric analysis. A further aim was to determine the ability of the triple mutant lacking RgpA, RgpB and Kgp to grow with hemoglobin as the only source of iron.

Materials and methods

Bacterial strain and mutants

Lyophilized cultures of *P. gingivalis* W50 and the extracellular proteinase/adhesin mutants W501 [$RgpA^-$ (20)] and W50AB [$RgpA^-$, $RgpB^-$ (36)] were obtained from the culture collection of the Centre for Oral Health Science, the University of Melbourne. The triple mutant W50ABK ($RgpA^-$, $RgpB^-$, Kgp^-) was generated by insertional inactivation of *kgp* in W50AB as follows. A pUC18 construct containing a *kgp* insert of W50 [pNS1 (28)] was insertionally inactivated with an erythromycin resistance cassette to generate pNSE1 (2). pNSE1 was linearized with *Aat*II and electrophorated into *P. gingivalis* W50AB to generate W50ABK following transformation procedures previously described (36). Positive transformants were selected for on horse blood agar (HBA; Oxoid Blood Agar Base 2 supplemented with 10% (v/v) defibrinated horse blood) containing 10 μg/ml erythromycin. *P. gingivalis* W50ABK colonies were non-pigmented. Integration of the insertionally inactivated *kgp* into the W50AB chromosome was confirmed by Southern blot analysis (data not shown). *P. gingivalis* W50 and mutants were grown anaerobically and maintained on HBA containing appropriate antibiotics with weekly subculturing. Culture purity was assessed regularly by Gram-stain and colony morphology.

Growth studies

Iron-limited growth studies were conducted in batch culture under anaerobic conditions in Mycoplasma Broth Base media (MBB; Becton Dickinson, BBL, Cockeyville, MD) supplemented with 0.5 g/l cysteine-HCl (4). Iron-limited cells of *P. gingivalis* W50 and W50ABK were produced by growth in MBB supplemented with 5.0 μM 2,2′-dipyridyl (Sigma, St Louis, MO), an iron chelator. A 5% (v/v) inoculum of the iron-limited cells was added to sterile media with added hemin (7.7 μM; Sigma) or human hemoglobin (1.0 μM; Sigma) as heme sources or with no added iron source. Growth was determined by monitoring optical density at a wavelength of 650 nm.

Hemoglobin hydrolysis by *P. gingivalis*

Batch cultures of *P. gingivalis* W50 and mutants for use in the hemoglobin hydrolysis assays were grown anaerobically at 37°C in 3.7% w/v brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK), supplemented with 0.5 g/l cysteine-HCl and 5.0 mg/l bovine hemin (Sigma). Cells were harvested by centrifugation (5500 × *g*, 20 min, 4°C) during exponential growth phase. *P. gingivalis* cells were washed and resuspended to a density of approximately 1.0 mg dry weight/ml in Pga buffer (10.0 mM NaH_2PO_4; 10.0 mM KCl; 2.0 mM citric acid; 1.25 mM $MgCl_2$; 20.0 μM $CaCl_2$; 25.0 μM $ZnCl_2$; 50.0 μM $MnCl_2$; 5.0 μM $CuCl_2$; 10.0 μM $CoCl_2$; 5.0 μM H_3BO_3; 1 μM Na_2MoO_4 with the pH adjusted to 7.5 with 5 M NaOH at 37°C), which was based on the chemically defined medium of Milner et al. (16). All cell manipulations and incubations were carried out in an anaerobe chamber (MK3 Anaerobic workstation, Don Whitley Scientific Ltd., Sydney, NSW, Australia) in an atmosphere of 5% hydrogen, 10% carbon dioxide and 85% nitrogen.

To start the assay, a *P. gingivalis* cell suspension was warmed to 37°C with stirring. To this was added hemoglobin in Pga buffer to give a final concentration of 1.0 mg/ml. Cells were separated from the buffer at specified times after hemoglobin addition by centrifugation (16,000 × *g*, 60 s) through 500 ml silicon oil with a specific gravity of 1.015 g/ml (7). An aliquot (0.4 ml) of the supernatant was immediately transferred to a vial containing 44 ml of 0.46 M acetic acid which lowered the pH to ~3.5, thereby inhibiting any residual proteolytic activity. A control of *P. gingivalis* with no added hemoglobin was used to determine endogenous production or release of peptides from the bacterium. Another control of hemoglobin without *P. gingivalis* cells was used to confirm the purity and stability of the protein under assay conditions.

Peptide analysis

Peptides produced by *P. gingivalis* hydrolysis of hemoglobin were identified by mass spectrometry. Mass spectrometric analysis of acid-treated supernatants was performed using a Voyager DE™ linear matrix assisted laser desorption/ionisation time of flight mass spectrometer (MALDI-MS) equipped with delayed extraction (Applied Biosystems, CA, USA). Samples were mixed (1 : 1 v/v) on the sample analysis plate with a saturated solution of 2,5 dihydroxybenzoic acid in 30% aqueous acetonitrile, containing 0.1% v/v trifluoracetic acid and left to dry. All spectra were obtained in linear, positive and negative mode with an accelerating voltage of 20 kV and pulse delay time of 125 ns. To assign the peaks in the mass spectra an *in silico* tryptic digestion of the α- and β-chains of human hemoglobin was conducted using the General Protein Mass Analysis for Windows (GPMAW; Lighthouse Data, Odense, Denmark) and MS Digest programs. Mass spectra of the *P. gingivalis* hemoglobin digest were then compared with the theoretical masses expected in a tryptic digest of hemoglobin. Peaks of the spectra were only assigned to hemoglobin peptides if the observed mass matched the theoretical mass to within 0.05%. More than 90% of the matches were within 0.03%.

Results

Hemoglobin hydrolysis

Mass spectrometric analysis of supernatants of whole cell suspensions of *P. gingivalis* W50 incubated at 37°C in Pga buffer for 30 min under strictly anaerobic conditions revealed between two to four major peaks with m/z values of between 900 and 1300. When hemoglobin was incubated without *P. gingivalis* cells only four peaks with *m/z* values of 7567.4, 7982.1, 15,127.2 and 15,868.5 could be detected. These values were within 0.01% of that expected for the singly and doubly charged ions of intact α- and β-chains of hemoglobin (15,126.0 and 15,867.3 Da, respectively). No hemoglobin-derived peptides were detected.

Mass spectrometric analysis of the supernatants from *P. gingivalis* W50 cells incubated with hemoglobin under strictly anaerobic conditions at 37°C revealed the presence of peaks that could be assigned to hemoglobin-derived peptides (for example see Fig. 1A). The four peaks with the highest *m/z* values were identified as the intact α- and β-chains of hemoglobin as the singly and doubly charged ions. All of the remaining peaks observed in the spectra could be assigned to hemoglobin peptides with C-terminal Arg or Lys residues (Fig. 2). Two peaks could not be unambiguously assigned as each of the observed *m/z* values could correspond to either, or both, of two α-chain hemoglobin 'tryptic' peptides [α(32–60) and α(62–92) both have masses of 3266.7; α(32–61) and α(61–92) both have masses of 3394.9]. Eight peptides from the β-chain and 12 peptides from the α-chain were recovered

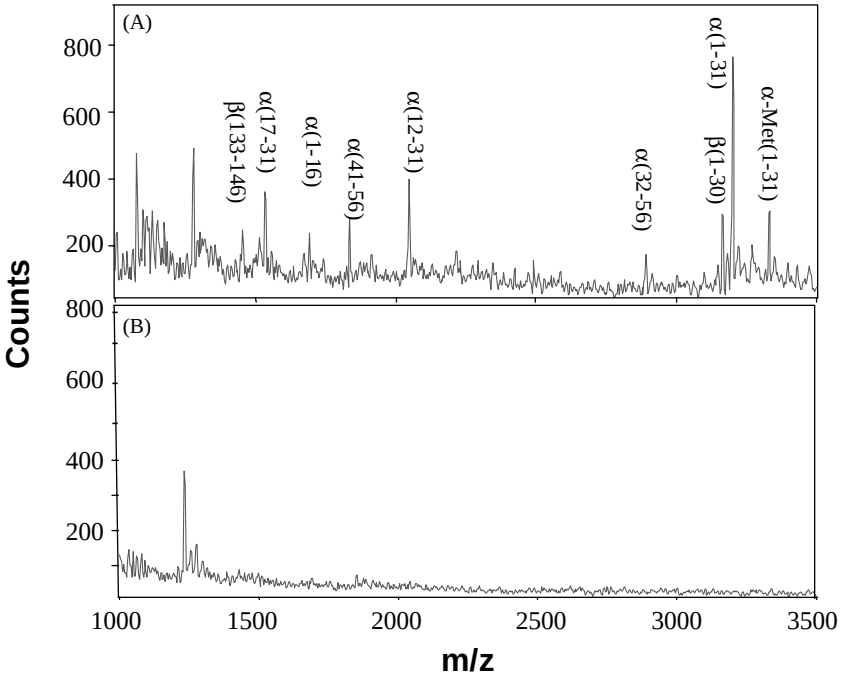

Fig. 1. Mass spectra of the supernatant of a *P. gingivalis* whole cell suspension in Pga buffer incubated at 37°C under anaerobic conditions with hemoglobin: A) Strain W50 after 5 min. B) The triple proteinase mutant W50ABK after 30 min.

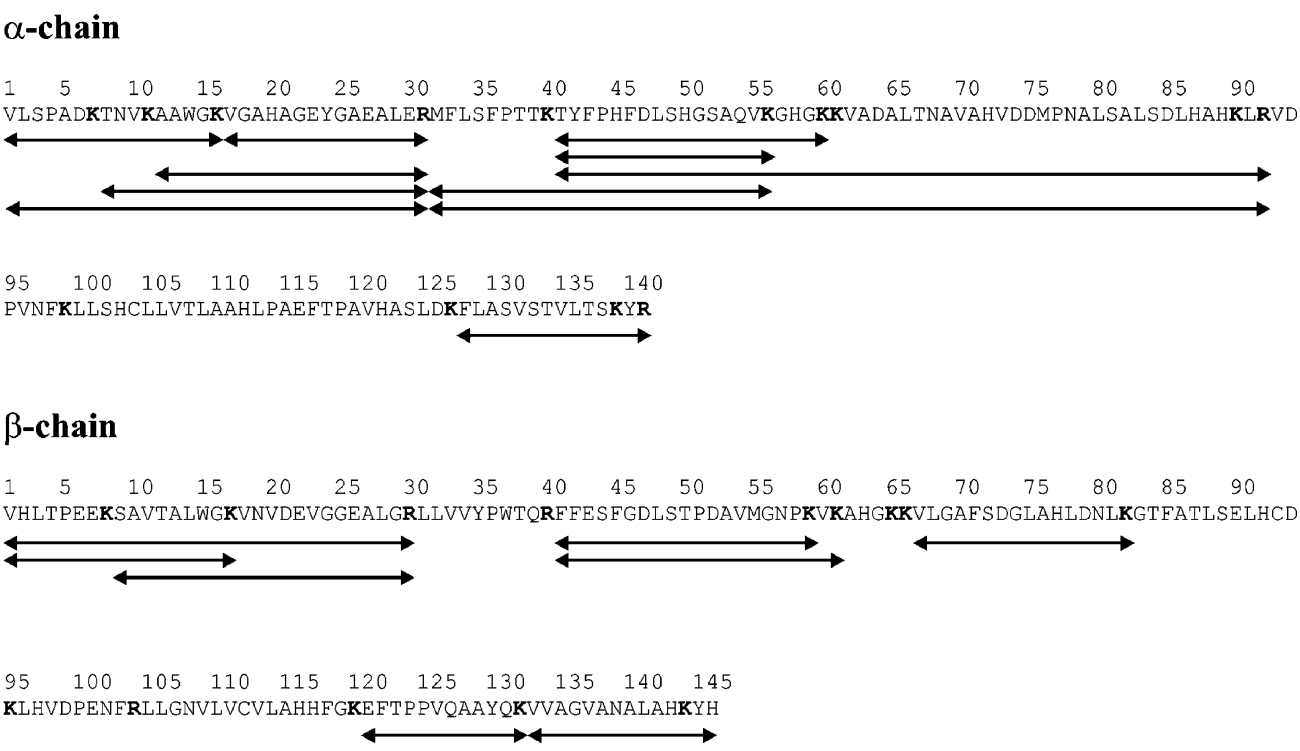

Fig. 2. Amino acid sequence of α- and β-chains of human hemoglobin showing all peptides identified during a 30 min incubation with *P. gingivalis* W50. Arg and Lys residues are highlighted.

Table 1. Mass spectrometric analysis of hemoglobin digestion by whole cells of *P. gingivalis* W50. The mass spectral data were collected from repeated analyses of cell suspension supernatants at six time points during 30 min incubations of whole cells of *P. gingivalis* W50 with 1.0 mg/ml hemoglobin at 37 C under strict anaerobic conditions.

	Time (min)					
Peptide	1	3	5	10	20	30
β(1–30)	–	✓	✓	✓	–	–
β(1–17)	–	–	–	–	–	✓
β(9–30)	–	–	–	✓	✓	✓
β(41–59)	–	–	–	–	✓	✓
β(41–61)	–	–	–	–	✓	✓
β(67–82)	–	–	–	–	–	✓
β(121–132)	–	–	–	–	✓	✓
β(133–146)	–	–	✓	–	✓	✓
α(1–16)	–	✓	✓	✓	–	–
α(1–31)*	✓	✓	✓	–	–	–
α(12–31)	✓	✓	✓	✓	✓	✓
α(17–31)	–	✓	✓	✓	✓	✓
α(8–31)	–	–	–	✓	✓	✓
α(32–56)	–	✓	✓	–	–	–
α(41–56)	✓	✓	✓	✓	✓	✓
α(41–60)	–	–	–	–	✓	–
α(41–92)	–	–	–	✓	–	–
α(32–92)	–	–	–	✓	–	–
α(128–141)	–	–	–	–	–	✓

*Includes both α(1–31) and α-Met(1–31).

in the supernatant over the time course of the hydrolysis for 30 min (Fig. 2). Of the total of 20 hemoglobin peptides identified, four were detected from the α-chain 1 min after the commencement of incubation with whole *P. gingivalis* cells and seven were recovered after 3 min of incubation, showing the progressive hydrolysis of the hemoglobin α-chain (Table 1). The β-chain hydrolysis was slower, with no peptides detected after 1 min of incubation with *P. gingivalis*, one peptide was recovered after 3 min, two after 5 min, five after 20 min and seven after 30 min (Table 1).

The digestion of hemoglobin by *P. gingivalis* W501 lacking the RgpA proteinase resulted in a total of 12 hemoglobin-derived peptides with C-terminal Arg or Lys residues being detected by mass spectrometry. Seven were assigned to α-chain peptides and five to β-chain peptides. Most of the detected peptides were the same as those found in the W50 digest; however, three previously undetected peptides were also found [β(83–95), β(121–146), β(9–59)]. Incubation of *P. gingivalis* W50AB, lacking both the Arg-specific proteinases RgpA and RgpB, with hemoglobin produced a total of 13 major peaks that could be assigned to α- or β-chain peptides. These 13 hemoglobin peptides all had a Lys residue at the C-terminus and immediately preceding the N-terminal residue of the peptide in the α- or β-chain sequence. Eight of these peptides were not detected in the W50 digest and six of these contained at least one internal Arg residue. Apart from the four peaks representing the intact α- and β-chains no hemoglobin-derived peptides were detected when whole cells of *P. gingivalis* W50ABK ($RgpA^-$, $RgpB^-$, Kgp^-) were incubated with hemoglobin for 30 min (Fig. 1B).

Growth studies

P. gingivalis W50 and W50ABK were iron-depleted in MBB medium containing the iron chelator 2,2'-dipyridyl. These cells were then used to inoculate MBB containing a) 7.7 mM hemin, b) 1.0 mM hemoglobin or c) no additions. The basal iron content of this medium was 9.9 mM, as determined by atomic absorption spectrophotometry (6). The wild-type and the triple mutant exhibited very limited growth in the MBB medium with no added iron but both strains grew well in the MBB medium containing hemin or hemoglobin (Fig. 3). The growth of the triple proteinase mutant was similar to that of the wild-type in all media.

Discussion

P. gingivalis is an anaerobic, asaccharolytic bacterium that relies on amino acid fermentation for energy production. Although *P. gingivalis* has been shown to directly transport and utilize some amino acids such as threonine, serine and arginine, it is believed to prefer short peptides (7, 14, 34). *P. gingivalis* becomes more numerous at sites where bleeding is occurring and hemoglobin is believed to be an important source of not only peptides and amino acids but also iron and protoporphyrin IX (13). Hemoglobin is a tetrameric protein composed of two α- and two β-chain subunits and each of these chains contains 11 Lys and three Arg residues (Fig. 2). In the current study we investigated the hydrolysis of hemoglobin by *P. gingivalis* W50 whole cells and mutants lacking the Arg-specific RgpA/B and Lys-specific Kgp proteinases using mass spectrometry, in an attempt to determine the proteinases involved in hemoglobin assimilation. When hemoglobin was incubated without *P. gingivalis* cells only the intact α- and β-chains of hemoglobin were detected. This

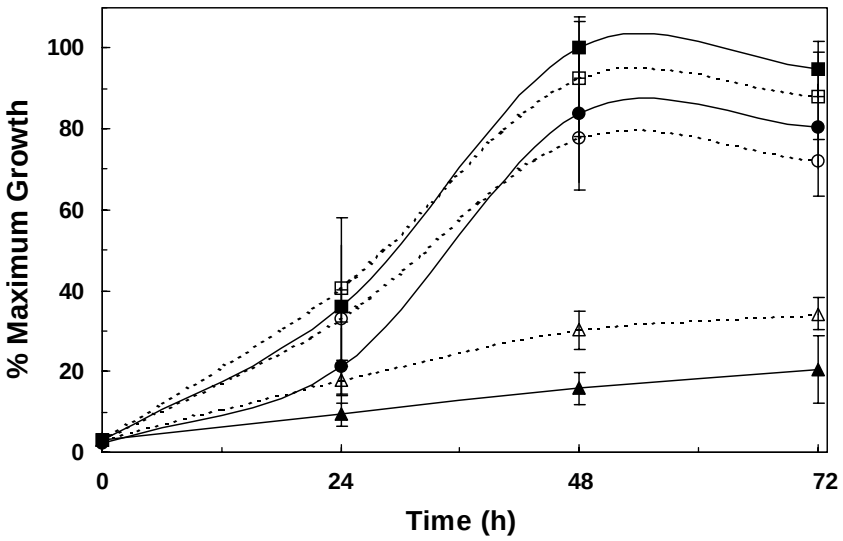

Fig. 3. P. gingivalis W50 (solid symbols) and W50ABK (open symbols) batch culture growth in MBB media containing no additions (△, ▲), hemoglobin (□, ■) or hemin (○, ●). Points represent the means and standard deviations from three experiments.

indicated that no breakdown of hemoglobin occurred under the assay conditions without *P. gingivalis*. When *P. gingivalis* cells alone were incubated under the assay conditions, up to four peaks with variable *m/z* values between 900 and 1300 were detected, these peaks were not studied further. Incubation of washed cells of *P. gingivalis* W50 with hemoglobin resulted in rapid hydrolysis of the multimeric protein. Of the 24 major peaks detected, all could be assigned to hemoglobin-derived peptides with C-terminal Arg or Lys residues or the unhydrolyzed α- and β-chains. These data are consistent with the Arg- and Lys-specific proteinases, RgpA/B and Kgp, providing the major proteolytic activity against hemoglobin on the cell surface of *P. gingivalis* W50.

The detection of a range of hemoglobin-derived peptides in the supernatant indicates that these peptides had not been bound to, or transported into, the *P. gingivalis* cell or further hydrolyzed. Some peptides may not have been detected by the mass spectrometric analysis; however, we have previously detected peptides covering the entire sequence of the hemoglobin β-chain, except the region -^{59}KVKAHGKK^{66}-, from a tryptic digest of hemoglobin using the same mass spectrometry analytical method (5). Of the 20 hemoglobin peptides identified in the current study, five contained no internal Arg or Lys residues [α(17–31), α(41–56), β(41–59), β(67–82) and β(121–132)]. No further cleavage of these peptides by the *P. gingivalis* Arg- and Lys-specific extracellular proteinases would have been possible and due to their length it is unlikely that they would have been transported through the outer membrane porins and then into the cell. All five of these peptides could be detected in the supernatant at the end of the 30 min incubation period. Therefore it is possible that *P. gingivalis* only metabolizes a subset of the peptides produced by its hydrolysis of hemoglobin.

The results on the kinetics of hemoglobin hydrolysis by *P. gingivalis* W50 can be rationalized using the crystal structure of hemoglobin [deoxyhemoglobin, structure 1A3N, Protein Data Bank (35)] . The Lys residues (e.g., K^{7}, K^{11}, K^{16}, K^{56}, K^{60}, and K^{61} of the α-chain) in the structure of human hemoglobin are surface exposed and are located on segments that are highly mobile. Mobile segments are usually not important for structural integrity, but are required to link rigid, structurally important features such as α-helices. Because the Lys residues are highly mobile and surface-exposed, the cleavage of the peptide backbone at these residues would allow access to the underlying residues without loss of the tertiary structure. The R^{31} and K^{40} residues of the α-chain are protected from proteolytic activity by two surface loops, one from the α-chain, ^{48}LSHGS^{52}, and one from the β-chain, ^{120}KEFTP^{124}. Proteinase access to R^{31} and K^{40} would require that these loops to be at least partially removed. The detection of the hemoglobin peptides α(1–31), α(12–31), and α(41–56) within 1 min of incubation with *P. gingivalis* suggests that partial cleavage had already occurred at K^{11} and K^{56} of the α-chain and K^{120} of the β-chain, thereby allowing cleavage at the underlying R^{31} and K^{40} residues of the α-chain. Once these peptides had been released R^{92} and K^{127} would then have become more exposed. Cleavage at these residues would then have resulted in the release of peptide fragments of the form α(x–92) and α(128–141), thus completing the hydrolysis at the susceptible Arg and Lys residues of the hemoglobin α-chain.

The first stage of the hemoglobin β-chain proteolysis would have been similar to that of the α-chain. Initially, cleavage would have occurred at the surface-exposed Lys residues, which then would have exposed the helices containing R^{30} and K^{132}. However, these helices are orientated such that access to the side-chains would have been restricted and further processing would have been slow, which is consistent with the experimental observations (Table 1). The β-chain would be expected to retain its native structure despite the loss of N-terminal and C-terminal peptides. The rapid appearance of β-chain fragments after 20 min of incubation with *P. gingivalis* coincides with the hydrolysis of the α-hemoglobin chains that would have shielded the crucial residues in the β-chains.

Interestingly, peptides from the C-terminal segments of the α-chain [α(93–127)] and β-chain [β(83–120)] were not recovered from the hydrolysis of hemoglobin by *P. gingivalis* W50. This may indicate that these sequences are involved in the binding of hemoglobin to the RgpA/Kgp proteinase/adhesin complexes or other cell-surface proteins and are therefore not immediately released as peptides into the supernatant. Five residues from the α-chain, His^{112}, Pro^{114}, Ala^{115}, Glu^{116} and Thr^{118}, and four residues from the β-chain, His^{116}, His^{117}, Phe^{118} and Lys^{120}, form an exposed surface area on the hemoglobin tetramer that may be a *P. gingivalis* binding site (Fig. 4). Binding of the RgpA–Kgp proteinase–adhesin complexes to hemoglobin has been attributed to interactions between the adhesin domains of the complexes and the hemoglobin protein subunits, although the exact binding site has not yet been identified (17, 19). The interface between the α- and β-chains of hemoglobin in the tetramer involves residues His^{103} to His^{122} of the α-chain and Asn^{108} to Gly^{119} of the β-chain. These residues interact to stabilize the tetramer and interactions that stabilize this interface may persist and continue to stabilize the two peptides bound to the *P. gingivalis* cell surface RgpA–Kgp complexes.

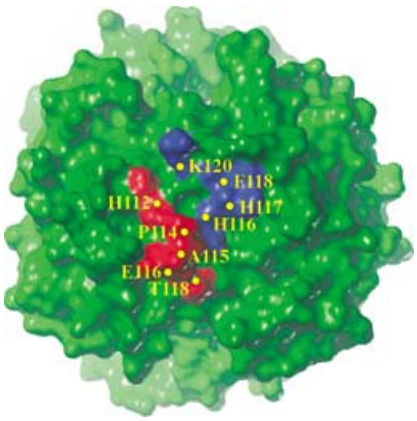

Fig. 4. Hemoglobin, space filling model. The surface-exposed residues His^{112}, Pro^{114}, Ala^{115}, Glu^{116} and Thr^{118} of the α-chain are colored red and the surface-exposed residues His^{116}, His^{117}, Phe^{118} and Lys^{120} of the β-chain are colored blue. Together these form a surface-exposed area that may be involved in hemoglobin binding to *P. gingivalis*.

P. gingivalis W501, which lacks the RgpA proteinase, produced hemoglobin peptides that resulted from the cleavage of hemoglobin at Arg and Lys residues suggesting that the RgpB proteinase was involved in hydrolysis. *P. gingivalis* W50AB, which lacks both extracellular Arg-specific proteinases produced only hemoglobin peptides that contained C-terminal Lys residues. The triple mutant *P. gingivalis* W50ABK was unable to hydrolyze hemoglobin at all, consistent with the previously demonstrated inability of a similar mutant to grow on bovine or human serum albumin as the sole source of amino acids/peptides (11, 24).

Kgp has been shown to be essential for the formation of the black pigmentation associated with *P. gingivalis* growth on blood agar plates (2). This black pigmentation has been attributed to the deposition of heme, as a μ-oxo dimer, on the cell surface (31). The results of the current study suggest that Kgp is essential for the rapid hydrolysis of hemoglobin at the

surface-exposed Lys residues. This rapid hydrolysis and subsequent release of heme at the cell surface may promote the formation of the μ-oxo dimer, resulting in its deposition on the cell surface.

As we demonstrated in this study that the triple proteinase mutant, W50ABK, is unable to hydrolyze hemoglobin we examined its ability to grow in medium containing hemoglobin as the only iron source. A medium containing low levels of iron that is unable to support the growth of *P. gingivalis* (4) was used as the basal medium for the growth studies. Similar growth of *P. gingivalis* W50 and W50ABK was obtained in the medium supplemented with either hemin or hemoglobin (Fig. 3), demonstrating that the triple proteinase mutant was able to utilize the heme from hemoglobin as the only source of iron. Therefore, *P. gingivalis* must have other mechanisms, apart from α- and β-chain hydrolysis, for liberating iron from hemoglobin. Some outer membrane TonB-linked receptors of gram-negative bacteria have been shown to bind hemoglobin, extract the heme and transport it into the periplasm (33). Genco and coworkers have characterized a *P. gingivalis* hemoglobin-binding, TonB-linked outer membrane receptor (HmuR) that may perform this function (26). There are up to four other recently identified putative TonB-linked outer membrane receptors of *P. gingivalis* that may also play a role in the extraction of iron/heme from hemoglobin and its transport into the periplasm (1, 8, 29, 36).

In conclusion, we have demonstrated, using whole cells of *P. gingivalis*, that the extracellular proteinases RgpA/B and Kgp can account for all of the hydrolytic activity against hemoglobin but are not essential for iron removal from this protein.

Acknowledgments

This study was supported by NH&MRC Project Grant No. 145831 and an Australian Dental Research Fund summer scholarship to P.A. The excellent assistance of Peter Riley and Rita Paolini is gratefully acknowledged.

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