

Sequential action of R- and K-specific gingipains of *Porphyromonas gingivalis* in the generation of the haem-containing pigment from oxyhaemoglobin

John W. Smalley[§], Andrew J. Birss[§], Borys Szmigielski[†] and Jan Potempa[†]

[§]Department of Clinical Dental Sciences, The University of Liverpool, Liverpool L69 3GN, U.K., [†]Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Krakow, Poland

Key words: *Porphyromonas*, gingipains, oxyhaemoglobin, methaemoglobin, protease, μ -oxo dimer, haem.

Short title: Haem pigment formation by *P. gingivalis*

Author for correspondence: John W Smalley. Tel: 0044151 706 5272. FAX: 0044151 706 5809.

e-mail: josmall@liv.ac.uk

Abstract

The arginine- and lysine-specific gingipains of *Porphyromonas gingivalis* have been implicated in the degradation of haemoglobin from which the black μ -oxo haem dimer-containing pigment is generated. Here we examined interactions of oxyhaemoglobin (oxyHb) with the Arg-(R)-specific (HRgpA) and Lys-(K)-specific (Kgp) gingipains. Incubation of oxyHb with HRgpA resulted in formation of methaemoglobin (metHb), which could be prevented by the R-gingipain specific inhibitor leupeptin. OxyHb-Kgp interactions resulted in formation of a haemoglobin haemichrome. This was inhibited by the lysine-specific protease inhibitor Z-Phe-Lys-acyloxymethylketone (Z-FKck). MetHb, formed by treatment of oxyHb with either NaNO_2 or by pre-incubation with HRgpA, was rapidly degraded by Kgp compared to oxyHb. MetHb degradation by Kgp was also inhibited Z-FKck. Together these data show that R-gingipain activity is crucial for converting oxyHb into the metHb form which is rendered more susceptible to Kgp degradation for the eventual release of iron(III) protoporphyrin IX and production of the μ -oxo haem dimer. This explains previous observations [1] of the requirement for a combination of both R- and K-gingipains for pigment production from oxyhaemoglobin by *P. gingivalis*.

Introduction

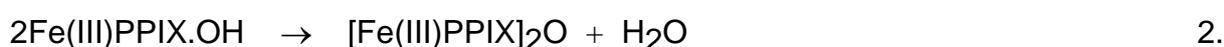
The black haem-pigment of *Porphyromonas gingivalis*, which serves ultimately as an iron source, is composed of the μ -oxo bishaem of iron(III) protoporphyrin IX, $[\text{Fe(III)PPIX}]_2\text{O}$, derived from haemoglobin [2, 3]. This haem species (also called μ -oxo dimer or haem dimer) is composed of two iron(III) protoporphyrin IX molecules covalently joined by an oxygen atom inter-bridge [4]. Its presence imparts the green colour to colonies of *P. gingivalis* during initial development on blood agar, and which become black as the concentration of $[\text{Fe(III)PPIX}]_2\text{O}$ increases, absorbing light across the visible spectrum.

The $[\text{Fe(III)PPIX}]_2\text{O}$ complex which becomes deposited on the cell surface can protect *P. gingivalis* against hydrogen peroxide because of its intrinsic catalase activity and because a cell surface layer acts as a sacrificial molecule oxidisable by hydrogen peroxide [5].

P. gingivalis generates the μ -oxo bishaem complex from haemoglobin via two routes [3]. Firstly, Fe(II) haems, proteolytically released from oxyhaemoglobin (oxyHb) and deoxyhaemoglobin (deoxyHb), combine with di-oxygen according to the following reaction:



a process which both removes, and hence detoxifies haem monomers, and consumes oxygen, facilitating anaerobiosis. Secondly, $[\text{Fe(III)PPIX}]_2\text{O}$ is generated from OH-bearing Fe(III) haem groups derived from methaemoglobin (metHb) which is the oxidised form of haemoglobin [3]. This process is described by the reaction:



It is important to emphasise that the finding that $[\text{Fe(III)PPIX}]_2\text{O}$ is formed from metHb reveals that *P. gingivalis* can generate the protective pigment from haemoglobin in an oxygenated environment.

Oxidation of oxyHb which is brought about by cells of *P. gingivalis* is inhibited by gingipain inhibitors N-ethylmaleimide and tosyl-Lys-chloromethane [3], suggesting that either cell-associated Lys- and/or Arg-specific proteases mediate this crucial step. It was previously considered that Kgp, and not Rgp, was singly responsible for haemoglobin degradation [6], an observation consistent with the fact that the globin chains contain multiple lysine residues which present ideal targets for attack by Kgp. However, it has been shown that a combination of either or both RgpA and RgpB, in addition to Kgp activity, is required by cells to produce the μ -oxo dimer from oxyHb *in vitro* or during growth on blood-containing media [1]. Inhibition of cellular Arg-Xaa protease activity by leupeptin was shown to prevent metHb formation, resulting in an alternate product identified as a haemoglobin haemichrome [1]. These findings suggest that either RgpB or HRgpA mediates metHb production and facilitates haem release for the eventual production of the μ -oxo dimer complex. To investigate this further we have examined the interactions of oxyHb with purified high molecular mass Arg-(R)-specific gingipain (HRgpA) and Lys-(K)-specific gingipain (Kgp) to determine the roles these enzymes play in haem-pigment formation.

Materials and methods

Gingipain purification

The culture medium of *P. gingivalis* strain HG66 was used as a source of soluble gingipains, HRgpA and Kgp, which were purified as described previously using gel filtration and arginine-sepharose chromatography [7]. The protein content

in each batch was measured using the BCA method with bovine albumin as the standard, whilst the concentration of active proteases was determined by active-site titration employing D-Phe-Phe-Arg-chloromethane as described previously [8]. Enzyme purity was checked using SDS-PAGE, in which both HRgpA and Kgp resolved into four major bands and one minor on SDS-PAGE [7] the identities of which were confirmed by N-terminal sequence analysis. For the arguments presented in this paper it is important to remember that Kgp and HRgpA from strain HG66 differ only in the catalytic domain which, although related, share only 27% identity of the primary structure, while the remaining haemagglutinin/adhesion domains (HAs) derived from the C-terminal part of the nascent polypeptide chains are practically identical in their amino acid sequences.

Preparation of oxyhaemoglobin

OxyHb was prepared from fresh horse erythrocytes [3] and for use in protease incubation experiments, diluted in 0.14M NaCl, 0.1M Tris-HCl, pH 7.5, to give a working concentration of 4 μ M (on a tetramer basis). At this concentration approximately 50% of the haemoglobin tetramers are dissociated into $\alpha\beta$ dimers. Methaemoglobin (methHb) was prepared by treatment of oxyHb (4 μ M) with a 4-fold molar excess of NaNO₂ in 0.14M NaCl, 0.1M Tris-HCl, pH 7.5 [9].

Incubation of haemoglobin with R- and K-gingipains

Stock solutions of HRgpA and Kgp were diluted in 0.14M NaCl, 0.1M Tris-HCl, pH 7.5, to give a working concentration of 0.2 μ M and incubations with oxyhaemoglobin (at 4 μ M) were carried out at 37°C. UV-visible spectra were recorded hourly in 1cm pathlength semi-micro cuvettes as previously described [1].

Where appropriate, samples of the incubation mixtures were withdrawn for SDS-PAGE.

Analysis of oxyHb and metHb concentrations

The concentrations of both oxyHb ([oxyHb]) and metHb ([metHb]) were determined spectroscopically from $A_{577\text{nm}}$ and $A_{631\text{nm}}$ measurements using the equations:

$$A_{577\text{nm}} = \epsilon_{\text{met}577\text{nm}} \cdot [\text{metHb}] + \epsilon_{\text{oxy}577\text{nm}} \cdot [\text{oxyHb}] \quad 3.$$

$$A_{631\text{nm}} = \epsilon_{\text{met}631\text{nm}} \cdot [\text{metHb}] + \epsilon_{\text{oxy}631\text{nm}} \cdot [\text{oxyHb}] \quad 4.$$

as described by Winterbourne *et al.* [10]. The millimolar extinction coefficients at 577nm and 631nm for oxyHb ($\epsilon_{\text{oxy}577\text{nm}} = 65$, and $\epsilon_{\text{oxy}631\text{nm}} = 1$) and for methaemoglobin ($\epsilon_{\text{met}577\text{nm}} = 15$ and $\epsilon_{\text{met}631\text{nm}} = 14$) were determined at pH 7.5 in 0.14M NaCl, 0.1M Tris-HCl, from 4 μ M reference samples as prepared above. All values are expressed on a haemoglobin tetramer basis. Equations 3. and 4. were solved giving the following expressions for calculation of the concentrations of oxyHb and metHb:

$$[\text{oxyHb}] = 0.0156 \cdot A_{576\text{nm}} - 0.0168 \cdot A_{631\text{nm}} \quad 5.$$

$$[\text{metHb}] = 0.0726 \cdot A_{631\text{nm}} - 0.0011 \cdot A_{576\text{nm}} \quad 6.$$

Rates of metHb formation were calculated by linear regression analysis from plots of [metHb] versus time over a seven hour period.

SDS-PAGE

SDS-electrophoresis was carried out on 15% polyacrylamide separating gels as previously described [11] and the gels stained for protein with coomassie blue.

Protease inhibitors

The protease inhibitors leupeptin and Z-Phe-Lys-acyloxymethylketone (Z-FKck) were obtained from Sigma Chemicals Ltd. and Bachem (St. Helens, UK), respectively.

Results

Interaction of HRgpA and Kgp with oxyHb

Incubation of control oxyHb resulted in increases in $A_{500\text{nm}}$ and $A_{631\text{nm}}$, and decreases in the intensities of the 577 and 541nm Q bands (Fig. 1a), consistent with auto-oxidation to the methaemoglobin form [10]. In the presence of HRgpA, similar changes to the Q bands regions were observed (Fig. 1b), and isosbestic points were seen at 475, 525 and 592nm indicating the direct change from oxyHb to metHb without generation of any intermediary product. The rates of metHb formation calculated from the concentrations of metHb using the absorbance changes at 577 and 630nm in equation 6, were $0.11 \text{ nmol ml}^{-1} \text{ h}^{-1}$ for control and $0.30 \text{ nmol ml}^{-1} \text{ h}^{-1}$ for the HRgpA-oxyHb incubation. To test whether metHb formation in the presence of the R-gingipain was proteolytically mediated, oxyHb was incubated with HRgpA in the presence of the specific R-gingipain protease inhibitor leupeptin (2mM). Under these conditions metHb formation was inhibited by 83% compared to control. After 24h the spectrum of the product of oxyHb-HRgpA incubation showed a 406nm Soret band and prominent 500 and 631nm bands. This was confirmed as metHb by its conversion to deoxyhaemoglobin (Soret λ_{max} of 429nm and 555nm visible band) by the addition of 10mM sodium dithionite to simultaneously reduce the haem iron to the Fe(II) state and remove dioxygen (data not shown).

Interaction of Kgp with oxyHb

Incubation of oxyHb with Kgp resulted in spectra showing progressively slight decreases in intensity and blue shift of the Soret band (Fig. 2 a). This was accompanied by decreases in $A_{576\text{nm}}$ and $A_{541\text{nm}}$ and increases in $A_{500\text{nm}}$ and $A_{630\text{nm}}$ (Fig. 2a, inset). However, unlike that for HRgpA, the oxyHb- Kgp incubation, gave a spectrum after 24h which was characterised by a 409nm Soret and a broad visible band at $\approx 535\text{nm}$ (Fig. 2b and inset, dotted line). Addition of 10mM sodium dithionite to this incubation mixture gave rise to a spectrum with a 422nm Soret and visible bands at ~ 530 and 558nm (Fig. 2b and inset, solid line), indicative of an iron(II) haemochrome species [1, 12, 13], confirming that an iron(III) haemichrome had initially been formed.

Degradation of metHb and oxyHb by Kgp

OxyHb and metHb (produced by oxyHb treatment with NaNO_2) were incubated with Kgp over 7h and spectra recorded periodically. Samples were also taken for SDS-PAGE. There was a pronounced decrease in Soret band intensity for the metHb incubated with Kgp compared to control metHb (Figs 3 a and c, respectively), showing haem loss from the protein [14]. However, as previously shown in Fig. 2a, incubation of oxyHb with Kgp produced only a small reduction in Soret intensity compared to control oxyHb (Figs 3 b and d, respectively). The loss of metHb Soret band intensity in the presence of Kgp was prevented by the lysine-specific gingipain protease inhibitor Z-FKck (10 μM) (Fig. 3e). In addition, when Z-FKck was present in the Kgp-oxyHb incubation a spectrum with a 406nm Soret band was obtained (Fig. 3e), which was identical to that of the control (Fig. 3f). Addition of

10mM sodium dithionite to the Kgp-oxyHb incubation mixture resulted in a haemochrome spectrum identical to that in Fig. 2b (data not shown), whilst reduction of the Z-FKck-Kgp-oxyHb incubation mixture with sodium dithionite yielded a spectrum with a 429nm Soret and a 555nm visible band indicative of the presence of deoxyhaemoglobin (Fig. 4). This showed that metHb had originally been formed. These data thus revealed that inhibition of Kgp activity could prevent haemichrome formation to result instead in the generation of metHb.

In a separate experiment Kgp was incubated with metHb which had been produced by HRgpA pre-treatment of oxyHb for 7h. Crucially, the metHb spectrum also displayed a reduction in Soret band intensity (Fig. 5) showing a loss of haem from the protein as observed in Fig. 3a for the incubation of Kgp with metHb which had been generated chemically using NaNO_2 .

SDS-PAGE analysis was carried out on 15% gels under reducing conditions to reveal the separated monomeric haemoglobin subunits (Fig 6). This showed that Kgp had completely digested the metHb α and β chains after 3h. However, UV-visible spectroscopy (Fig. 3a) had revealed that the Soret band had not completely collapsed after this time. The spectra at 3h and beyond most probably represent the presence of a small amount of undegraded metHb, partially degraded lower molecular weight α and β chain fragments carrying haem moieties, along with a mixture of iron protoporphyrin IX monomer and μ -oxo dimer. In contrast, oxyHb was not degraded even after 7h (Fig. 6). The lysine-specific gingipain protease inhibitor Z-FKck totally inhibited the Kgp-mediated breakdown of metHb. There was also no breakdown of oxyHb by Kgp in the presence of Z-FKck (Fig. 6).

Discussion

To shed further light on the mechanisms by which *P. gingivalis* generates the μ -oxo bishaem-containing black pigment, interactions of oxyHb with purified gingipains were examined. For this we employed HRgpA and Kgp multidomain proteases which, while differing in their catalytic domains, share identical haemagglutinating/adhesion domains [15, 16]. These studies revealed that HRgpA promotes metHb formation from oxyHb, whilst Kgp-oxyHb interactions result in a haemoglobin haemichrome. The data herein confirm and support our previous observations [1] of the need for oxidation of oxyHb into the met form for it to be degraded to eventually yield the μ -oxo bishaem. This is an important finding as it reveals that despite its strict anaerobic growth requirement, *P. gingivalis* can produce the defensive pigment under aerobic conditions.

Experimental support for production of $[\text{Fe(III)PPIX}]_2\text{O}$ via metHb comes from the observation that the haem pigment is composed of 10-15% iron protoporphyrin IX in the monomeric, un-dimerised form (Fe(III)PPIX.OH) [2]. Whilst the reaction of the reduced haem species Fe(II)PPIX with O_2 to give $[\text{Fe(III)PPIX}]_2\text{O}$ (equation 1) is rapid, μ -oxo bishaem formation via dimerisation of Fe(III)PPIX.OH monomers (equation 2) is much slower [17]. This slower rate of dimerisation may be circumvented by the interaction of Fe(III)PPIX.OH with the HA2 haem/haemoglobin binding adhesins of both Kgp and HRgpA which can promote μ -oxo dimer formation from the iron(III) haem monomers [18].

The R-specific gingipain inhibitor leupeptin inhibited HRgpA-mediated metHb formation showing this effect was dependent upon protease activity. Arginine residues are located at the C-terminus of both α and β chains, at positions 31 and 92 in the α chain, and at positions 30, 40 and 104 in the β chain (in addition to position

116 in the α chain of horse Hb; [19]). Their surface location may render them susceptible to Rgp attack to bring about structural changes within the haem pocket facilitating electron transfer between the haem iron and ligated O_2 to give the metHb form.

In the presence of Kgp, oxyHb was converted into a haemichrome. Haemoglobin haemichromes cannot be re-oxygenated and are physiologically inactive [13]. They form via the actions of protein denaturants such as urea and fatty acids, which bring about structural changes distorting the haem pocket geometry to result in the co-ordination of the haem Fe atom by another amino acid ligand in addition to the proximal histidine [12, 13]. Kgp-mediated haemichrome formation was inhibited by Z-FKck, and may therefore be assumed to be related to structural changes resulting from proteolytic activity. Kgp digestion of haemoglobin yields the proteolytic fragment VVAGVANALAHK from the β chain which lacks the C-terminal -YH dipeptide [20]. The proteolytic attack on the C-terminal -KYR and -KYH residues of α and β chains, respectively, may contribute to breakdown of the tetramer as it is known that the C-terminus in general [21], and the tyrosine residues of these in particular [22, 23], are important in stabilising the quaternary structure. In addition, it should be pointed out that at a substrate concentration of $4\mu\text{M}$ as used in this study, approximately 50% of the haemoglobin is dissociated into $\alpha\beta$ dimers. It is not known whether dissociation into $\alpha\beta$ dimers renders them more susceptible to attack by Kgp and HRgpA compared to tetramers. Studies are in hand to determine the initial sites of attack on oxyHb by HRgpA and Kgp which lead to metHb and haemichrome formation, respectively.

Importantly, Kgp rapidly degraded metHb which had been produced by either treatment of oxyHb with either NaNO_2 or pre-incubation with HRgpA. This supports

our previous observations [1] of the need for a combination of both Lys- and Arg-specific protease activity to generate the μ -oxo dimer from oxyHb. It is noteworthy however, that other workers have used commercial preparations of haemoglobin for gingipain studies. Such preparations can contain as much as 95% in the methaemoglobin form, and provides the explanation for why Kgp alone has been shown to be effective in degrading haemoglobin [6, 20].

Since haem (and hence iron) acquisition is crucial to the metabolism and survival of *P. gingivalis*, a mechanism which ensures iron protoporphyrin IX release and μ -oxo dimer formation from both oxygenated and deoxygenated haemoglobin would be an advantage for cells living under both aerobic and anaerobic environments.

Acknowledgements

The authors would like to thank Professor Victor Flynn of the Department of Pure Mathematics, The University of Liverpool, for kindly providing the solution to the simultaneous equations for the multi-component spectroscopic analysis, and Professor Michael Wilson and Dr Brandon Reeder, Department of Biological Sciences, The University of Essex, for helpful comments. This work was supported by the National Institutes of Health Grant DE 09761 and a grant from the MNiSW (Warsaw, Poland) (to J.P.). J.P. is a recipient of an award *SUBSYDIUM PROFESORSKIE* from the Foundation for Polish Science (FNP, Warszawa, Poland).

References

- [1] J.W. Smalley, M.F. Thomas, A.J. Birss R. Withnall, J. Silver, *Biochem. J.* 379 (2004) 833-840.
- [2] J.W. Smalley, , J. Silver, P.J. Marsh, A.J. Birss, *Biochem. J.* 331(1998) 681-677.
- [3] J.W. Smalley, A.J. Birss, R. Withnall, J Silver, *Biochem. J.* 362 (2002) 239-245.
- [4] J. Silver, B. Lukas, *Inorg. Chim. Acta* 78 (1983) 219-224.
- [5] J.W. Smalley, A.J. Birss, J. Silver, *FEMS Microbiol. Lett.* 183 (2000) 159-164.
- [6] A. Sroka, M. Sztukowska, J. Potempa, J. Travis, C.A. Genco, *J. Bacteriol.* 183 (2001) 5609-5616.
- [7] R. Pike, W. McGraw, J. Potempa, J. Travis, *J. Biol. Chem.* 269 (1994) 406-411.
- [8] J. Potempa, R. Travis, *Biol. Chem.* 378 (1997) 223-230.
- [9] H. Kosaka, K. Imaizumi, H. Imai, I. Tyuma, *Biochim. Biophys. Acta* 581 (1979) 184-188.
- [10] C.C. Winterbourne, B.M. McGrath, R.W. Carrell, *Biochem. J.* 155 (1976) 493-502.
- [11] J.W. Smalley, P. Charalabous, C.A. Hart, J. Silver, *Microbiology* 149 (2003) 843-853.
- [12] E.A. Rachmilewitz, J. Peisach, W.E. Blumberg, *J. Biol. Chem.* 246 (1971) 3356-3366.
- [13] J.M. Rifkind, O. Abugo, A. Levy, J. Heim, *Meth. Enzymol.* 231 (1994) 449-480.
- [14] M. Rousselot, D. Le Guan, C. Chabasse, F. Zal, *FEBS Journal* 273 (2006) 1582-1596.
- [15] N. Pavloff, J. Potempa, N.R. Pike, V. Prochazka, M.C. Kiefer, J. Travis, P. Barr, *J. Biol. Chem.* 270 (1995) 1007-1010.

- [16] N. Pavloff, P.A. Pemberton, J. Potempa, W.C.A. Chen, N.R. Pike, V. Prochazka, M.C. Kiefer, J. Travis, P. Barr, *J. Biol. Chem.* 272 (1997) 1595-1600.
- [17] Y. Inada, K. Shibata, *Biochem. Biophys. Res. Commun.* 9 (1962) 323-327.
- [18] J.W. Smalley, A.J. Birss, B. Szmigielski, J. Potempa, *Microbiology.* 152 (2006) 1839-1845.
- [19] E. Antonini, M. Brunori, in: A. Neuberger, E. L. Tatum, Eds., *Hemoglobin and myoglobin in their reactions with ligands*, North-Holland Publishing Company, Amsterdam, 1971, pp. 55-97.
- [20] J.P. Lewis, J.A. Dawson, J.C. Hannis, D. Muddiman, F.L. Macrina, *J. Bacteriol.* 181(1999) 4905-49013.
- [21] M.F. Perutz, *Nature* 228 (1970) 726-739.
- [22] D. Wang, T.G. Spiro, *Biochemistry* 37 (1998) 9940-9951.
- [23] J. Kneipp, G. Balakrishnan, R. Chen, T.-J. Shen, S.C. Sahu, N.T. Ho, J.L. Giovannelli, V. Simplaceanu, C. Ho, T.G. Spiro, *J. Mol. Biol.* 356 (2006) 335-353.

Figure Legends

Fig. 1:

UV-visible spectra of oxyHb incubated with HRgpA. a) control oxyHb; b) oxyHb plus HRgpA. Buffer was 0.14M NaCl, 0.1M Tris-HCl, pH 7.5, and temperature 37°C. OxyHb, HRgpA and leupeptin concentrations were 4 μ M and 0.2 μ M, respectively. Arrows denote changes in absorbance intensities with time.

Fig. 2:

UV-visible spectra showing the effect of incubation of horse oxyhaemoglobin (4 μ M) with Kgp (0.2 μ M) over a 7h period (panel a), and after 24h (dotted line, panel b). Solid line in panel b shows the result of adding 10mM Na₂S₂O₄. Incubation conditions were the same as described for Fig. 1. Insets in a and b: ordinate expansion of the spectra between 500nm and 700nm. Substrate and enzyme concentrations were 4 μ M and 0.2 μ M, respectively. For clarity not all spectra are shown

Fig. 3:

UV-visible spectra of metHb(a) and oxyHb(b) incubated with Kgp (c) and (d) are metHb and oxyHb controls, respectively. Panels (e) and (f) show incubations of oxyHb and metHb, respectively, in the presence of the lysine-specific protease inhibitor Z-FKck. Buffer and incubation conditions were as for Fig. 1. MetHb was prepared by treatment of oxyHb with NaNO₂. Kgp concentration was 0.2 μ M, and metHb and oxyHb concentrations were 4 μ M.

Fig. 4: Effect of sodium dithionite on the product of a 7h incubation of oxyhaemoglobin with Kgp in the presence of the specific lysine protease inhibitor Z-FKck (10 μ M). Substrate and enzyme concentrations and incubation conditions were the same as described for Fig. 3.

Fig. 5: Effect of Kgp (0.2 μ M) on methaemoglobin (4 μ M) produced by pre-treatment of oxyhaemoglobin with the R-gingipain HRgpA. Incubation conditions were the same as for Fig. 3.

Fig. 6: SDS-PAGE of the α and β chains of metHb and oxyHb during incubation with Kgp in the presence and absence of the lysine-specific gingipain inhibitor Z-Phe-Lys-acyloxymethylketone (Z-FKck). Incubation conditions were as for Fig. 4. Samples (\approx 5 μ g haemoglobin per track) were electrophoresed on 15% polyacrylamide gels and stained with coomassie blue.

Fig. 1

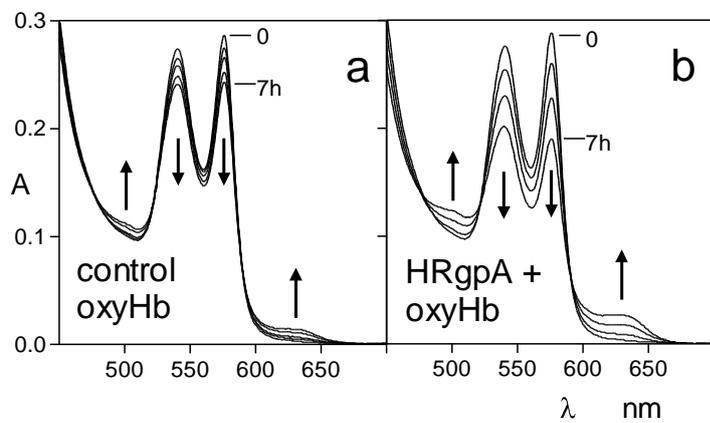


Fig. 2

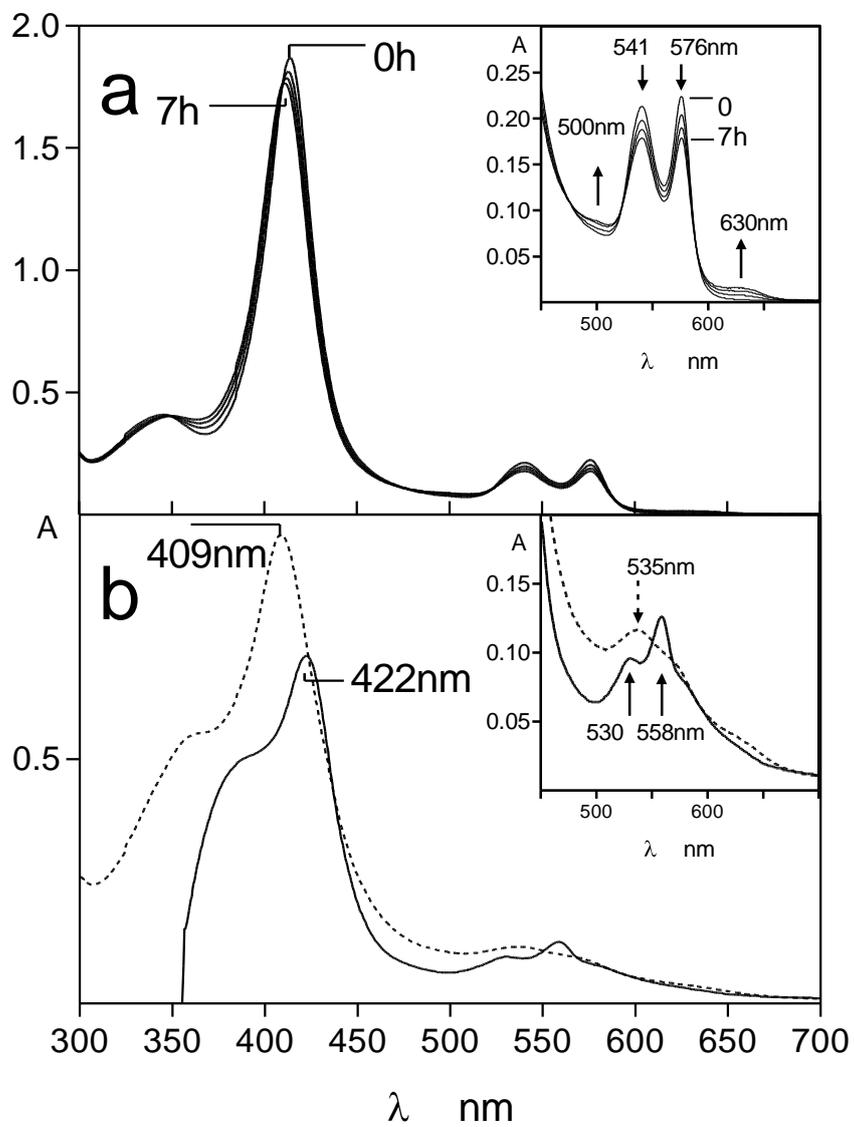


Fig. 3

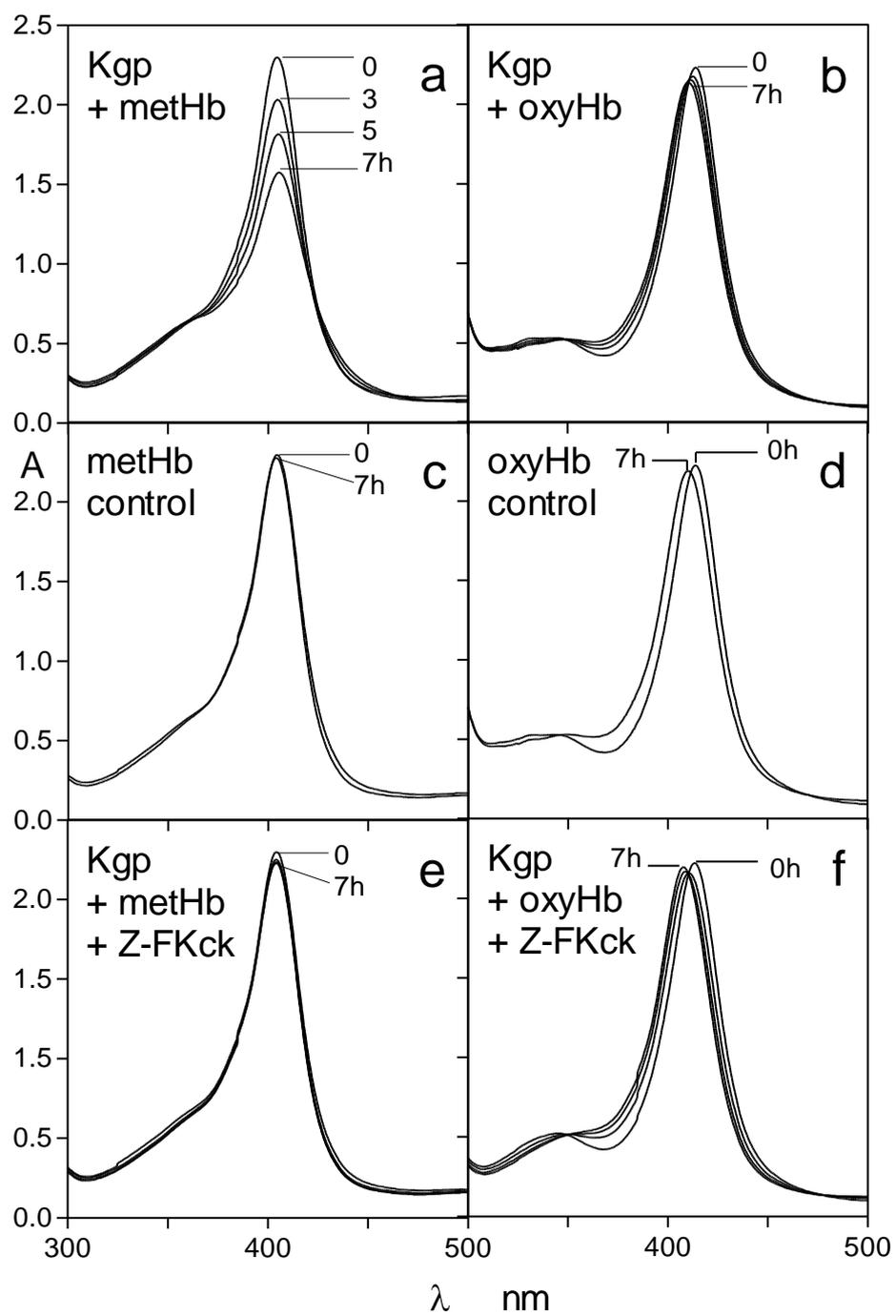


Fig. 4

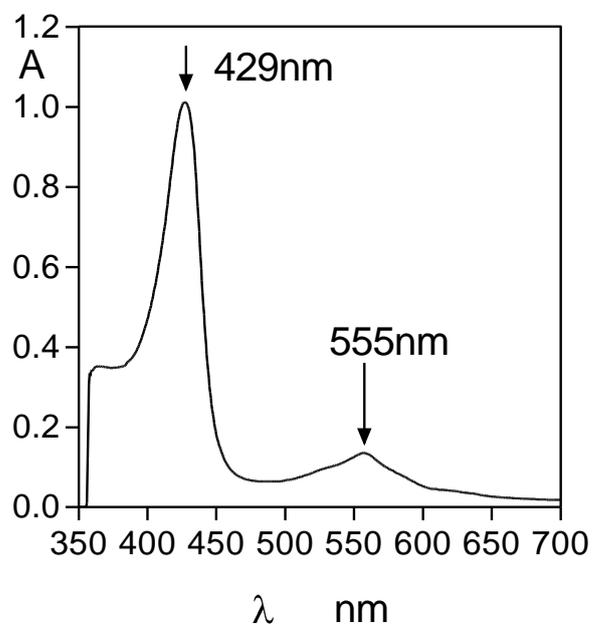


Fig. 5

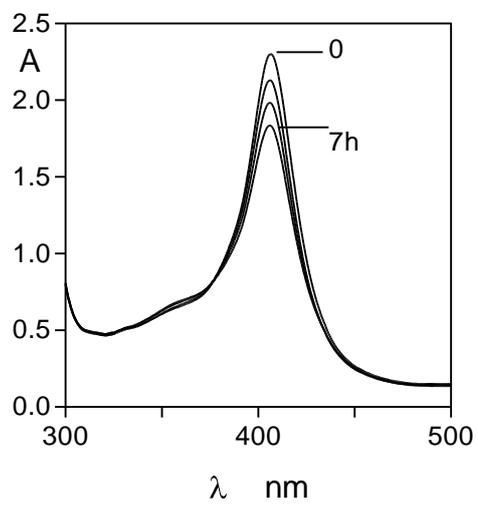


Fig. 6

