Interactions of *Porphyromonas gingivalis* with oxyhaemoglobin and deoxyhaemoglobin

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When grown on blood-containing solid media, the anaerobic periodontal pathogen *Porphyromonas gingivalis* produces a haem pigment, the major component of which is the μ-oxo bishaem of iron protoporphyrin IX [Smalley, Silver, Marsh and Birss (1998) Biochem. J. **331**, 681–685]. In this study, μ-oxo bishaem generation by *P. gingivalis* from oxy- and deoxyhaemoglobin was examined. Bacterial cells were shown to convert oxyhaemoglobin into methaemoglobin, which was degraded progressively, generating a mixture of both monomeric and μ-oxo dimeric iron protoporphyrin IX. The rate of methaemoglobin formation was accelerated in the presence of bacterial cells, but was inhibited by *N*-ethylmaleimide and tosyl-lysylchloromethylketone. Interaction of cells with deoxyhaemoglobin resulted in formation of an iron(III) haem species (Soret λ_{max}, 393 nm), identified as pure μ-oxo bishaem.

Key words: iron protoporphyrin IX, methaemoglobin, oxidation, μ-oxo bishaem, protease.

INTRODUCTION

Porphyromonas gingivalis is a Gram-negative black-pigmenting anaerobe that is most strongly associated with progressive periodontal (gum) disease in adults. It is the major focus of a genome project to identify its pathogenic characteristics (see http://www.pgingivalis.org). Periodontal diseases affect the supporting tissues of the tooth in 80–90 % of adults and are a major cause of tooth loss in the Western world [1]. They occur with increased frequency in patients with Down's syndrome and with systemic diseases such as diabetes mellitus, AIDS, leukaemia, neutropenia and Crohn's disease.

When grown on blood-containing media, *P. gingivalis* produces a black haem pigment, the major component of which is the μ-oxo bishaem of iron protoporphyrin IX [2]. The formation of μ-oxo bishaem is a mechanism through which haemoglobin-derived haem ties up oxygen and oxygen intermediates to generate an anaerobic microenvironment [2]. The fact that μ-oxo bishaem in both soluble form and as a cell-surface layer can protect *P. gingivalis* against hydrogen peroxide, by virtue of its inherent catalase activity [3], makes it an important virulence factor for this organism.

Generation of μ-oxo bishaem is presumed to involve the proteolytic release of iron protoporphyrin IX from haemoglobin by the Lys-specific gingipain (kgp) [4]. The importance of this enzyme in iron porphyrin acquisition is highlighted by the fact that *kgp*-deficient cells do not bind or degrade haemoglobin and also fail to pigment when cultured on blood agar [5]. Peptides have been demonstrated to result from the action of kgp on α- and β-globin chains at lysine residues flanking the haem-histidine co-ordination sites [4]. We have proposed that μ-oxo bishaem is produced by the progressive attack on dioxygen by proteolytically freed monomeric iron(II) protoporphyrin IX [Fe(II)PPIX] molecules [2]. However, the exact interactions between haemoglobin and the bacterial cell that lead to iron protoporphyrin IX release are unclear. In an attempt to resolve these problems we have examined the interactions of whole *P. gingivalis* cells with oxy- and deoxyhaemoglobin.

EXPERIMENTAL

Bacterial strain and growth conditions

P. gingivalis W50 was grown as described previously [2] in Schaedler anaerobe broth (Oxoid, Basingstoke, Hants, U.K.) and harvested at the end of the exponential growth phase (2 days). The cells were harvested by centrifugation (5000 ***g*** for 45 min at 4 °C), washed three times in 0.14 M NaCl buffered at pH 7.5 with 0.1 M Tris/HCl (NaCl/Tris) to remove contaminating growth-medium constituents, and re-suspended in NaCl/Tris.

Haemoglobin

For these studies, haemoglobin prepared as a haemolysate from horse erythrocytes was chosen, primarily because *P. gingivalis* obtains haemoglobin via haemolysis and also because previous studies on the identification of haem-pigment components have utilized horse blood [2]. Erythrocytes from fresh horse blood (TCS Biologicals, Wirral, Cheshire, U.K.) were pelleted by centrifugation at 5000 ***g*** for 5 min at 5 °C, washed three times in NaCl/Tris buffer to remove plasma components, and lysed by re-suspension in 1 mM Tris/HCl, pH 7.0, for 20 min at 20 °C [6]. The cell membranes were removed from the haemolysate by centrifugation (20000 ***g*** for 20 min at 5 °C) and the haemoglobin-containing supernatant re-centrifuged to remove any residual

Abbreviations used: Fe(II)PPIX, iron(II) protoporphyrin IX; Fe(III)PPIX, iron(III) protoporphyrin IX; Fe(III)PPIX.OH, haematin; NEM, *N*-ethylmaleimide; TLCK, tosyl-lysylchloromethylketone.

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cell ghosts. SDS/PAGE under reducing conditions revealed that the haemoglobin preparation was essentially stroma-free. The haemoglobin was diluted by a factor of 1 in 100 in NaCl/Tris, pH 7.5, and the concentration determined from the extinction coefficient of the Soret band.

Preparation of oxy- and deoxyhaemoglobin

Oxyhaemoglobin was prepared by oxygenation of the above haemoglobin solution in NaCl/Tris for 20 min at a flow rate of 3 ml·min^{-1}. The UV-visible spectrum was recorded immediately to confirm the presence of the oxygenated protein.

Deoxyhaemoglobin solutions were prepared in 10 ml batches by adding freshly prepared $Na_2S_2O_4$ (final concentration, 10 mM) to the haemoglobin stock solution, followed by bubbling with N_2 for 20 min at a flow rate of 3 ml·min^{-1}. The deoxyhaemoglobin solution was then sealed in a sterile capped plastic tube and transferred immediately to a Mark III anaerobic cabinet (Don Whitley Scientific, Shipley, W. Yorks, U.K.), where the solution was uncapped in the anaerobic atmosphere (80 % N_2/10 % CO_2/10 % H_2, by vol.). The UV-visible spectrum of a sample of the solution was also recorded immediately to confirm the presence of the deoxygenated species. The deoxyhaemoglobin was fully functional with respect to oxygen carriage as it could be converted into the oxygenated protein upon aeration or by bubbling with oxygen for 2 min (results not shown). Both oxy- and deoxyhaemoglobin preparations were used immediately.

Incubation of *P. gingivalis* cells with oxy- and deoxyhaemoglobin

For the cell/haemoglobin incubation experiments, NaCl/Tris buffer, pH 7.5, was sparged for 20 min with either oxygen or nitrogen, as appropriate, and equilibrated at 37 °C before use. Bacterial cells were suspended in either oxygenated or deoxygenated NaCl/Tris buffer, pH 7.5, to give a final concentration of 75 μg of protein·ml^{-1} when mixed with oxy- or deoxyhaemoglobin. This concentration of bacterial cells was chosen to ensure a minimal background light scattering over the UV-visible range 300–800 nm, and to reduce the amount of cellular protein for subsequent SDS/PAGE analysis. The final concentration of deoxy- and oxyhaemoglobin in the incubation mixtures was 12 μM (with respect to haem, 225 μg of protein·ml^{-1}). Incubations were carried out in a total volume of 10 ml at 37 °C, in either air or the anaerobic cabinet in an 80 % N_2/10 % CO_2/10 % H_2 atmosphere, as appropriate. The deoxygenated incubation mixtures contained freshly prepared sodium dithionite at a concentration of 10 mM. Control incubations contained either cells or deoxy- or oxyhaemoglobin only in oxygenated or deoxygenated buffer with 10 mM sodium dithionite, as appropriate. At time zero and then at timed intervals, samples were removed from the incubation mixtures, placed in optical cuvettes and the UV-visible spectra recorded immediately. In the case of the anaerobic incubations, the samples were transferred in the anaerobic atmosphere to optical microcuvettes, which were then sealed hermetically before the spectra were recorded. The UV-visible spectra obtained for the incubation mixtures containing cells and oxy- or deoxyhaemoglobin were corrected for light scattering due to the bacterial cells in suspension, by electronically subtracting the spectra of the control cell incubations in each of the respective buffers.

Raman spectroscopy

Raman spectroscopy was performed as described previously [7] with a Labram Raman spectrometer using a helium/neon laser to excite the samples at 514.5 nm. The laser was focused on the liquid samples with an Olympus ×50 ultra-long-working-distance microscope objective, which also collected the back-scattered light. The Rayleigh radiation was rejected by means of a holographic notch filter and the Raman light was dispersed on to a Peltier-cooled CCD detector with a grating of 1800 grooves/mm in the spectrograph.

Measurement of the rate of oxyhaemoglobin oxidation

The oxidation of oxyhaemoglobin to methaemoglobin was confirmed by the decrease in the λ_{max} of the Soret band. The rate of oxidation was assessed quantitatively by measurement of the decrease in A_{576} [8].

Preparation of μ-oxo bishaem-containing pigment from *P. gingivalis*

P. gingivalis strain W50 was cultured for 8 days on horse blood agar plates as a lawn growth which was scraped gently from the agar surface with a sterile plastic loop. The pigment was extracted as described previously [2].

Effect of protease inhibitors on oxyhaemoglobin oxidation

Incubations of bacterial cells (75 μg of protein·ml^{-1}) with oxyhaemoglobin were carried out in NaCl/Tris with 0.1 mM tosyllysylchloromethylketone (TLCK) and 2 mM *N*-ethylmaleimide (NEM) to inhibit cellular gingipain activity [9]. The changes in both the Soret band intensity and λ_{max} value, and the decrease in A_{576}, were monitored with time at 37 °C as described above. These readings were compared with incubations of cells with haemoglobin without protease inhibitors, and with oxyhaemoglobin in the presence and absence of both NEM and TLCK.

Protease assays

The synthetic substrates L-α-benzoyl-L-arginine-*p*-nitroanilide (0.8 mM) and α-*N*-acetyl-L-lysine-*p*-nitroanilide (0.4 mM) were used to determine the Lys- and Arg-gingipain activities, respectively, of whole cells. The reactions were carried out at 37 °C in 0.14 M NaCl/0.1 M Tris/HCl, pH 7.5, and the rate of hydrolysis was monitored at 405 nm in 1 ml cuvettes with a pathlength of 1 cm.

SDS/PAGE

Samples of the bacterial cell/haemoglobin incubation mixtures were solubilized at 100 °C for 5 min in reduced application buffer containing 2 M urea, 1 % SDS and 50 mM dithiothreitol. They were electrophoresed on 15 % polyacrylamide gels as described previously [10].

Pyridine-haemochrome assay for iron(III) protoporphyrin IX [Fe(III)PPIX]

Concentrations of Fe(III)PPIX were determined by reaction with 1 M pyridine in the presence of fresh $Na_2S_2O_4$ (10 mM) to produce the bispyridine-haemochrome, which is formed by biaxial ligation of Fe(II)PPIX with pyridine after reduction of iron(III) haem. Bovine haemin [Fe(III)PPIX chloride; Sigma, product number H-2250] was used as a standard, which was initially dissolved in 0.14 M NaCl/0.1 M Tris (pH $\approx$ 9.8), and the pH of the solution was then adjusted to pH 7.5 by drop-wise addition of dilute HCl.

Spectrophotometry

UV-visible spectra were recorded from samples in quartz or plastic microcuvettes (Elkay UtraVu, Shrewsbury, MA, U.S.A.) having a volume of 1 ml and a pathlength of 1 cm in a Pharmacia Biotech Ultrospec 2000 scanning spectrophotometer.

RESULTS

After 72 h of incubation, mixtures of bacterial cells with oxyhaemoglobin changed colour, from red to green. The oxyhaemoglobin spectrum was replaced by one with a broad bimodal Soret band (Figure 1). The μ-oxo bishaem had a λ_{max} value of 384.3 nm and the monomeric haematin [Fe(III)PPIX.OH] had a λ_{max} value of $\approx$ 365 nm [11,12]. It should be noted that the presence of the monomer's Soret band at $\approx$ 365 nm causes that of the μ-oxo bishaem to appear at 384.3 nm. The latter would be expected at a longer wavelength but it was displaced by being convoluted with the spectrum of the monomer. In a previous study [11] the μ-oxo bishaem was not made pure (i.e. without monomer present), hence only the apparent position of the Soret band was noted. This is in agreement with the literature value at pH 8.2 for this haem species [11]. This spectrum was also identical to that of the μ-oxo bishaem-containing pigment extracted from blood-agar-grown cells and to a solution of Fe(III)PPIX at pH 7.5 containing a mixture of both the μ-oxo bishaem and the monomeric species (Figure 1b).

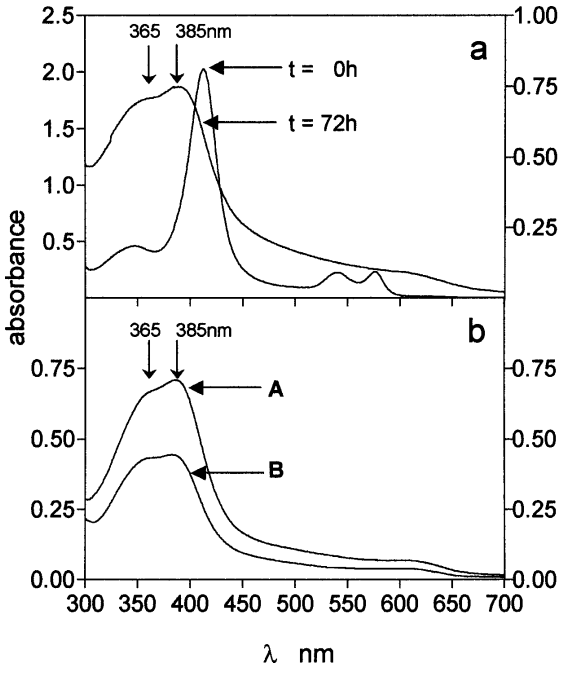

Figure 1 UV-visible spectra of horse oxyhaemoglobin incubated with cells of *P. gingivalis* W50 for 72 h (a) and of the μ-oxo bishaem-containing pigment and Fe(III)PPIX (b)

(**a**) The cell-protein concentration was $\approx$ 75 $\mu g \cdot ml^{-1}$ and the oxyhaemoglobin concentration was 225 $\mu g \cdot ml^{-1}$ (12 μM with respect to haem). Incubation was carried out in 0.14 M NaCl/0.1 M Tris/HCl, pH 7.5. The absorbance scale on the left-hand *y*-axis refers to the spectrum obtained at 0 h, and the scale on the right refers to that obtained after 72 h of incubation. (**b**) The spectra of the μ-oxo bishaem-containing haem pigment (curve A; 125 $\mu g \cdot ml^{-1}$) from blood-agar-grown cells of strain W50 in 0.14 M NaCl/0.1 M Tris/HCl, pH 7.5, and a 6 μM solution of Fe(III)PPIX (curve B) at pH 7.5 (containing both the μ-oxo bishaem and the monomeric species) are included for comparison.

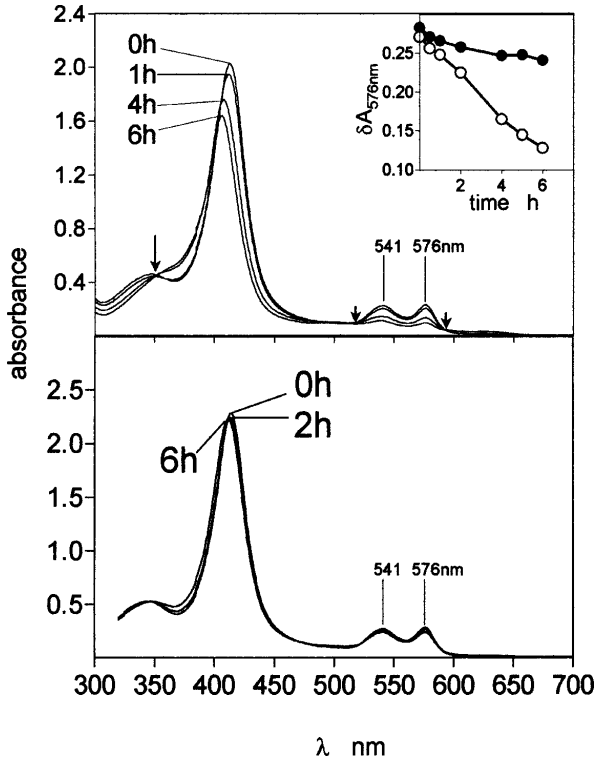

Figure 2 UV-visible spectra of oxyhaemoglobin during incubation with (top panel) and without (bottom panel) *P. gingivalis* W50 over 6 h

Top panel: the inset depicts the rates of cell-mediated oxidation of oxyhaemoglobin (○) and auto-oxidation of the haem protein alone (●), as measured by the decrease in intensity of the 576 nm Q band. The cell-protein and oxyhaemoglobin concentrations and incubation conditions were as in Figure 1. Bottom panel: spectra of the oxyhaemoglobin controls. The isosbestic points are indicated by arrows.

When the cell/oxyhaemoglobin incubation was studied over a shorter time period, a gradual change was observed in the λ_{max} of the Soret band (from 414 to 406.3 nm), along with a reduction in the band intensity (Figure 2) and a noticeable change in the colour of the solution, to a darker red. These changes were also accompanied by a decrease in the intensity of the haemoglobin/haem Q bands at 541 and 576 nm, indicating the progressive conversion of oxyhaemoglobin into methaemoglobin [8]. After 24 h there was no evidence for the presence of methaemoglobin, but the spectrum was characterized by a broad Soret band, indicating the appearance of μ-oxo bishaem. This was confirmed by Raman spectroscopy, which showed the presence of strong μ-oxo bishaem bands at 1370, 1565 and 1620 cm^{-1} (results not shown), as observed in the black haem-pigmented colonies of *P. gingivalis* strain W50 [7]. In contrast, for the control oxyhaemoglobin there was only a minimal change in the reduction in the λ_{max} value of the Soret band or A_{576}, reflecting the low auto-oxidation rate. Isosbestic points at $\approx$ 350, $\approx$ 520 and $\approx$ 590 nm were observed in the UV-visible spectra of the cell/oxyhaemoglobin incubation mixture from each time period (Figure 2, top panel, arrows). This indicates that the first step in the generation of μ-oxo bishaem was a direct conversion of oxyhaemoglobin into methaemoglobin. However, spectra with characteristics of deoxyhaemoglobin were not observed at any stage during the incubation.

The progressive digestion of the intact haem protein was demonstrated by SDS/PAGE (results not shown). Densitometry revealed that $\approx$ 30 % of the haemoglobin was degraded between

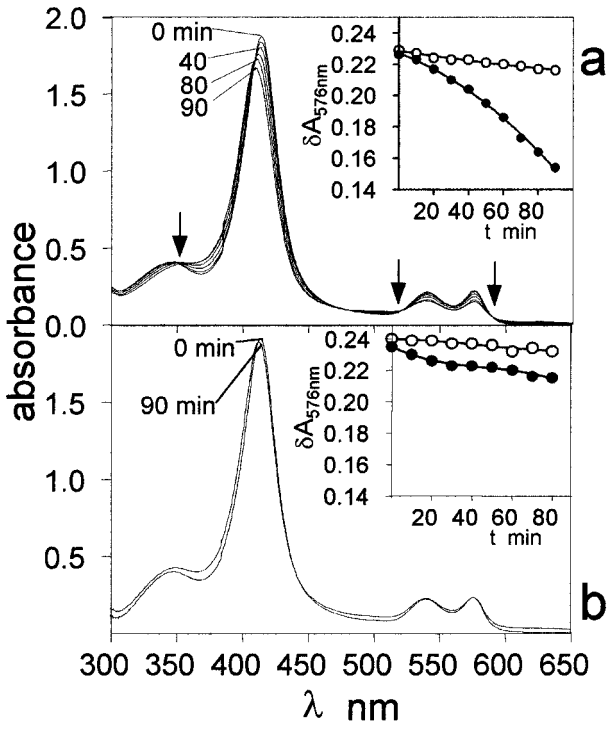

Figure 3 ***P. gingivalis*** **cell-mediated oxidation (a) and auto-oxidation (b) of oxyhaemoglobin**

Insets in (**a**) and (**b**) show rates of oxidation in the presence (○) and absence (●) of a mixture of the proteinase inhibitors NEM and TLCK. The haemoglobin and cell-protein concentrations were 225 and 75 $\mu g \cdot ml^{-1}$ in (**a**) and (**b**) respectively. Isosbestic points are shown by arrows.

0 and 60 min, and that 55% had been degraded after 6 h. SDS/PAGE also confirmed the absence of intact α- and β-globin chains or any smaller-molecular-mass breakdown peptides in the incubation mixture after 24 h (results not presented). After 72 h, the incubation mixture was centrifuged at 10000 ***g*** for 10 min at 20 °C to separate the supernatant from the cells, and the amounts of cell-associated and soluble Fe(III)PPIX were determined by pyridine-haemochromogen assay. This showed that the majority of Fe(III)PPIX released from the haemoglobin during incubation (65%) was cell-associated.

The conversion of oxyhaemoglobin into methaemoglobin was examined in more detail over the initial 90 min period of incubation (Figure 3a). In the presence of bacterial cells the Soret band λ_{max} value decreased from 414 to 409.4 nm and the intensity was reduced by 0.2 absorbance units. In contrast, there was minimal change in either the intensity or the Soret band λ_{max} of the control haemoglobin (results not shown). Plots of A_{576} against time (Figure 3a, inset) showed an 8-fold higher rate of haemoglobin oxidation in the presence of cells compared with the auto-oxidation rate of the haem protein. Again, isosbestic points were observed in the spectra (Figure 3a, arrows), indicating the direct oxidation to methaemoglobin in both the presence and absence of bacterial cells. Addition of NEM and TLCK to bacterial cells completely inhibited Lys- and Arg-gingipain activities (results not shown). During incubation of bacterial cells with oxyhaemoglobin in the presence of NEM and TLCK (Figure 3b) the λ_{max} of the Soret band remained unchanged and the rate of methemoglobin formation decreased to a level similar to that of the normal auto-oxidation rate in the absence of inhibitors (Figure 3b, inset).

The results of incubation of bacterial cells with deoxyhaemoglobin under anaerobic conditions are shown in Figure 4. Deoxyhaemoglobin prepared by treatment of oxyhaemoglobin with sodium dithionite and N_2 gassing gave a typical spectrum with characteristic Soret and Q band absorbances at 429 and 555 nm, respectively. After 24 h, during which the control haemoglobin remained in the deoxy form, the λ_{max} of the Soret band of haemoglobin incubated with bacterial cells decreased to 393 nm, and this peak represented the only detectable product (Figure 4a). These spectral changes were accompanied by a colour change of the solution from dark red to straw-like yellow. SDS/PAGE revealed a complete degradation of the α- and β-haemoglobin chains after 24 h (results not shown). The intensity of the 393 nm Soret band increased to a maximum at 72 h, and this spectrum remained unchanged up to 120 h (results not shown). A small amount of Fe(III)PPIX monomer was present after 24 h, as indicated by the slight shoulder at around 360 nm. All the spectra from each time period passed through isosbestic points at approximately 340, 370 and 400 nm (Figure 4a, arrows), indicating that the deoxyhaemoglobin was converted directly into the 393 nm-absorbing product. The presence of sodium dithionite in the buffer (seen as the steeply rising absorbance below 400 nm) held the haemoglobin in the deoxygenated state over the course of the experiment, as shown by the control haemoglobin spectrum (Figure 4b, 72 h). This deoxyhaemoglobin control could be converted into the oxygenated form by pipetting a small amount of oxygenated buffer or air into the sample. However, it is unclear why sodium dithionite was absent from the incubation mixture comprising cells and haemoglobin (Figure 4a), but we speculate that it was consumed both as a result of the reduction of bacterial cellular components and through reaction with residual oxygen in the system. Therefore, although the level of dioxygen may have been sufficiently low in the anaerobic incubation to yield deoxyhaemoglobin, it is possible that sufficient O_2 was present to react with released Fe(II)PPIX molecules to form the μ-oxo bishaem; this only occurred when all the dithionite had been consumed.

Separation of the 72 h cell/deoxyhaemoglobin incubation mixture by centrifugation revealed that only a minor fraction of the 393 nm-absorbing component was cell-associated (results not shown). Bubbling of O_2 for 30 min at a flow rate of 3 $ml \cdot min^{-1}$ into either the incubation mixture or the supernatant did not change the λ_{max} value from 393 nm. Treatment of the supernatant solution with 1 M pyridine did not give a pyridine-iron(II) haemochrome spectrum, although λ_{max} increased from 393 to 402.5 nm, whereas addition of 10 mM $N_2S_2O_4$ increased λ_{max} from 393 to 409.8 nm. Addition of 1 M pyridine plus 10 mM $N_2S_2O_4$ resulted in a pyridine-iron(II) haemochrome spectrum with its major peak at 411.5 nm, and minor peaks at 522 and 548 nm (results not presented). These data indicated that the 393 nm-absorbing product was an iron(III) haem species. The explanation for these observations is that the 393 nm absorbance band originates from pure μ-oxo bishaem, which is almost unaffected by the presence of Fe(III)PPIX monomer and thus shows the true position of its Soret band. This was confirmed by the presence of a band at $\approx$ 610 nm (Figure 4a, inset) which agrees with the value in the literature for μ-oxo bishaem [11]. In the presence of 1 M pyridine the 393 nm product gave a band at 402.5 nm, which is due to the bis(pyridyl)protoporphyrinate IX iron(III) species $[Fe(III)PPIX(py)_2]^+$. This species only forms from the μ-oxo bishaem in the presence of a large excess of pyridine (J. Silver, unpublished work). The Raman spectrum of the 24 h incubation mixture (obtained by 514.5 nm laser excitation) showed the presence of some μ-oxo dimer (results not shown) and confirmed the above interpretation. In addition, the

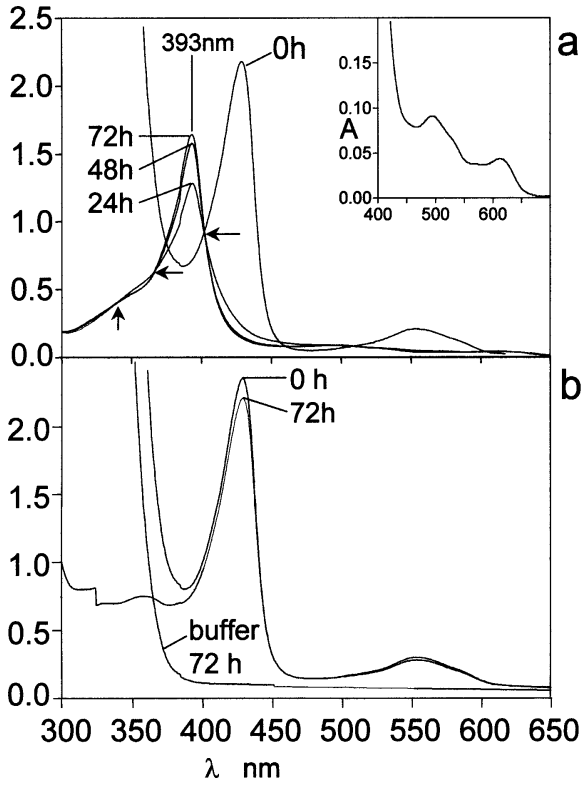

Figure 4 UV-visible spectra of deoxygenated horse haemoglobin incubated over 72 h with (a) or without (b) whole cells of *P. gingivalis* W50

Deoxyhaemoglobin was prepared by reduction of oxyhaemoglobin with sodium dithionite and equilibration with N_2. The spectra for the deoxyhaemoglobin controls at 0 h and after 72 h incubation in the absence of bacterial cells, and for the reduced buffer after 72 h, are shown in (**b**). The incubations were carried out in an anaerobic atmosphere (see text for details). The inset shows the ordinate expansion of the spectrum for the bacterial cell/deoxyhaemoglobin incubation after 72 h. The arrows in (**a**) indicate the apparent isosbestic points.

sharp 393 nm Soret band suggested that the soluble pure μ-oxo bishaem produced as a result of the interaction of bacterial cells with deoxyhaemoglobin had a low degree of aggregation. It should be pointed out that in this context aggregation should not be confused with the formation of the μ-oxo bishaem. Instead, the term refers to the 'stacking' of haem molecules in solution, probably due to a combination of weak π-bonding interactions with the aggregates segregating from solution due to hydrophobic effects. It is anticipated that aggregation can be suppressed by buffering or dilution of the solutions.

DISCUSSION

The μ-oxo bishaem of iron protoporphyrin IX is the major haem component of the black pigment of *P. gingivalis* [2,7], and its accumulation accounts for the green-black colouration of pigmented colonies during prolonged growth of *P. gingivalis* on blood-containing media. The precise steps in the formation of the μ-oxo bishaem have remained unclear but in this study we have shown that the μ-oxo bishaem complex is generated by *P. gingivalis* from both oxy- and deoxyhaemoglobin.

During incubation with *P. gingivalis* under aerobic conditions, methaemoglobin was first generated from oxyhaemoglobin, before conversion into the μ-oxo bishaem. This was demonstrated spectrophotometrically by a decrease in the λ_{max} value of the Soret band, reductions in the intensities of 541 and 576 nm Q bands, and the observation of isosbestic points in the UV-visible spectra during incubation. Methaemoglobin formation was accelerated in the presence of bacterial cells at a rate $\approx$ 8-fold greater than the rate of auto-oxidation. This is the first report documenting this phenomenon. The protease inhibitors TLCK and NEM, which are inhibitory towards Arg- and Lys-gingipains [9], prevented haemoglobin oxidation. It must be concluded that either or both classes of gingipain play a role in accelerating methaemoglobin formation and, hence, μ-oxo bishaem generation. Oxyhaemoglobin oxidation may occur as a result of denaturation of the protein [13], and it is likely that combined Lys- and Arg-gingipains initiate structural changes within the globin molecules, as both α- and β-chains contain numerous lysine and arginine residues. Methaemoglobin forms naturally within the erythrocyte but is re-reduced by membrane-associated methaemoglobin reductase and cellular reducing agents (glutathione and ascorbate) to render it capable of oxygen carriage [14]. *P. gingivalis* acquires haemoglobin from erythrocyes through haemolysis [15,16]. Extracorpuscular release of oxyhaemoglobin would favour methaemoglobin formation since it may not be re-reduced enzymically. Moreover, Lys- and Arg-gingipains may inactivate methaemoglobin reductase and erythrocytic reducing agents may be lost to the extracellular milieu during haemolysis.

Haemoglobin, presented in the methaemoglobin form, can be degraded [10,17] and utilized as a source of iron protoporphyrin IX by *P. gingivalis* [18]. It is noteworthy that whole cells of *P. gingivalis* have a greater binding capacity for haemoglobin in the oxidized form compared with the reduced, deoxygenated species [19], but it is not known whether methaemoglobin is degraded at a higher rate than deoxyhaemoglobin. In this regard, cellular haemoglobin binding is mediated partly by Lys-gingipain [20], and Kuboniwa et al. [21] have found that haemoglobin binding to isolated recombinant Lys-gingipain is increased under anaerobic conditions.

Formation of methaemoglobin from oxyhaemoglobin is related to the uneven distribution of electrons along the Fe–O_2 bond. This confers a 'superoxo' character to the oxyhaemoglobin, making it possible for the ligand to dissociate from haemoglobin as superoxide [22]. Under physiological conditions O_2^- can be displaced from methaemoglobin by OH^-, Cl^- and H_2O [23,24]. Thus the slightly alkaline pH found *in vivo* in the inflamed gingival crevice and diseased periodontal pocket [25,26] would favour μ-oxo bishaem formation, since the displacement of superoxide from methaemoglobin by OH^- would result in haemoglobin subunits containing Fe(III)PPIX.OH molecules. Proteolytically released Fe(III)PPIX.OH species would react together spontaneously, with the elimination of H_2O, to form the μ-oxo bishaem {$[Fe(III)PPIX]_2O$}:

$$2Fe(III)PPIX.OH \rightarrow H_2O + [Fe(III)PPIX]_2O \quad (1)$$

The formation of μ-oxo bishaem via this route would not account for consumption of dioxygen *per se*. However, it should be noted that deoxyhaemoglobin was not formed during incubation of oxyhaemoglobin with bacterial cells and it must be concluded that the ligated oxygen was used up during the process of μ-oxo bishaem generation from the oxygenated protein. The periodontal pocket, which is the preferred habitat of *P. gingivalis* [27], is not totally anaerobic [28], and partial pressures of O_2 of $\approx$ 20 mmHg have been measured [29]. This would yield a haemoglobin oxygen saturation of $\approx$ 25%. We propose that under such conditions proteolysis of the haemoglobin would result in the release of iron porphyrins from the superoxide-bearing methaemoglobin subunits [$HbFe(III)O_2^-$] which would react with iron(II) haem species [Fe(II)PPIX] from the remaining deoxygenated subunits to yield the μ-oxo bishaem.

It is conceivable under conditions of high oxygen saturation, and when there is insufficient iron(II) porphyrin or erythrocytic and/or bacterial reducing power available to form μ-oxo bishaem, that the bacterial cell may have to endure a flux of superoxide released from methaemoglobin. Erythrocytic superoxide dismutase is important in defence against O_2^- generated as a result of normal intracellular methaemoglobin formation. It is thus significant that *P. gingivalis* possesses superoxide dismutase to break O_2^- down to H_2O_2 [30]. Whereas this enzyme is instrumental in protecting the cell during exposure to atmospheric oxygen and from macrophage-derived oxidants [31,32], it is likely that superoxide dismutase is an essential pre-requisite for haem acquisition from oxyhaemoglobin. Any H_2O_2 generated by *P. gingivalis* through O_2^- dismutation would be destroyed by both monomeric and μ-oxo dimeric Fe(III)PPIX molecules by virtue of their inherent catalase activities [33].

Haemoglobin oxidation has other important consequences. It should be noted that the presence of methaemoglobin in a mixture with oxyhaemoglobin increases the oxygen-binding affinity of the latter, which is thought to occur through an allosteric effect as a result of exchange of ferrous and ferric haem $\alpha\beta$-subunits [34]. This phenomenon hinders oxygen unloading at sites where it is required for normal cellular metabolism (as exemplified by cyanosis resulting from chemical and drug-induced methaemoglobinaemia), and could thus compromise periodontal tissue repair. Importantly, from an ecological perspective, methaemoglobin formation would favour growth of other anaerobic bacterial species, first by withholding oxygen, which may otherwise be converted into reactive oxygen species as a result of metabolism of aerobic species, and secondly by facilitating oxygen consumption in the form of the μ-oxo bishaem.

P. gingivalis also degraded deoxyhaemoglobin to yield a 393 nm-absorbing product that was identified as Fe(III)PPIX in the form of pure μ-oxo bishaem. The μ-oxo bishaem remained in solution and did not bind to the cell surface; an observation possibly explained by the low degree of aggregation, as indicated by the narrow Soret band of the 393 nm product [35]. Aggregation does not occur in dilute concentrations and cell-surface binding may also be poor under such conditions. This may also be evidence that the cell surface binds already stacked (aggregated) μ-oxo bishaem molecules rather than isolated ones. In contrast to incubation with oxyhaemoglobin, only small amounts of Fe(III)PPIX monomer were formed, as indicated by the slight shoulder on the lower wavelength side of the 393 nm Soret band after 24 h of incubation. This finding indicates the possibility that an oxidizing species is engendered by the cells which oxidizes deoxyhaemoglobin to methaemoglobin, which under alkaline conditions would pick up OH^-. Proteolytic digestion of this would release Fe(III)PPIX.OH molecules, which would react with the elimination of water to form the μ-oxo bishaem, as in eqn (1).

The interaction of *P. gingivalis* with oxyhaemoglobin resulted in formation of a mixture of both monomeric and dimeric Fe(III)PPIX, whereas pure μ-oxo bishaem was generated from the deoxygenated protein. Detection of the monomeric species in cell/oxyhaemoglobin incubation mixtures is consistent with the presence of Fe(III)PPIX monomer [presumed to be Fe(III)PPIX.OH] in the haem pigment, as revealed previously by Mössbauer spectroscopy [2]. Whereas some monomer may arise from the dimer as a result of the pH-dependent equilibrium between the monomeric and dimeric forms [11], it is possible that it also originates as iron(III) monomers proteolytically released from methaemoglobin subunits. These monomeric Fe(III)PPIX species, when not participating in μ-oxo bishaem formation, are nevertheless important defensively as they are more active in degrading hydrogen peroxide than the μ-oxo bishaem [3,33]. In addition to confirming previous observations [2,7], this study has shown that the protective component of the haem pigment, the μ-oxo bishaem complex, is generated both from oxy- and deoxyhaemoglobin, and that this process can also be mediated by non-viable bacterial cells.

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