The periodontopathogen *Porphyromonas gingivalis* binds iron protoporphyrin IX in the μ-oxo dimeric form: an oxidative buffer and possible pathogenic mechanism

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Mössbauer spectroscopy was used to re-evaluate iron protoporphyrin IX, FePPIX, binding and the chemical nature of the black iron porphyrin pigment of *Porphyromonas gingivalis*. We demonstrate that FePPIX is bound to the cell in the μ-oxo dimeric form, $[Fe(III)PPIX]_2O$, and that the iron porphyrin pigment is also composed of this material. *P. gingivalis* also assimilated monomeric Fe(II)- and Fe(III)PPIX into μ-oxo dimers *in vitro*. Scatchard analysis revealed a greater binding maximum of cells for μ-oxo dimers than for monomeric Fe(III)- or Fe(II)PPIX, although the relative affinity constant for the dimers was lower. Formation of $[Fe(III)PPIX]_2O$ via reactions of Fe(II)PPIX with oxygen, and its toxic derivatives, would serve as an oxidative buffer and permit *P. gingivalis* and other black-pigmenting anaerobes to engender and maintain a local anaerobic environment. Tying up of free oxygen species with iron protoporphyrin IX would also reduce and limit Fe(II)PPIX-mediated oxygen-radical cell damage. More importantly, formation of a cell-surface μ-oxo dimer layer may function as a protective barrier against assault by reactive oxidants generated by neutrophils. Selective interference with these mechanisms would offer the possibility of attenuating the pathogenicity of *P. gingivalis* and other iron protoporphyrin IX-binding pathogens whose virulence is regulated by this reactive molecule.

INTRODUCTION

Periodontal (gum) diseases affect 80–90 % of adults and are a major cause of tooth loss in the Western world now that caries (tooth decay) incidence is in decline. The Gram negative, black-pigmenting anaerobes of the genera *Prevotella* and *Porphyromonas* are important pathogens associated with these conditions. Their characteristic black pigment is thought [1–3] to result from intracellular accumulation of iron protoporphyrin IX, Fe(?)PPIX, identified in reduced-pyridine extracts of cells grown on blood-containing media [2]. *Porphyromonas gingivalis* has a growth requirement for iron protoporphyrin IX [4,5] derived proteolytically from haemoglobin [6–8] and the haem-carrying plasma proteins haptoglobin, albumin and haemopexin [9,10] in the micro-ulcerated gingival crevice and anaerobic periodontal lesion. Its virulence is regulated by this iron porphyrin, which increases pathogenicity [4,11] and induces cellular Fe(?)PPIX binding [12–14]. Other than its accepted role as the prosthetic group of a *b*-type cytochrome [1–3], the biological functions of accumulated Fe(?)PPIX (43 % of dry weight [1]) are obscure since, paradoxically, FePPIX stored either in or on the bacterial cell surface poses a major problem because of its involvement in oxygen-radical-mediated damage to DNA, proteins and lipids [15]. We have speculated that cell-surface Fe(?)PPIX binding results from the close packing of monomeric iron porphyrin through hydrophobic interactions [13], but the exact mechanism of this binding, the chemical nature of the iron porphyrin-containing pigment and the oxidation state of the iron are not known. We have re-evaluated this problem using ^{57}Fe Mössbauer spectroscopy, which is particularly suitable for studying iron even when it is present in small amounts. The technique gives information on valent state, spin state and co-ordination number, and, under conditions where model compounds exist for comparison, it is invaluable for characterization of the electronic environment of biological iron [16–19].

EXPERIMENTAL

Bacterial growth conditions

P. gingivalis W50 was grown in liquid culture as previously described [20] under anaerobic reducing conditions (80 % N_2, 10 % CO_2, 10 % H_2) in Schaedler anaerobe broth (Oxoid Ltd., Basingstoke, U.K.), which contains a basal 10 mg of iron protoporphyrin IX chloride, [Fe(III)PPIX]Cl, per litre. Bacteria were pelleted by centrifugation (5000 ***g*** for 45 min at 4 °C) at the end of log-phase growth (3 days). Bacteria were also grown anaerobically on 5 % horse-blood agar (Blood Agar Base No 2; Lab M Ltd., Bury, U.K.) on which they develop the characteristic black pigment after 5–6 days.

Treatment of liquid-culture-grown cells

Bacterial cell pellets harvested as described above were treated in the following ways: they were (A) suspended in 50 ml of 0.14 M NaCl/0.1 M Tris/HCl, pH 7.4 (NaCl-Tris; pre-equilibrated for 18 h in the anaerobic reducing atmosphere), and incubated anaerobically, for 4 h; (B) suspended in NaCl-Tris as described above and incubated anaerobically with 2 mM [Fe(III)PPIX]Cl; (C) suspended in 50 ml of NaCl-Tris, as described above, containing 10 mM sodium dithionite and incubated anaerobically for 4 h at 37 °C with 2 mM [Fe(III)PPIX]Cl; and (D) suspended in NaCl-

Abbreviations used: FePPIX, iron protoporphyrin IX; Fe(?)PPIX, iron protoporphyrin IX in which the valent state of the iron is not established; [Fe(III)PPIX]Cl, iron protoporphyrin IX chloride; NaCl-Tris, 0.14 M NaCl/0.1 M Tris/HCl, pH 7.4; $[Fe(III)PPIX]_2O$, μ-oxo dimeric form of FePPIX.

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Tris containing 0.26 mM potassium ferricyanide and 2 mM [Fe(III)PPIX]Cl and incubated aerobically for 4 h at 37 °C. After incubation, cells were washed once in the respective reducing or oxidizing buffer (pre-equilibrated anaerobically or aerobically as appropriate) to remove excess unbound [Fe(III)PPIX]Cl and once in 50 ml of deionized water, and concentrated by freeze-drying.

Treatment of blood-agar-grown cells

Black-pigmented colonies (5–6 days old) were washed once in NaCl-Tris and in water, and concentrated by centrifugation and freeze-drying (treatment E).

Extraction of iron porphyrin-containing pigment

Blood-agar-grown cells, as described above, were washed in NaCl-Tris for 10 min. The iron porphyrin pigment was then extracted by incubation in 0.14 M NaCl/0.1 M Tris, pH 9.8, for 10 min at 20 °C. The cells were pelleted by centrifugation, the pH 9.8 buffer extraction was repeated, the combined supernatant liquids were dialysed at 4 °C for 24 h against deionized water, and then freeze-dried (treatment F). The pigment yield was 40 % of cell dry weight and, although it was unlikely to be pure iron porphyrin, for the purposes of calculations in this paper it was taken as a maximum and agreed well with previous estimates [1].

Mössbauer spectroscopy

The freeze-dried bacterial samples of treatments A–F (60–100 mg) were packed into aluminium blanks 1.3 mm thick and 10 mm in diameter, and Mössbauer spectra were recorded at 78 K as described previously [16]. The spectra were computer fitted and referenced to natural iron at room temperature.

Scatchard binding analysis

Scatchard analysis was carried out as previously described [13,14] using liquid-cultured cells in NaCl-Tris. Solutions of the μ-oxo dimer were prepared by dissolving [Fe(III)PPIX]Cl (1 mM) in 0.1 M Tris/0.14 M NaCl (approx. pH 9.8) and then readjusting the pH to 7.4 with dilute HCl. $K_3Fe(CN)_6$ (0.25 mM) or $Na_2S_2O_4$ (10 mM) were also added to these solutions to yield the oxidized and reduced monomeric iron porphyrin species, respectively. All ligand solutions were prepared immediately before use.

RESULTS AND DISCUSSION

The Mössbauer parameters were compared with those of a reference sample of a solution of the μ-oxo dimeric form of FePPIX, $[Fe(III)PPIX]_2O$ (sample G; Table 1). All bacterial samples had poor statistics, due to the fact they contained natural proportions of ^{57}Fe iron. Cells grown in liquid culture (treatment A) with no additional iron porphyrin other than the basal 10 mg of [Fe(III)PPIX]Cl per litre, and which were light brown in colour (indicating the possible presence of only small amounts of bound iron porphyrin), gave no Mössbauer spectra even when the sample was run for three weeks when the source was new (at full strength). We interpret this to mean that signals were not received from any cellular haem proteins (i.e. cytochromes [1]). Only the pigment (treatment F) and cells exposed to higher concentrations of iron protoporphyrin IX (treatments B–E) gave rise to observable Mössbauer spectra. In all cases the Mössbauer parameters were indicative of Fe(III)PPIX present as μ-oxo dimers {$[Fe(III)PPIX]_2O$}. Thus, cells of *P. gingivalis* were able to bind FePPIX as μ-oxo dimers when grown on blood agar (treatment E), i.e. with haemoglobin as the major source of FePPIX, and when exposed to both iron(II) and iron(III) porphyrin (treatments B, C and D, respectively). Treatments A–D were carried out using exogenous [Fe(III)PPIX]Cl. It is likely that some μ-oxo dimer would form in treatments A, B and D (as a result of the equilibrium existing between monomer and dimer [18,19]), as we found that the incubation conditions themselves (i.e. in the absence of bacterial cells) were unable to reduce the [Fe(III)PPIX]Cl. Whereas cells in treatment C would have carried some surface μ-oxo dimer initially (i.e. from growth in liquid culture), this would have been reduced by subsequent incubation in the presence of $Na_2S_2O_4$. Thus, contrary to our previous assumptions [13], even under the anaerobic reducing conditions of treatment C and during anaerobic growth on blood agar (treatment E), the iron porphyrin was deposited on the cell surface not as Fe(II)PPIX monomers but in the dimeric form. All the Mössbauer spectra (computer-fitted as two Lorentians) were asymmetric, whereas usually, for reasons discussed previously [19], those of pure μ-oxo dimer should be symmetric. This would indicate the presence of a small proportion of some monomeric high-spin iron(III) species [19]. This material is likely to be a $[Fe(III)PPIX]OH.H_2O$ monomer (haematin) and from the Mössbauer spectra statistics we estimate this to be a maximum of 15 % of the total Fe(III)PPIX in treatment D and much less in treatments B, C, E and F.

Table 1 Mössbauer parameters (at 78 K) of cells and iron porphyrin pigment derived from *P. gingivalis* W50

See text for details of samples. The isomer shift is referenced to natural iron foil. Values in parentheses are the total error of the previous last digit (s). –, No Mössbauer absorbance observed. Γ, Half width at half height.

Sample	Isomer shift, δ ($mm \cdot s^{-1}$)	Quadruple splitting ΔE_Q ($mm \cdot s^{-1}$)	Γ ($mm \cdot s^{-1}$)	Percentage of total area
A	–	–	–	–
B	0.38 (11)	0.54 (11)	0.26 (5)/0.39 (16)	57.2 (13.6)/42.8 (19.2)
C	0.39 (2)	0.59 (2)	0.28 (3)/0.17 (3)	69.1 (8.1)/30.9 (6.3)
D	0.43 (4)	0.59 (3)	0.32 (4)/0.20 (6)	74.7 (11.0)/25.3 (8.8)
E	0.37 (4)	0.54 (4)	0.24 (4)/0.18 (7)	70.4 (12.6)/29.6 (11.7)
F	0.41 (2)	0.55 (2)	0.23 (3)/0.22 (4)	59.3 (7.6)/40.7 (8.0)
G*	0.39 (1)	0.60 (1)	0.23 (1)	100.0 (4)

*, Reference sample of μ-oxo dimer [19]; frozen solution, pH 8.

The electronic spectrum of the black pigment had the characteristics of $[(Fe(III)PPIX]_2O$ in the absence of a reducing agent (Figure 1). Electronic spectra could not be obtained from any of the cell suspensions due to scatter.

Further evidence for the presence of iron porphyrin in the μ-oxo dimeric form (and not as the reduced monomeric species) came from the observation that no colour reaction occurred upon the addition of pyridine to freshly isolated liquid-cultured cells or blood-agar-grown cells taken directly from the anaerobic cabinet or to the extracted black pigment. However, a strong red coloration was immediately obtained when a few crystals of either sodium dithionite or dithiothreitol were added; an observation explained by the fact that protoporphyrin IX–iron(II) bispyridine forms only via biaxial ligation of pyridine with the iron in the II state [21]. Moreover, as the $[Fe(III)PPIX]_2O$ structure does not dissociate easily in pyridine, using this as an extractant [2] will only be effective if the dimer is first reduced with dithionite. This would explain why the $[Fe(III)PPIX]_2O$

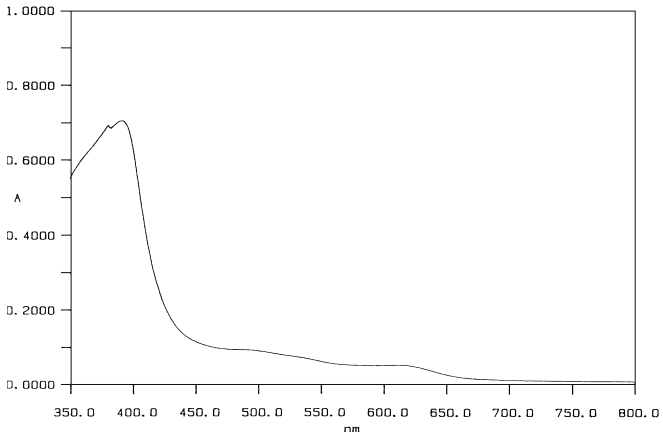

Figure 1 Electronic spectrum of the extracted black pigment of *P.gingivalis* W50 grown on blood agar

The spectrum was recorded in distilled water at pH 7.0 in a Perkin–Elmer Lambda 2 spectrophotometer.

species has not been recognized previously to be associated with cells of *P. gingivalis* or other black-pigmenting oral anaerobes.

During growth of *P. gingivalis in vivo* or *in vitro* on blood agar, proteolysis of haemoglobin and other iron(II) porphyrin-carrying plasma proteins would generate free Fe(II)PPIX in the vicinity of the cell surface. At pH 7.4 in the presence of residual oxygen (from the tissues, gingival sulcus fluid or from disrupted oxyhaemoglobin molecules) these Fe(II)PPIX monomeric species would oxidize to μ-oxo dimers. At pH 7.4, [Fe(III)PPIX]OH.H_2O would also be converted to μ-oxo dimers [18,19]. Thus, first, if only iron(III) monomers were present in solution, these would quickly dimerize and bind to the cell as dimers. A putative iron porphyrin-binding site would have to bind the dimer itself if it could not break such a dimer into monomers. If several binding sites were close together, extra dimer molecules would be able to bind to those already bound as in the crystal structure of the μ-oxo dimer. We note that only the crystal structure of μ-oxo-bis(5,15-dimethyl-2,3,7,8,12,13,17,18-octaethylporphinato)-iron(III) molecules is known [22], but that solid [Fe(III)PPIX]$_2$O can be prepared in microcrystallite form [23]. It is also noteworthy that *in vivo*, in the reduced near-anaerobic periodontal pocket [24,25], the bacterium would have to deal with both iron(II) and iron(III) porphyrin species, and therefore must also bind iron(II) species. The above would explain the increased iron porphyrin-binding capacity of cells grown under conditions of iron porphyrin excess in the chemostat [12–14], which would already be carrying a pellicle of μ-oxo dimer.

It is unclear why the pigmentation phenotype is restricted to the above species or whether this phenomenon involves some catalytic process involving iron porphyrin-binding cell-surface components to generate μ-oxo dimer-containing pigment. It is currently accepted that specific outer-membrane haem-binding proteins expressed by *P. gingivalis* during growth under iron porphyrin limitation [26,27] and the lipid A moiety of lipopolysaccharide [28] mediate cellular iron porphyrin binding, although their binding affinities and stoichiometries are not known. As these components are only present in low amounts [26,27,29] they may function as a surface on which dimerization could occur and/or as a seed for further crystal growth.

The observations that cells pigment black when grown on blood agar, and that freeze-dried metabolically inert cells and isolated outer membranes bind iron porphyrin from solution to become coloured dark brown [12–14,28], suggest that cells have a surface covering of [Fe(III)PPIX]$_2$O. Since the cells were able to assimilate both iron(II) and iron(III) porphyrin, once converted into [Fe(III)PPIX]$_2$O, we compared the relative binding affinities of these monomeric species with that for μ-oxo dimers. Scatchard analysis (Figure 2) revealed negatively sloped plots for

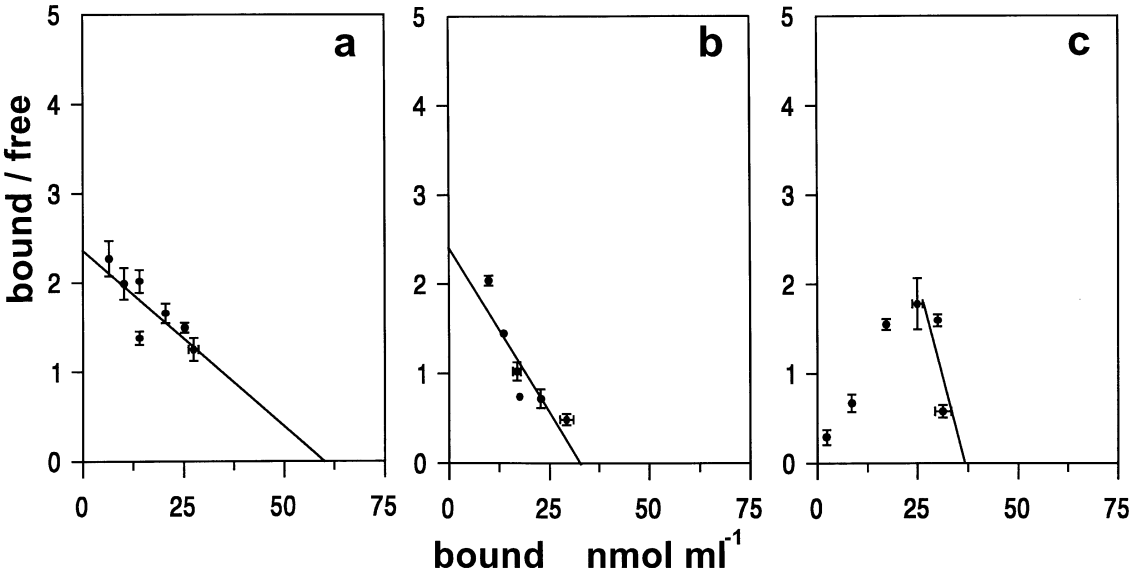

Figure 2 Scatchard analysis of (a) μ-oxo dimer, (b) Fe(III)PPIX and (c) Fe(II)PPIX binding to cells of *P. gingivalis* W50

Assays were performed using liquid-cultured cells (3×10^7) at 37 °C in a total volume of 1 ml of NaCl-Tris. Data points are mean $\pm$ S. D. of four replicate determinations of both ratios of bound/free and bound ligand (nmol · ml^{-1}). Association constants (K_a) were determined by linear regression analysis. A K_a for Fe(II)PPIX binding (**c**) was derived from a plot through the last three data points.

Table 2 Association constants (K_a) and binding maxima (B_{max}) for μ-oxo dimer, and Fe(III)PPIX and Fe(II)PPIX monomer binding to whole cells of *P.gingivalis* W50

See Figure 2 and text for details of binding assay.

Iron porphyrin species	K_a (M^{-1})	B_{max} (nmol · ml^{-1})
μ-oxo Dimer	4×10^4	60
Fe(III)PPIX monomer	7.4×10^4	30
Fe(II)PPIX monomer	14×10^4	37

Table 3 Calculated maximum number of μ-oxo dimers bound per cell, and volume and depths of μ-oxo dimer surface layers formed after exposure of cells to either μ-oxo dimer or Fe(III)PPIX and Fe(II)PPIX monomers

Depth of dimer layer is based on a uniform covering of μ-oxo dimers of estimated dimensions $1.4 \times 1.4 \times 0.8$ nm and a molecular volume of approx. 1.5 nm^3, and coccal cells of 0.5 μm average radius and volume of approx. 5×10^8 nm^3.

Iron porphyrin species	Number of dimers/cell*	Volume of μ-oxo dimer layer (nm^3)	Depth of μ-oxo dimer layer (nm)
μ-oxo Dimer	12×10^8	18×10^8	320
Fe (III)PPIX monomer	3×10^8	4.5×10^8	110
Fe(II)PPIX monomer	3.7×10^8	5.5×10^8	130

* Determined by Scatchard analysis (see Figure 2 and Table 2).

binding of $[Fe(III)PPIX]_2O$ and Fe(III)PPIX, whereas co-operative kinetics were observed for Fe(II)PPIX [13]. The B_{max} for the μ-oxo dimer in this binding system was two-fold compared with that for either Fe(II)PPIX or Fe(III)PPIX, although the relative association constants for the reduced and oxidized monomer were higher (Table 2). The maximum number of dimers bound per cell (calculated from B_{max} values) was approximately four-fold greater when the cells were exposed to μ-oxo dimers compared with exposure to either Fe(II)PPIX or Fe(III)PPIX in the monomeric form (Table 3). This equates to a layer two to three-fold deeper when formed from μ-oxo dimer compared with monomers (Table 3). However, the thicknesses of such layers may also vary according to the packing orientation of the dimer molecules, but are likely to be thinner depending on the presence of iron porphyrin-binding sites on outer-membrane vesicles [12], which would increase the cell surface area [20,30]. It is difficult to accurately enumerate blood-agar-grown cells (for which the pigment is 40 % of the dry weight), but for these cells we calculate that the μ-oxo-dimer layer would be at least 2 μm deep. Despite their iron content we have been unable to detect μ-oxo dimer layers electron microscopically, either within or on the surface of glutaraldehyde-fixed sections of these cultures. This is not surprising, as the spatial separation of the dimers would be at least 8 Å, and insufficient to yield an electron opacity. Aggregation of μ-oxo dimers may give rise to amorphous rather than crystalline layers, as these have the potential to hydrogen-bond with at least five water molecules per dimer (one on each carboxy group and one on the oxygen of the μ-oxo bridge). It is unlikely that bulky μ-oxo dimer layers could be accommodated within the periplasmic space or stored intracellularly. We speculate that μ-oxo dimers may not totally cover the surface, as a continuous layer would present a physical barrier to uptake of peptides and amino acids and excretion of toxic metabolites, and would inhibit essential cell-surface-associated proteases [31].

It is important to consider the biological function of iron porphyrin binding and storage as a μ-oxo dimer pigment layer. Binding of iron(II) porphyrin could be used to mop up excess oxygen via the overall reaction:

$$4Fe(II)PPIX + O_2 \rightarrow 2[Fe(III)PPIX]_2O$$

which would tie up any oxygen in the immediate vicinity of the cell surface to engender and maintain a local anaerobic micro-environment. Formation of μ-oxo dimers would occur through the stepwise attack on the O_2 molecule by Fe(II)PPIX so that active oxygen species, such as O_2^-, O_2^{2-}, $O^\bullet$, and O^-, would also form dimers, being intermediates in the stepwise reaction. In addition, active oxygen species such as OH^-, which could be precursors of the above species via reactions of the type:

$$2OH^- \rightarrow H_2O + O^{2-}$$

would also be mopped up in dimer formation. We speculate that the above reactions could function as an 'oxidative buffer' to protect the cell from the potentially lethal effects of oxygen derivatives and thus guard against assault from reactive oxidants generated by neutrophils in the gingival sulcus and periodontal lesions [32]. Further, binding of iron porphyrins (excess to metabolic requirements) in the form of μ-oxo dimers would be a way of reducing and indeed limiting any oxygen radical-mediated damage associated with these reactive molecules. A μ-oxo dimer layer would also provide a barrier to distance the outer membrane from the above-mentioned injurious effects. Importantly, from an ecological perspective, these mechanisms would allow black-pigmenting anaerobes and other co-aggregating oxygen-sensitive species [33,34] to withstand oxidative stresses within a dental plaque biofilm. The knowledge that FePPIX molecules are bound as μ-oxo dimers could aid design of strategies to selectively prevent cellular acquisition of this essential factor and, thus, to abrogate the pathogenicity of *P. gingivalis*. In this regard, it would also be pertinent to examine for the presence of the above phenomenon in other medically important FePPIX-binding pathogens [35] whose virulence is also regulated by this reactive molecule.

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