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## Evidence of mutualism between two periodontal pathogens: Co-operative haem acquisition by the HmuY haemophore of *Porphyromonas gingivalis* and the cysteine protease interpain A (InpA) of *Prevotella intermedia*

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### SUMMARY

Haem (iron protoporphyrin IX) is both an essential growth factor and virulence regulator of the periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia*, which acquire it through the proteolytic degradation of haemoglobin and other haem-carrying plasma proteins. The haem-binding lipoprotein HmuY haemophore and the gingipain proteases of *P. gingivalis* form a unique syntrophic system responsible for capture of haem from haemoglobin and methaemalbumin. In this system, methaemoglobin is formed from oxyhaemoglobin by the activities of gingipain proteases and serves as a facile substrate from which HmuY can capture haem. This study examined the possibility of cooperation between HmuY and the cysteine protease interpain A (InpA) of *P. intermedia* in the haem acquisition process. Using UV-visible spectroscopy and polyacrylamide gel electrophoresis, HmuY was demonstrated to be resistant to proteolysis and thus capable to cooperate with InpA to extract haem from haemoglobin which was proteolytically converted to methaemoglobin by the protease. Spectroscopic pH titrations showed that both the iron(II) and iron(III) protoporphyrin IX-HmuY complexes were stable over the pH range 4 to 10, demonstrating that the haemophore could function over a range of pH which may be encountered in the dental plaque biofilm. This is the first demonstration of a bacterial haemophore working in conjunction with a protease from another bacterial species to acquire haem from haemoglobin and may represent mutualism between *P. gingivalis* and *P. intermedia* co-inhabiting the periodontal pocket.

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## Keywords

haemophore; interpain A; protease; haem; Porphyromonas; Prevotella

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## INTRODUCTION

Black-pigmenting anaerobic bacteria, including *Porphyromonas gingivalis* and *Prevotella intermedia*, are among the major pathogens associated with severe periodontitis (Holt and Ebersole, 2005), an inflammatory disease which damages the periodontal tissues supporting the teeth. Both *P. gingivalis* and *P. intermedia* have an absolute requirement for haem<sup>1</sup>, which they can store as an extracellular pigment, and which is composed of iron(III) protoporphyrin IX (Fe(III)PPIX) derived mainly from the proteolytic breakdown of haemoglobin, and from haem-carrying plasma proteins albumin and haemopexin. The haem pigment of *P. gingivalis* is composed of Fe(III)PPIX in the  $\mu$ -oxo bishaem or dimeric form, [Fe(III)PPIX]<sub>2</sub>O (Smalley et al., 1998), whereas that from both *P. intermedia* and *Prevotella nigrescens* is in the monomeric form, haematin, Fe(III)PPIX.OH (Smalley et al., 2003). Development of the haem-containing black pigments by these organisms serves an important defensive role as the intrinsic catalase activity of ferrihaems destroys hydrogen peroxide (Smalley et al., 2000). Furthermore, formation of the  $\mu$ -oxo bishaem-containing pigment by *P. gingivalis* is a chemical process which consumes oxygen and would thus promote anaerobiosis (Smalley et al., 1998, 2002). Neither *P. gingivalis* nor *P. intermedia* possess the genes encoding the biosynthesis of iron protoporphyrin IX, and are thus reliant on exogenous sources of haem such as haemoglobin.

The paradigm for haem acquisition from iron(II) oxyhaemoglobin by both *P. gingivalis* and *P. intermedia* involves a crucial initial haemoglobin oxidation stage which concomitantly reduces the affinity of the globin for the iron(III) protoporphyrin IX and renders the protein susceptible to further proteolytic degradation (Smalley et al., 2007, 2008; Byrne et al., 2009). In the case of *P. gingivalis*, the oxidized or methaemoglobin form of haemoglobin is produced proteolytically by the action of the arginine-specific cysteine protease gingipain, Rgp, and which is subsequently degraded by the lysine-specific gingipain, Kgp (Smalley et al., 2007; 2008). For *P. intermedia*, the proteolytic oxidation step is mediated by interpain A (InpA) (Byrne et al., 2009), a major extracellular cysteine protease (Mallorquí-Fernández et al., 2008), which is capable of disarming the host defences by degrading complement (Potempa et al., 2009; Potempa and Potempa, 2012). InpA can promote formation of both hydroxy- and aquomethaemoglobin under alkaline and acid conditions, respectively, which are also susceptible to degradation and haem release by InpA (Byrne et al., 2009). Whilst the agents responsible for haem deposition at the cell surface of *P. gingivalis* and *P. intermedia* have not been elucidated, binding of haem for purposes of internalisation by *P. gingivalis* is mediated by cell-surface haem-binding proteins such as haemin-binding protein 35 (Shoji et al., 2010) and the haemophore-like protein, HusA (Gao et al., 2010). To date, the most well characterised haem uptake system of *P. gingivalis* comprises the iron(III)- and iron(II)-

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<sup>1</sup>As a common convention haem will be used to refer to iron protoporphyrin IX irrespective of iron oxidation state. Where necessary, the iron oxidation state will be denoted as either iron(III) or iron(II).

haem-binding protein haemophore HmuY and its cognate receptor HmuR (Olczak et al., 2005; Wojtowicz et al., 2009b). Previously, we demonstrated that Rgp-mediated oxidation of iron(II) oxyhaemoglobin, facilitates the capture of the iron(III) protoporphyrin IX moiety by HmuY (Smalley et al., 2011).

Early work has shown that *P. gingivalis*, a member of the “red complex” of plaque organisms, requires the more prevalent “orange complex” members, including *P. intermedia*, for colonization (Socransky et al., 1998). Although other studies conflict with this view (Kolenbrander et al., 1985, 2006), more recent papers strongly show that such interactions exist. For example, co-aggregation between *P. gingivalis* and *P. intermedia* is shown to be mediated by adhesins encoded by the genes for gingipain (Kamaguchi et al., 2003). It has been widely accepted that co-aggregation promotes the generation of complex nutrient webs connecting species with distinct metabolic capabilities and increasing the pathogenicity of tightly interacting species. In this respect, a recent study has shown that bacterial loads of *P. intermedia* and *P. gingivalis* in plaque are strongly associated one with the other, and this co-colonisation is significantly linked to increased probing depth of diseased periodontitis sites (Nadkarni et al., 2012). These associations may be attributable to acquisition of haem, the essential growth factor for these two bacterial species. Given that haemoglobin oxidation is crucial to proteolytic haem release, and also the propensity for iron(III) protoporphyrin IX-containing haemoglobin to relinquish their haem to HmuY (Smalley et al., 2011), together with the demonstration that InpA can mediate oxidation of haemoglobin (Byrne et al., 2009), it is possible that HmuY, and by extension *P. gingivalis*, could benefit from the proteolytic activity of co-colonising or co-aggregating *P. intermedia* cells in acquiring haem. Here we support this statement by demonstrating that methaemoglobin formed by the activity of InpA is a facile substrate for transfer of iron(III) protoporphyrin IX to HmuY.

## MATERIALS AND METHODS

### Purification of HmuY

*P. gingivalis* apoHmuY (NCBI accession number CAM 31898) lacking the first 25 residues was expressed using a pHmuY11 plasmid and *Escherichia coli* ER2566 cells (New England Biolabs) and purified from a soluble fraction of the *E. coli* lysate as previously described (Olczak et al., 2008; Wojtowicz et al., 2009a).

### Interpain A purification

InpA was expressed recombinantly in *E. coli* and purified by affinity chromatography on Fast Flow Ni-NTA (Ni<sup>2+</sup>-nitrilo-triacetate)–Sephacel (Qiagen) followed by anion-exchange chromatography (MonoQ, GE Healthcare) as described previously (Mallorquí-Fernández et al., 2008). Before use, InpA was pre-activated by incubation for 15 min in 2 mM dithiothreitol (DTT) in 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.5. For use in haemoglobin degradation assays, the above buffer was replaced with that without DTT by ultrafiltration using 10 kDa cut-off Microcons (Amicon).

## Polyacrylamide gel electrophoresis (PAGE and SDS-PAGE)

SDS-PAGE was carried out as previously described (Charalabous et al., 2007). Gels were firstly stained for protein-bound haem with tetramethylbenzidine-H<sub>2</sub>O<sub>2</sub> (TMB-H<sub>2</sub>O<sub>2</sub>) and counterstained for protein with Coomassie Brilliant Blue R-250 (CBB) (Byrne et al., 2009). Samples were solubilised in application buffer at 37°C for 1 h without DTT. For native PAGE, urea and SDS were omitted from the separating gel, and the sample solubilisation was also performed in application buffer without SDS, urea and DTT as previously described (Smalley et al., 2011).

## Haemoglobin preparations

Oxyhaemoglobin, prepared from fresh horse erythrocytes as previously described (Smalley et al., 2008), was stored in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 at -80°C until required. Methaemoglobin was prepared proteolytically by treatment of oxyhaemoglobin (16 µM with respect to haemoglobin subunit) with InpA (2 µM) for 7 h at 37°C in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 (Byrne et al., 2009). This haemoglobin preparation comprised 44 % methaemoglobin as calculated from A<sub>577nm</sub> and A<sub>630nm</sub> values (Smalley et al., 2007).

## UV-visible spectroscopy

UV-visible spectra were recorded using an Ultrospec 2000 spectrophotometer (Biochrom Ltd) using 1 cm pathlength cuvettes.

## HmuY-haem complex formation

The HmuY-ferrihaem complex was prepared by reacting equimolar amounts of iron(III) protoporphyrin IX with HmuY at 0.14M NaCl, 0.1M Tris-HCl, pH 7.5, at 37°C, the reaction being monitored by UV-visible spectroscopy which showed a spectrum with a 411nm Soret and lower intensity Q bands at 527 and 558 nm as previously reported for the six-coordinate, bis-histidine ligated, low-spin complex (Smalley et al., 2011; Wojtowicz et al., 2009b).

## Spectrophotometric pH titrations of haem with HmuY

For UV-visible absorption analysis, 10 µl of the iron(III) protoporphyrin IX-HmuY complex (1:1 molar protein:haem ratio; 20 [proportional]M HmuY) were diluted into 1 ml of either 50 mM citrate buffer adjusted to pH 2.4, 2.7, 2.9, 3.1, 3.4, 50 mM acetate buffer pH 3.3, 5.2, 50 mM MES buffer pH 6.1, 50 mM phosphate buffer pH 7.1, 50 mM Tris/HCl buffer pH 7.9, 9.4, or 20 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer pH 9.2 and 12.0; all buffers also containing 0.14 M NaCl. HmuY samples were equilibrated in the above buffers at 4°C for 16 h, after which the pH was checked. Samples of the iron(II) protoporphyrin IX-HmuY complex were made in the above series of buffers after addition of an excess of sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) as reported previously (Wojtowicz et al., 2009b). Non-linear curve fitting was carried out using OriginPro 7.5 software.

## RESULTS

### HmuY resistance to InpA proteolysis

We observed that both the holo- and apo-forms of HmuY were completely resistant to InpA activity (Fig 1, panels A and B), and in the case of haem-liganded form, there was no evidence of haem loss as revealed by TMB-H<sub>2</sub>O<sub>2</sub> staining (Fig 1A). However, in keeping with previous observations (Wójtowicz et al., 2009a), we found that denaturation by heating at 100°C for 10 min rendered both apo- and holoHmuY susceptible to InpA (Fig 1, panel C). Additionally, pre-treatment of apoHmuY for 24 h with InpA at 37°C did not affect subsequent haem pickup as demonstrated by rapid formation of a spectrum typical of the iron(III) protoporphyrin IXHmuY complex (Fig 2).

### HmuY-haem complex formation during incubation of oxyhaemoglobin pre-treated with InpA

To experimentally verify whether haem extraction by HmuY is aided by InpA (through mediating oxyhaemoglobin oxidation), oxyhaemoglobin was proteolytically oxidised by 7 h pre-incubation with InpA (2 µM) to yield a starting substrate comprising 44 % methaemoglobin. This was incubated with HmuY (16 µM), which resulted in the rapid formation of an iron(III) protoporphyrin IX-HmuY spectrum (411 nm Soret  $\lambda_{\max}$  and 527 and 558 nm Q bands; Fig 3). This spectrum remained stable over 24 h. Importantly, iron(III) protoporphyrin IX-HmuY formation from oxyhaemoglobin was greatly reduced in the absence of InpA (data not shown; starting methaemoglobin concentration in the preparation = 15 %), which is likely a direct result of the low rate of auto-oxidation observed at this pH (Tsuruga et al., 1998).

However, to confirm that haem was in complex with HmuY and not still present as haemoglobin, the incubation mixture was treated with 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> to concomitantly remove dioxygen and to reduce the haem iron. This yielded a spectrum with a 423 nm Soret<sup>2</sup>, plus visible bands at 526 and 556 nm (data not presented), indicative of the iron(II) protoporphyrin IX-HmuY complex (Smalley et al., 2011; Wojtowicz et al., 2009). This unequivocally demonstrated that haem had become complexed to HmuY during incubation with the InpA-treated oxyhaemoglobin.

It is noteworthy that the InpA-induced methaemoglobin substrate showed little haem loss relative to the auto-oxidized control as judged by the Soret band intensity (data not shown) and agrees with the previous observation of limited proteolysis at pH 7.5 during which time InpA-mediated oxidation is taking place (Byrne et al., 2009). SDS-PAGE and densitometry confirmed that incubation of InpA with oxyhaemoglobin at pH 7.5 resulted in immeasurably low levels of haem release after 7 h (data not shown). Therefore it is unlikely that binding by HmuY of the low levels of any haem liberated proteolytically as a result of InpA activity could account for the strong intensity of the iron(III) protoporphyrin IX-HmuY spectrum produced upon addition of HmuY to the InpA-oxyhaemoglobin incubation. From this it was

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<sup>3</sup>The presence of a 429 nm Soret and single 555 nm Q band would have signified the presence only of haem in the form of deoxyhaemoglobin.

confirmed that holoHmuY formation was, in the main, due to the direct acquisition of haem from methaemoglobin induced by InpA activity.

Previously we reported that under native PAGE, apoHmuY migrates as a single band with an  $R_f$  greater than that of the diffusely-stained  $\alpha$  and  $\beta$  haemoglobin chains (Smalley et al., 2011). In addition, the mobility of the haem-complexed HmuY band is increased relative to apoHmuY, which is attributed to the increased negative charge of the iron(III) protoporphyrin IX-HmuY complex imparted by the ionised carboxylate groups of the ligated haem (Smalley et al., 2011). In the current study during native PAGE of HmuY incubated with InpA-pre-treated oxyhaemoglobin, the CBB and TMB-H<sub>2</sub>O<sub>2</sub> staining of the slightly faster migrating HmuY-haem band increased in intensity while that of the apoHmuY displayed a reciprocal reduction (Fig 4, upper panels, arrowed) indicating the progressive formation of the iron(III) protoporphyrin IX-haemophore complex. This was accompanied by reduced TMB-H<sub>2</sub>O<sub>2</sub> staining of the haemoglobin  $\alpha$  and  $\beta$  chains indicating loss of haem (Smalley et al., 2008). HoloHmuY formation occurred at a greater rate in the presence of InpA than without it, which corroborated the UV-visible spectroscopic data. Formation of some haem-stained holoHmuY was also observed for oxyhaemoglobin incubated in the absence of InpA (Fig 4, lower panels, arrowed), which is attributable to formation of methaemoglobin *via* natural auto-oxidation during incubation.

The facile transfer of iron(III) protoporphyrin IX from haemoglobin to HmuY was further corroborated by assessment of the relative amount of iron(III) protoporphyrin IX-HmuY formed with time during incubation of HmuY with oxyhaemoglobin pre-incubated with or without InpA for 7 h. Difference spectra were derived by subtracting the control time zero spectrum of the InpA-pre-treated oxyhaemoglobin plus HmuY from the spectra at each subsequent time interval (Fig 5 A). Negative absorbances (troughs) relating to loss of methaemoglobin at 500 nm and at 630 nm, and absorbance increases at 527 nm and 558 nm, attributable to the formation of the iron(III) protoporphyrin IX-HmuY complex, were observed. Note that the relative absorbance intensities were altered to those seen in the spectrum of the HmuY-iron(III) protoporphyrin IX as in Fig 2, which arises from subtraction of the control spectra from those of the tests (Smalley et al., 2011). In contrast there was little change in the difference spectra in both the Soret and Q band regions for the control spectra (oxyhaemoglobin not pre-treated with InpA and containing only auto-oxidised haemoglobin) over the same time period above (Fig 5 B). These data clearly demonstrated a greater degree of HmuY-iron(III) protoporphyrin IX complex formation from oxyhaemoglobin pre-treated with InpA, in turn a result of the greater rate of methaemoglobin formation mediated by the action of InpA.

### **HmuY-haem complex forms during co-incubation of oxyhaemoglobin with both InpA and HmuY**

Using native PAGE we monitored the effect of incubating equimolar amounts of HmuY and oxyhaemoglobin (16  $\mu$ M with respect to the monomer) in the presence of InpA (2  $\mu$ M). We observed a time-dependent increase in the amount of the faster migrating holoHmuY (CBB-stained panel, Fig 6), which also became progressively stained with TMB-H<sub>2</sub>O<sub>2</sub>, indicating transfer of haem to HmuY (Fig 6). Both the TMB-H<sub>2</sub>O<sub>2</sub> and CBB staining of the slower

migrating haemoglobin  $\alpha$  and  $\beta$  bands demonstrated a reciprocal decrease in intensity, which is typical during the formation of haem-free globin chains (Smalley et al., 2008). A smaller amount of the faster migrating holoHmuY was also generated (notably after 24 h) when oxyhaemoglobin was incubated only with HmuY (Fig 6), and is attributable to haem pickup by HmuY from auto-oxidised methaemoglobin.

### HmuY binds haem over a range of pH

We examined the haem binding ability of HmuY under different pH conditions which may be encountered in the oral cavity. UV-visible absorption spectral changes were measured over a range of pH between 2 and 12. This was done by spectroscopically titrating HmuY with a molar equivalent of haem and plotting the change in absorbance at 411nm and 425nm for the iron(III)- and iron(II) protoporphyrin IX-HmuY complexes, respectively. From this, the fraction of total haem bound to the haemophore was calculated (Fig 7). This revealed that both the iron(II) and iron(III) protoporphyrin IX-HmuY complexes were stable within the pH range 4 to 10, whereas significant decreases in Soret band intensities at 411 nm (for the iron(III)-form) and at 425 nm (for the iron(II)-form) were observed for pH values lower than ~4 and above ~10, indicating haem release (Fig 7). Nevertheless, these data showed that HmuY could remain functionally active over a range of pH which may be encountered in dental plaque.

## DISCUSSION

*P. gingivalis* exists predominantly in complex biofilm communities within the periodontal pocket, co-aggregating with other species such as *Actinomyces viscosus*, *Streptococcus mutans*, *Streptococcus gordonii*, and *Fusobacterium nucleatum* (Nonaka et al. 2001; Li et al., 1991; Kinder and Holt, 1989). It is also known that *P. gingivalis* gingipains mediate co-aggregation with *P. intermedia* (Kamaguchi et al., 2003). Such interactions are considered to provide growth substrates and reduce oxygen tension allowing anaerobic growth. In such an environment, *P. intermedia* plays an important role in the maturation of plaque by forming a complex with *P. nigrescens* and *F. nucleatum* which bridges early colonizing Gram-positive bacteria with Gram-negative species of the mature biofilm (Socransky et al., 1998), and thus contributes indirectly to periodontal disease progression.

Others have hypothesised that a degree of mutualism exists between *P. gingivalis* and *P. intermedia*. For example, binding of IgG by *P. intermedia* cell-surface Fc receptor may protect *P. gingivalis* from IgG (Labbé and Grenier, 1995), whilst fermentation of glutamic and aspartic acids by *P. intermedia* and *F. nucleatum* results in ammonia generation and thus a more neutral pH which could benefit acid-sensitive species including *P. gingivalis* (Takahashi, 2003). Cooperative inhibition of complement activation, and hence the defensive sequelae associated with the inflammatory response through the synergistic degradation of complement factor C3b by InpA of *P. intermedia* and Kgp of *P. gingivalis*, has also been described (Potempa and Potempa, 2012). It is also known that the stronger haemolytic activity of *P. intermedia* may advantage the growth of *P. gingivalis* by augmenting the levels of free haemoglobin (Okamoto et al., 1999). In this study, we have demonstrated that, in addition to deployment of its own gingipain proteases to engender

methaemoglobin formation (Smalley et al., 2011) to facilitate HmuY haemophore haem capture, *P. gingivalis* may also benefit from the *P. intermedia* haem acquisition system, i.e. the proteolytic activity of InpA.

For HmuY to be instrumental in co-operative haem acquisition, an important pre-requisite would be resistance to proteases, which could otherwise jeopardise haem binding ability. In this respect, Wójtowicz et al. (2009a) have shown that HmuY is completely resistant to the activities of trypsin, Kgp, and R-gingipains HRgpA and RgpB, making it a very durable agent to work in concert with proteases for haem extraction. It is noteworthy that higher levels of total serum IgG and IgG1 specific for *P. gingivalis* HmuY have been measured in chronic periodontitis patients compared to non-periodontitis controls (Trindade et al., 2012), demonstrating that HmuY is expressed *in vivo*. In addition, Potempa et al. (2009) found that the majority of *P. intermedia* strains freshly isolated from chronic and aggressive periodontitis sites carry and express the InpA gene. Since *P. gingivalis* and *P. intermedia* inhabiting the same environmental niche may concurrently deploy InpA and HmuY, it was relevant to investigate their concomitant effects on oxyhaemoglobin. Importantly, we found that both iron(III) protoporphyrin IX-complexed and apoHmuY were completely resistant to proteolysis by InpA and that apoHmuY pre-treated with InpA remained capable of forming the HmuY-iron(III) protoporphyrin IX complex. This indicated that HmuY could remain functionally active in a protease-rich environment, a feature which would be important in establishing a co-operative relationship between the two proteins and hence between the two microorganisms for haem capture.

For assimilation of haem by *P. gingivalis* from any oxyhaemoglobin released into a periodontal pocket as a result of bleeding, the haemoglobin must firstly be converted to the methaemoglobin form before haem sequestration by the HmuY haemophore (Smalley et al., 2011). However, at the slightly alkaline pH of these environments (Eggert et al., 1991; Bickel and Cimasoni et al., 1985) the natural auto-oxidation rates of both  $\alpha$  and  $\beta$  chains of oxyhaemoglobin are lowest (Tsuruga et al., 1998), which would limit HmuY haem extraction. This problem is overcome through the proteolytically mediated oxidation of oxyhaemoglobin by Rgp which facilitates both subsequent attack and haem release mediated by Kgp (Smalley et al., 2008) and iron(III) protoporphyrin IX extraction by HmuY (Smalley et al., 2011). Oxyhaemoglobin oxidation mediated by InpA is minimal at pH 7.5 (i.e., at a pH typical of the diseased periodontal pocket and inflamed gingival crevice) compared to that at lower pH (Byrne et al., 2009), at which the protease is optimally active against protein substrates (J. Potempa, unpublished findings). Nevertheless, pre-treatment of oxyhaemoglobin with InpA at pH 7.5 rendered it susceptible to haem extraction by the *P. gingivalis* haemophore and formation of a stable HmuY-iron(III) protoporphyrin IX complex. These data showed that InpA would be effective at slightly alkaline pH in “priming” oxyhaemoglobin for haem extraction by HmuY.

*P. gingivalis* may encounter fluctuations in environmental conditions since it exists in the oral fluids, colonizes oral surfaces, and invades host cells. One of the factors which may change is pH. Despite individual variations, plaque pH is the range 5.6-6.5, whereas the pH of resting saliva is about 6.7 (Kleinberg, 1970; Moreno and Margolis, 1988). The pH of the gingival crevice has been shown to be alkaline (Kleinberg and Hall, 1969) and development



of periodontitis is associated with a rise in pH above 8.0 (Bickel et al., 1985; Eggert et al., 1991), resulting in conditions favouring Gram-negative bacteria, including *P. gingivalis* (McDermid et al., 1988; Bickel and Cimasoni, 1985). *P. intermedia* also displays a saccharolytic metabolism generating an acidic terminal growth pH in both liquid (Takahashi & Yamada, 2000) and on solid media (Smalley et al., 2003). In contrast, *P. gingivalis* grows best in neutral to alkaline environments which suit its proteolytic activity (McDermid et al., 1988). Therefore, HmuY binding of both iron(II) and iron(III) protoporphyrin IX over the pH range 2.5 to 12 was determined. We observed that both the iron(II) and iron(III) protoporphyrin IX-HmuY complexes were stable over the pH range 4 to 10, demonstrating that the haemophore could actively bind haem under extremes of pH which might be encountered in the plaque biofilm. In light of these findings, we conclude that *P. gingivalis* may also be able to gain haem *via* the HmuY haemophore at pHs outside its growth optimum, and especially at acid pH where InpA is more efficient in oxidising haemoglobin (Byrne et al., 2009) compared to R-gingipain which has a neutral to alkaline pH optimum (Potempa et al., 1998). It is also noteworthy that *P. gingivalis* can elevate the pH of its environment through base production when in a medium which is initially poised in the acid range (Takahashi et al., 1997), and as a consequence may be capable of surviving local depressions in pH caused by acid generating species such as *P. intermedia*.

At the molecular level, oxyhaemoglobin oxidation to the met-state results in relaxation of the affinity of globin for iron(III) protoporphyrin IX compared to that of iron(II) species (Hargrove et al., 1996). In the circulation, iron(II)haemoglobin species released from damaged and senescent erythrocytes gradually transform into methaemoglobin, from which serum albumin and/or haemopexin having association constants for iron(III) protoporphyrin IX of  $\approx 10^8$  and  $2 \times 10^{14} \text{ M}^{-1}$ , respectively (Adams and Berman 1980; Hrkal et al. 1974), can extract the iron porphyrin. This enables both haem transport to the spleen and liver for reprocessing and limits its bioavailability to any invading bacteria. However, it would appear that both *P. intermedia* and *P. gingivalis* also take advantage of the above chemistry through evolution of a proteolytic mechanism to circumvent host haem sequestration by promoting methaemoglobin formation. Here we have shown that InpA of *P. intermedia*, through proteolytically oxidising haemoglobin, facilitates iron(III) protoporphyrin IX extraction by the HmuY haemophore of *P. gingivalis*. Of significance is the fact that both apoHmuY and its haem-ligated form are completely resistant to proteolysis by InpA, indicating that HmuY could function alongside the protease for haem acquisition. This co-operative action is not without precedent as synergistic acquisition of iron from haemoglobin has been shown during abscess formation for *Bacteroides fragilis* and *E. coli* which produces a haemoglobin protease (Otto et al., 2002). However, to our knowledge, this is the first documented demonstration of a bacterial haemophore acting syntrophically with a specific “haemoglobinase” from a different bacterial species. These findings support a potential mutualistic relationship between *P. intermedia* and *P. gingivalis* for haem acquisition. It is noteworthy that a HmuY-like outer-membrane lipoprotein has been reported in *P. intermedia* strain 17 (Lewis et al., 2006), which is dramatically up-regulated in iron-deplete conditions (Yu et al., 2007). It is likely that such a molecule may function in a similar fashion to HmuY by extracting haem from InpA proteolytically “primed” oxyhaemoglobin. Taken together, the apparent similarity of the haem uptake mechanisms suggests the

possibility of convergent evolution of the haem acquisition systems of *P. gingivalis* and *P. intermedia*. In a complex polymicrobial environment such a system would satisfy the nutritional needs of each species. As a consequence it would be expected that the efficiency of haem acquisition in co-aggregates of *P. gingivalis* and *P. intermedia* would be much higher, and would encourage a higher degree of inter species cooperativity.

## Acknowledgments

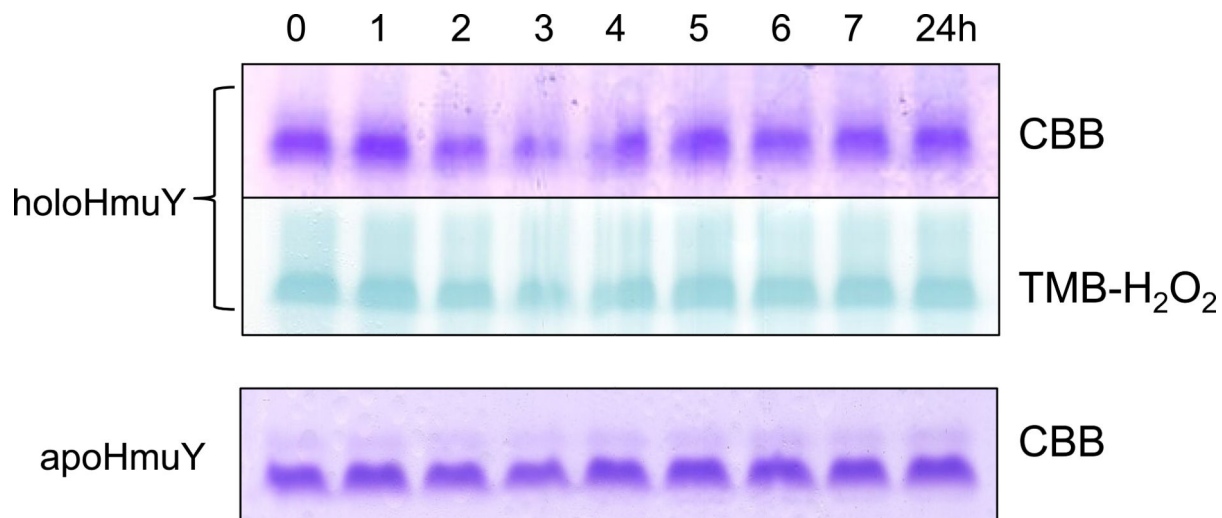
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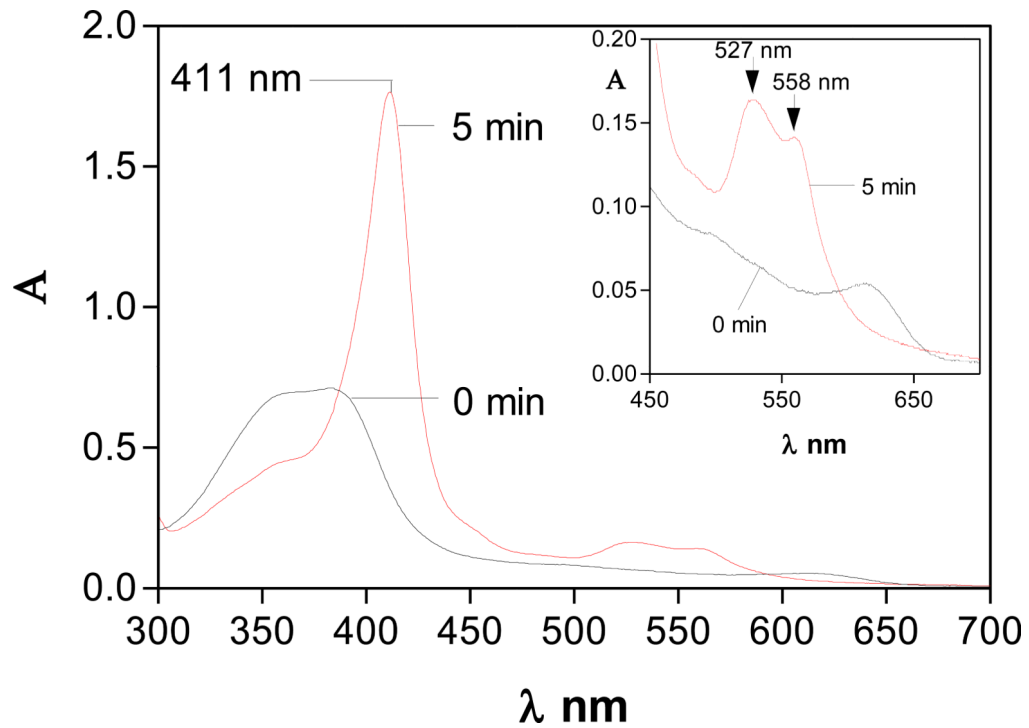
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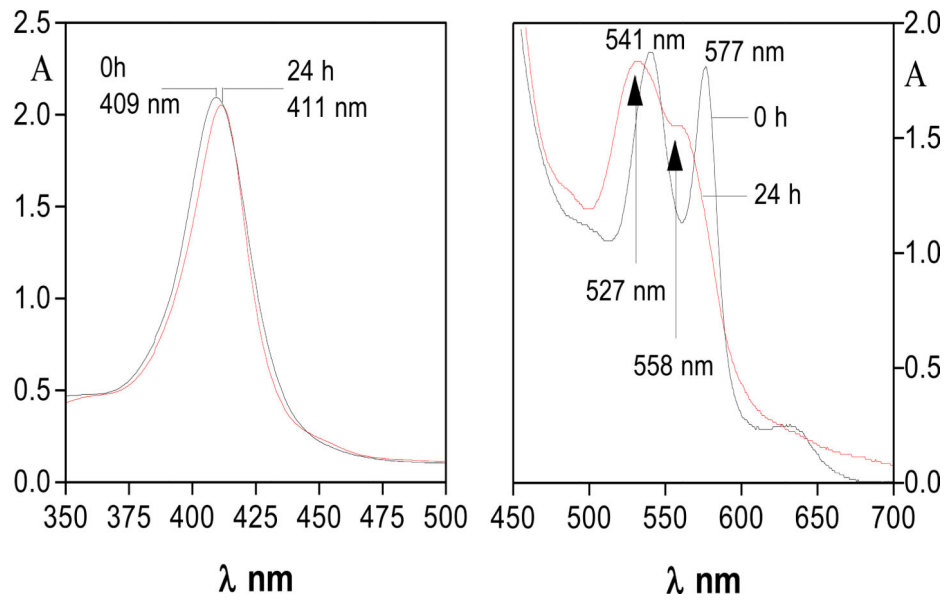
**Figure 1.**

Non-reducing SDS-PAGE showing apoHmuY and holoHmuY are resistant to InpA degradation. HoloHmuY and apoHmuY (both at 16  $\mu$ M) were incubated with 2  $\mu$ M InpA in 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.5, at 37°C, for the times indicated and subjected to non-reducing SDS-PAGE. Panels A and B, native holo- and apoHmuY plus InpA. Panels C and D, holo- and apoHmuY denatured by heating at 100°C for 10 min. The presence of haem complexed with HmuY is confirmed by haem peroxidase staining using tetramethylbenzidine-H<sub>2</sub>O<sub>2</sub> (TMB-H<sub>2</sub>O<sub>2</sub>) before counterstaining with Coomassie Brilliant Blue R-250 (CBB). 10  $\mu$ g of HmuY were loaded per track.

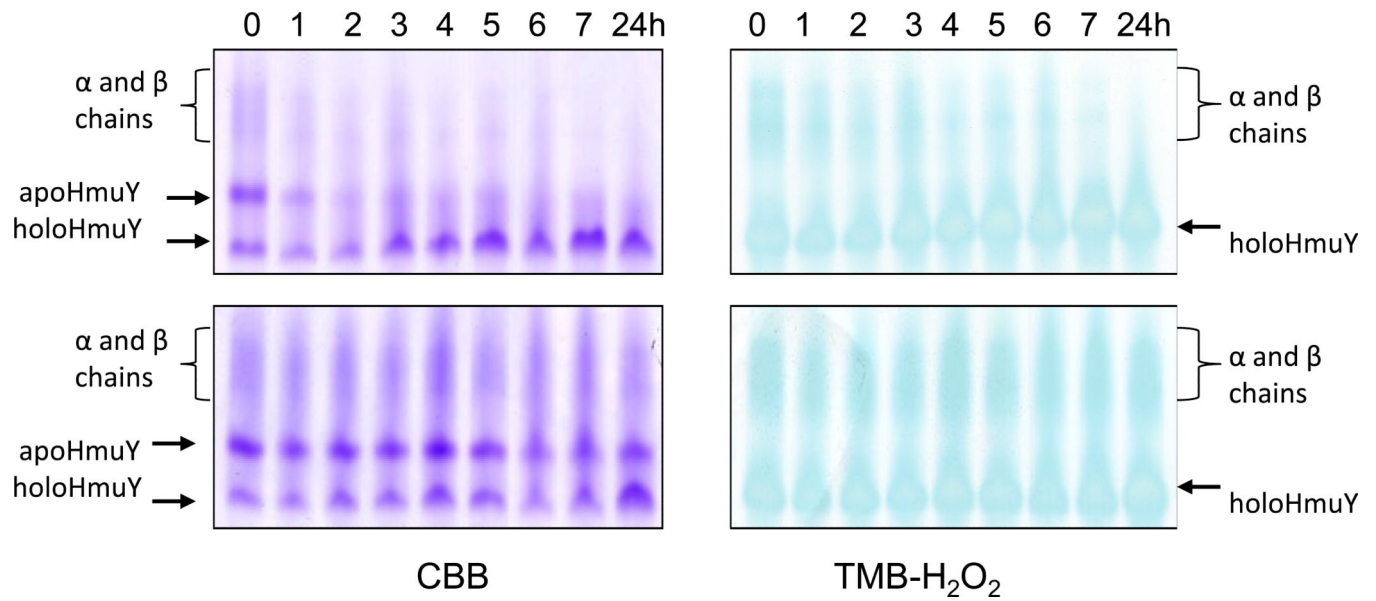


**Figure 2.**

UV-visible spectroscopic demonstration of the formation of the HmuY-iron(III) protoporphyrin IX complex by HmuY pre-treated with InpA. HmuY (16  $\mu$ M) was pre-incubated at 37°C for 24 h with InpA (2  $\mu$ M), before exposure to an equimolar amount of iron(III) protoporphyrin IX. Experimental conditions were as in Fig 1. Inset graph shows ordinate expansion of the Q band region.



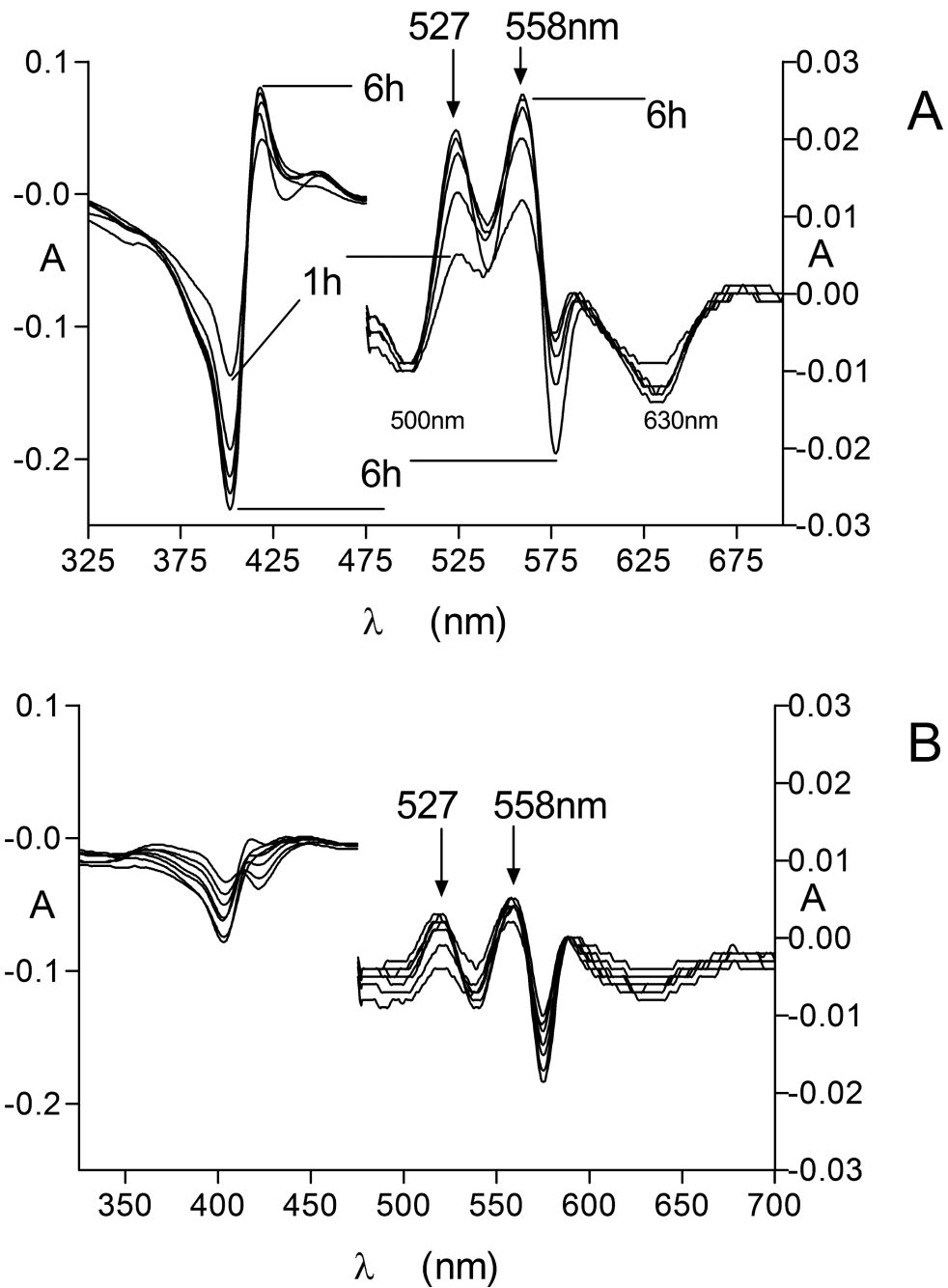
**Figure 3.** HmuY-iron(III) protoporphyrin IX formation from methaemoglobin induced by the action of InpA on oxyhaemoglobin. Black line, methaemoglobin at time zero; red line, 5 mins after addition of HmuY. Methaemoglobin was formed by incubation of oxyhaemoglobin (16  $\mu$ M; with respect to subunit) with InpA (2  $\mu$ M) at 37°C, in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5.



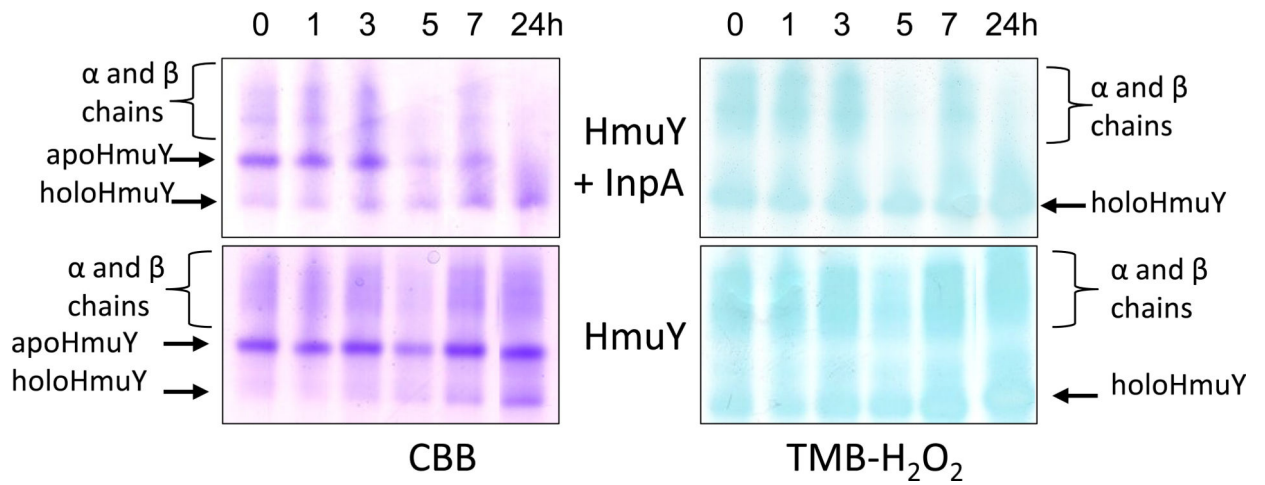
**Figure 4.**

Native PAGE showing the effect of HmuY on oxyhaemoglobin pre-treated with or without with InpA. Oxyhaemoglobin (16  $\mu$ M, with respect to subunit) was pre-incubated at 37°C with (upper panels) or without InpA (2  $\mu$ M) (lower panels) at pH 7.5 for 7 h in 0.1 M NaCl, 0.1 M Tris-HCl, and then exposed to HmuY (16  $\mu$ M) and sampled at the times shown. Gels were stained for the presence of haem with TMB-H<sub>2</sub>O<sub>2</sub> and counterstained with CBB.



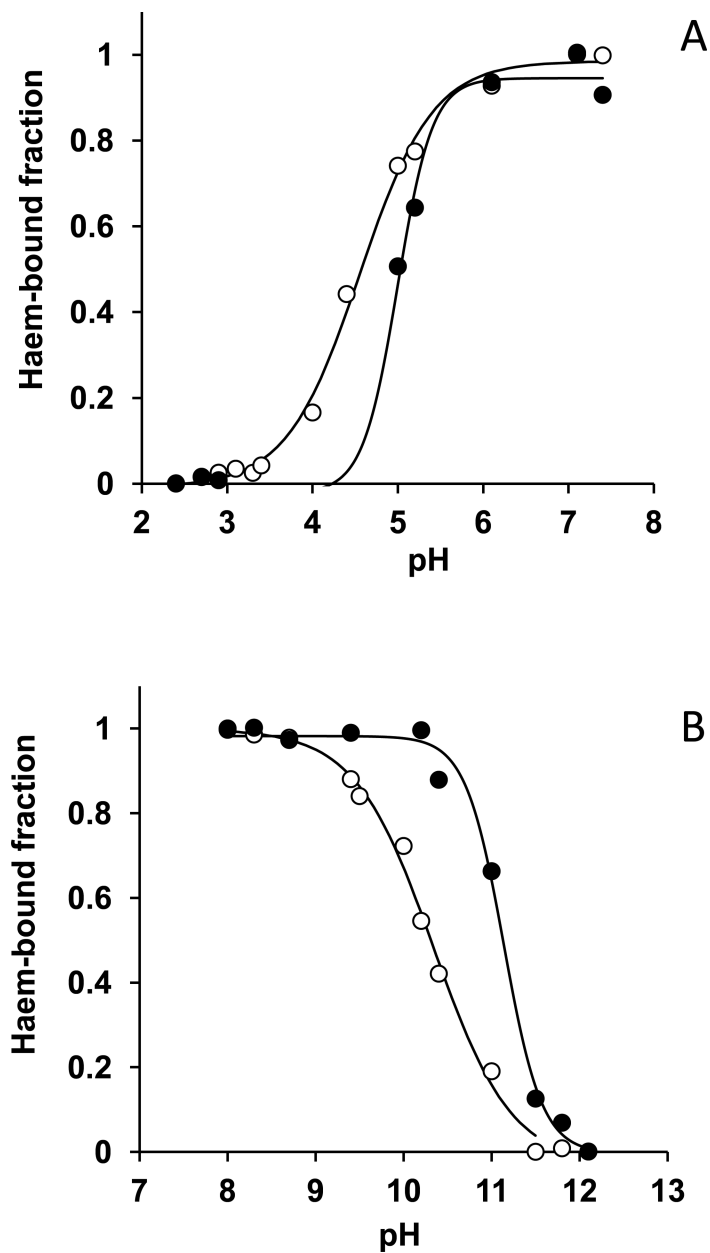


**Figure 5.** Difference spectra showing the generation of the HmuY-iron(III) protoporphyrin IX complex Q bands during incubation of HmuY with InpA-pre-treated oxyhaemoglobin. InpA-pretreated oxyhaemoglobin (A); auto-oxidised haemoglobin. Experimental conditions were as described in Figure 4. See text for details.



**Figure 6.**

Native PAGE showing the effect of co-incubating oxyhaemoglobin in the presence of both InpA and HmuY. Oxyhaemoglobin (16  $\mu$ M) was incubated together with InpA (2  $\mu$ M) plus HmuY (16  $\mu$ M) at 37°C in 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.5, for the times indicated and subjected to native PAGE before staining for the presence of haem with TMB-H<sub>2</sub>O<sub>2</sub> and then counterstained for protein with CBB.



**Figure 7.** Spectrophotometric titrations of HmuY with iron(II) and iron(III) protoporphyrin IX over a range of pH. Spectra of the acidic (upper panel) and basic (lower panel) species of HmuY-haem at a 1:1 molar ratio were analyzed under oxidized (empty circles) and reduced (filled circles) conditions. Soret band absorbance changes were monitored at 411 nm (oxidized) and 425 nm (reduced) and from these the haem-bound fractions of the HmuY complexes were calculated. Solid lines indicate the sigmoidal fits.