Fe-haem bound to *Escherichia coli* bacterioferritin accelerates iron core formation by an electron transfer mechanism

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BFR (bacterioferritin) is an iron storage and detoxification protein that differs from other ferritins by its ability to bind haem cofactors. Haem bound to BFR is believed to be involved in iron release and was previously thought not to play a role in iron core formation. Investigation of the effect of bound haem on formation of the iron core has been enabled in the present work by development of a method for reconstitution of BFR from *Escherichia coli* with exogenously added haem at elevated temperature in the presence of a relatively high concentration of sodium chloride. Kinetic analysis of iron oxidation by *E. coli* BFR preparations containing various amounts of haem revealed that haem bound to BFR decreases the rate of iron oxidation at the dinuclear iron ferroxidase sites but increases the rate of iron core formation. Similar kinetic analysis of BFR reconstituted with cobalt-haem revealed that this haem derivative has no influence on the rate of iron core formation. These observations argue that haem bound to *E. coli* BFR accelerates iron core formation by an electron-transfer-based mechanism.

Key words: bacterioferritin, *Escherichia coli*, haem reconstitution, iron oxidation, iron storage.

**INTRODUCTION**

Ferritin is an iron-storage protein found in plants, animals and microbes that ensures maintenance of adequate iron supplies while affording protection from the toxicity of iron [1–3]. Three types of bacterial ferritin or ferritin-like proteins are currently recognized: FTN (bacterial ferritin), BFR (bacterioferritin) and DPS (DNA-binding protein from starved cells) [4,5]. BFR is composed of 24 subunits that oligomerize to form a ∼450 kDa spherical shell that defines a central cavity in which as many as ∼2700 iron atoms can be stored as a ferric hydroxide core [6]. The BFR subunit is a four α-helical bundle protein with an additional short C-terminal α-helix, and each monomer possesses a centrally located dinuclear iron site that promotes iron core formation and is referred to as the ferroxidase centre [7].

One of the key differences between BFR and other ferritin or ferritin-like proteins is that BFR binds haem. Specifically, the BFR 24-mer possesses twelve haem-binding sites that are located between adjacent pairs of subunits in an arrangement that has been referred to as the ‘subunit dimer’. Each subunit binds to the haem iron by co-ordination of Met52 to result in a haem centre with a highly unusual bis-methionine axial ligation [8,9] and with the haem orientated such that the propionate groups are located on the inner surface of the spherical protein shell. At present, the only other example of such a haem-binding environment in a naturally occurring protein is in the surface protein SHP from *Streptococcus pyogenes* [10,11].

A functional role for haem in iron release was initially suggested by the finding that haem-free BFR accumulated more than four times as much iron by development of a method for reconstitution of BFR from *Escherichia coli* with exogenously added haem at elevated temperature in the presence of a relatively high concentration of sodium chloride. Kinetic analysis of iron oxidation by *E. coli* BFR preparations containing various amounts of bound haem revealed that haem bound to BFR decreases the rate of iron oxidation at the dinuclear iron ferroxidase sites but increases the rate of iron core formation. Similar kinetic analysis of BFR reconstituted with cobalt-haem revealed that this haem derivative has no influence on the rate of iron core formation. These observations argue that haem bound to *E. coli* BFR accelerates iron core formation by an electron-transfer-based mechanism.

**EXPERIMENTAL**

**Protein preparation**

Wild-type BFR was expressed from inoculation cultures of *E. coli* AL1 cells [BL21(DE3) bfr:kan] containing the plasmid pALN1 (pET21a possessing the *E. coli* bfr gene) that were grown in LB (Luria–Bertani) medium containing ampicillin (0.1 mg/ml) and kanamycin (0.05 mg/ml) [6,18]. Flasks (2 litre) containing

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**Abbreviations used:** BFD, bacterioferritin-associated ferredoxin; BFR, bacterioferritin; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; RD2, rubredoxin-2.

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LB medium (1 litre) and ampicillin (0.1 mg/ml) were inoculated with this culture and grown at 37°C with 250 rev./min shaking. IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) was added to induce expression when $D_{600} = 1$. Cells were harvested by centrifugation 10–15 h after induction. The cell pellet was washed with and resuspended in potassium phosphate buffer (buffer A: 50 mM potassium phosphate, pH 7.2) before lysis by two passes through an Avestin Emuliflex-C5 high-pressure homogenizer. The lysed sample was incubated at 65°C for 10–15 min and placed on ice prior to centrifugation to remove cell debris and precipitated contaminants. The supernatant fluid was applied to a column of Q-Sepharose Fast Flow (20 ml; GE Healthcare) equilibrated with buffer A containing sodium chloride (0.5 M). Fractions containing BFR were pooled, concentrated by ultrafiltration (Amicon Ultra, Millipore) and loaded on to a gel-filtration column of HiLoad Superdex-200 Preparation Grade (200 ml; GE Healthcare). The column was washed with buffer A, and the protein was eluted with a linear sodium chloride gradient (0–0.5 M). Fractions containing BFR were pooled, concentrated by centrifugal ultrafiltration (Amicon Ultra, Millipore) and loaded on to a gel-filtration column of HiLoad Superdex-200 Preparation Grade (200 ml; GE Healthcare) that had been equilibrated with buffer A containing sodium chloride (0.5 M). Fractions containing pure BFR, as identified by SDS/PAGE, were pooled. Iron and other metal ions were removed by repeated concentration and dilution by centrifugal filtration of the protein in Mes buffer (50 mM, pH 6.5) containing DTT (dithiothreitol; 2 mM), EDTA (1–10 mM) and 2,2′-bipyridyl (1–5 mM) (Sigma). The M52H variant of BFR was expressed and purified as described previously [19].

### Spectroscopy

CD measurements were recorded with a Jasco Model J-810 spectropolarimeter for BFR samples (0.1 μM) in Mes buffer (20 mM, pH 6.5) or Mes buffer containing NaCl (1 M). The temperature of the cuvette holder (0.2 cm pathlength) was controlled with a Peltier device that was operated under the control of a computer. Electronic absorption spectra were recorded with either a Varian Cary 4000 or a 6000i spectrophotometer or a PerkinElmer L-800 spectrophotometer and quartz cuvettes (1 cm pathlength). Haem binding to BFR (0.5 μM) in Mes buffer (0.1 M, pH 6.5) containing NaCl (1 M) at 80°C was monitored by electronic spectroscopy with a user-configured S2000 miniature fibre-optic spectrophotometer and a MINI-D2T miniature deuterium tungsten light source (Ocean Optics).

### Fe- and Co-protoporphyrin IX reconstitution of BFR

Haemin chloride (Frontier Scientific) was dissolved in sodium hydroxide solution (0.1 M), diluted (to 1.5 mM) with Mes buffer (0.2 M, pH 6.5) and centrifuged to remove insoluble material immediately before adding the haemin to the protein in Mes buffer (0.2 M, pH 6.5) containing NaCl (1 M) at 80°C. BFR was incubated at 80°C for 5–15 min after the addition of haem before cooling to room temperature (~22°C). Free and adventitiously bound haem were removed with an Amicon Ultra 30k centrifugal filter (Millipore) and with a PD-10 desalting column (GE Biosciences). The concentration of reconstituted haem-binding sites of wild-type BFR was determined on the basis of the Soret absorbance ($ε_{418nm} = 1.09 \times 10^5$ M$^{-1}$·cm$^{-1}$). Reconstitution and purification of BFR with cobaltic protoporphyrin IX chloride (Porphyrin Products) was performed with the same procedure. The concentration of haem-binding sites reconstituted with cobalt protoporphyrin IX (Co-haem) was determined by measuring the cobalt content of the reconstituted protein with a PerkinElmer NexION 300D inductively-coupled plasma mass spectrometer.

### Kinetic methods

Phase 2 kinetics of iron oxidation by BFR as defined by Le Brun et al. [20] was measured with an SFM-400 stopped-flow spectrometer (Bio-Logic SAS). Equal volumes of BFR (1 μM) in Mes buffer (0.2 M, pH 6.5) and ferrous ammonium sulfate solution (0, 10, 20, 30, 40, 50, 60, 80 and 100 μM) freshly prepared in HCl (6 mM) were mixed at 25°C. Pseudo-first-order rate constants were calculated from the plots of the initial rates of iron oxidation against the iron concentrations that do not exceed the saturation point of the dinuclear iron sites (0, 5, 10, 15 and 20 μM). A Varian Cary 4000 spectrophotometer was used to measure the kinetics of Phase 3 of iron oxidation. Fe$^{2+}$ solutions were prepared by dissolving ferrous ammonium sulfate solution (200 μM) were made to BFR solutions (0.5 μM) in Mes buffer (0.1 M, pH 6.5) at 25°C in 30 min intervals, and the change in absorbance at 340 nm was monitored to measure the oxidation of iron. Measurements were initiated 20 s after the initial iron addition.

### RESULTS

#### Thermal stability of *E. coli* BFR is greatly enhanced by 1 M NaCl

The thermal stability of BFR is expected to be significant in view of the fact that the purification protocols used in the present study and elsewhere (for example [21]) include removal of less-stable protein impurities by heating the impure BFR preparation to 65°C. This expectation is consistent with the transition observed at ~70°C during thermal denaturation of BFR as monitored by far-UV CD spectroscopy (Figure 1A, inset). Nevertheless, this Figure is also consistent with the conclusion that, upon heating to 80°C, the secondary structure of BFR undergoes significant changes and that the native structure of the protein is not restored when the temperature is returned to 25°C (Figure 1A). Thus, in the absence of added salt, BFR was irreversibly denatured and precipitation was observed following exposure to 80°C. Repeating this thermal denaturation experiment in the presence of 0.5 M NaCl (Figure 1B, inset) stabilizes BFR sufficiently that this thermal transition is not observed, and the negative ellipticity that reflects the α-helical structure of the protein is largely restored upon returning to 25°C (Figure 1B). In this case, however, the renaturation trace is distinctly different from the denaturation trace, implying that the paths for denaturation and renaturation are inequivalent in the presence of 0.5 M NaCl. On the other hand, increasing the concentration of NaCl to 1 M renders the thermal denaturation of BFR fully reversible (Figure 1C, inset), as confirmed by inspection of the far-UV CD spectra that were collected before and after these samples were exposed to elevated temperatures (Figure 1C, inset).

#### *E. coli* BFR can be reconstituted with haem at 80°C in 1 M NaCl

Addition of exogenous haem to BFR at 25°C in 1 M NaCl fails to produce a protein adduct with the electronic spectrum that is characteristic of BFR with haem bound (Figure 2A). In contrast, addition of haem to an identical sample of BFR at 80°C results in a change in electronic spectrum that is consistent with rapid and specific binding of haem to BFR, as demonstrated by the increase in absorbance of the expected Soret maximum at 418 nm (Figure 2B). Following this addition of haem, removal of free and non-specifically bound haem by passage through a PD-10 desalting column resulted in fractions of BFR containing various amounts of haem (Figure 2C). Binding of haem added to BFR...
Haem bound to bacterioferritin accelerates core growth

Figure 1 Influence of salt on reversibility of BFR thermal denaturation

CD spectra were obtained for samples of 0.1 μM BFR with 11% haem in Mes buffer (20 mM, pH 6.5) containing (A) no NaCl, (B) 0.5 M NaCl and (C) 1 M NaCl before (solid line) and after (broken line) heating to 80°C and cooling to 25°C (1°C/min). In each case, the thermal denaturation curves as monitored by the thermally induced changes in ellipticity at 220 nm are shown in the insets for both increasing (solid line) and decreasing (broken line) temperature.

in this manner to the previously identified haem-binding site was further confirmed by observation of an absorbance maximum at ∼739 nm that is indicative [12,22] of bis-methionine ligation of haem (Figure 2C, inset).

Figure 2 Haem binding to BFR observed by electronic absorption spectroscopy

(A) Addition of haem to BFR at 25°C does not show specific binding of haem to BFR. Spectrum of 0.5 μM BFR in 0.1 M Mes (pH 6.5) and 1 M NaCl before haem addition (solid line), 3 min after the addition of 6 μM haem (dashed line), and 24 h after the addition (dotted line). (B) Addition of haem to BFR at 80°C shows specific binding of haem, as shown by the increase in the Soret band at 418 nm (inset). (C) Spectra of BFR samples with various levels of haem content after the addition of haem at 80°C and passing through a desalting column to remove free or non-specifically bound haem. Bis-methionine binding of haem is shown by the peaks at ∼739 nm (inset). BFR containing 11% (solid line), 31% (dashed line), 50% (dotted line) and 86% (dashed-dotted line) haem content is shown.

The spectrum of the M52H variant following the addition of haem does not resemble the spectrum of wild-type BFR reconstituted with haem

To confirm that the haem bound to BFR is co-ordinated by Met52, a variant of BFR in which this residue is substituted for histidine was used. This substitution was previously shown to lead to haem-free BFR [12], even though histidine is a common haem axial ligand. Addition of haem to the protein, according to the method described above, led to precipitation of ∼25–33% of the protein following heating at 80°C. This instability was a consequence of heating the protein in the presence of haem at 80°C because treatment with haem without heating or heating in the absence of haem did not adversely affect protein stability. Addition of 12 mol of haem per BFR 12-mer at 65°C resulted in precipitation of some of the variant protein (<10%), and following elution of the protein over a gel-filtration column, the absorbance spectrum

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Figure 3 Phase 2 iron oxidation profiles of BFR with varying haem content

Ferrous ammonium sulfate was added to final concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 μM to 0.5 μM BFR containing (A) 18%, (B) 42%, (C) 62% and (D) 89% haem in 0.1 M Mes buffer (pH 6.5) at 25 °C using a stopped-flow instrument.

(Soret maximum = 405 nm) of the protein resembled that of haem oxygenase and metmyoglobin. In these proteins, the ferric haem iron is bound by a single histidine residue [23,24]. Assuming that the molar absorptivity for haem bound to this protein is the same as that reported for haem oxygenase (405 nm = 1.5 × 10³ M⁻¹·cm⁻¹; [25]), just 0.3–0.4 mol of haem is bound to the M52H 12-mer following this treatment. Attempts to concentrate the haem-bound form of M52H BFR for further analysis resulted in loss of some haem, indicating that it is not stably co-ordinated to this variant protein, consistent with our previous observation that expression in E. coli leads to isolation of this variant with no haem bound [12].

To ensure that the minimal haem-binding exhibited by the M52H variant was not a consequence of the lower temperature employed to promote haem-binding, the wild-type protein was also treated with 12 mol of haem at 65 °C. Although not as effective in promoting haem-binding as treatment at 80 °C, the spectrum of the resulting protein was essentially identical with that observed following reconstitution at higher temperature, but consistent with the binding of only ~8 mol of haem per BFR 12-mer. A low-intensity band was observed at ~630 nm, indicating the presence of some high-spin ferri-haem that could reflect heterogeneity of haem binding at the native binding site (i.e. co-ordination of just one Met52 rather than two). Overall, these results demonstrate that haem binding to wild-type BFR under the reconstitution conditions described above results in haem binding to the natural bis-methionine-co-ordinated site.

Iron oxidation kinetics at the dinuclear iron site as a function of BFR haem content

The ferroxidase activity of BFR that leads to formation of the iron core has been described in terms of three kinetic phases [20,26]. Phases 1 and 2 involve the binding and oxidation of Fe²⁺ respectively at the dinuclear iron sites. Following formation of dinuclear iron sites, iron core formation continues in Phase 3, which involves the binding and oxidation of iron at the inner core. The oxidation of iron in Phases 2 (fast) and 3 (slow) can be studied by monitoring the absorbance change at 340 nm following the addition of Fe²⁺ to BFR.

The kinetics of Phase 2 iron oxidation observed for BFR samples bearing various amounts of bound haem were studied by stopped-flow spectroscopy (Figure 3), and the results are shown as the dependence of the final absorbance change as a function of the added iron concentration (Figure 4A). Consistent with previous results obtained for BFR with low variable amounts of haem bound [20,27], we observed inflection points in each case at the approximate concentration of iron required for stoichiometric addition of iron to the 48 binding sites present in each BFR 24-mer. Thus the presence of haem did not change the stoichiometry of iron binding to BFR during Phases 1 and 2. However, the rate of Phase 2 iron oxidation was affected by the amount of haem bound to BFR (Figures 3 and 4B). Increasing amounts of haem bound to the BFR 24-mer decreased the rate of Phase 2 iron oxidation, which agrees with the conclusions of a study that compared the kinetics of iron oxidation by wild-type BFR having all haem-binding sites occupied to the M52L and M52H variants of BFR that are unable to bind haem [12].

Iron core formation kinetics as a function of BFR haem content

The effect of bound haem on Phase 3 of the BFR ferroxidase reaction was studied by monitoring the absorption change that resulted following four sequential additions of iron. In this way, any change in kinetics that accompanies the growth in size of the iron core could also be evaluated. With the first addition, the
kinetic burst in iron oxidation that is characteristic of Phase 2 caused an initial increase in absorbance, as iron quickly bound to and oxidized at the dinuclear iron site (Figures 5 and 6A). Iron added after assembly of the dinuclear iron sites binds to and oxidizes at nucleation sites or contributes to iron core growth at an already nucleated surface [18,28]. BFR with the greatest haem content displayed an apparent sigmoidal profile of iron core growth following the initial addition of iron (Figure 6A), as well as lower rates of nucleation and iron core formation (Figure 6B). For the second, third and fourth additions of iron to BFR, conditions under which a nascent iron core exists, BFR (Figure 6B). For the second, third and fourth additions of iron to BFR, conditions under which a nascent iron core exists, BFR samples with greater haem content exhibited higher rates of iron core formation (Figure 6B). Comparison of rates of iron oxidation observed following the final addition of iron to BFR samples possessing 11 % and 86 % haem bound reveals more than a two-fold increase in rate for the sample with greater haem content.

To gain insight into the mechanism by which iron core formation is enhanced by haem, the kinetics of Phase 3 of the ferroxidase activity of BFR samples reconstituted with various amounts of Co-protoporphyrin IX were assessed. The binding of Co-haem could be verified by the observation that the Soret band shifts from 418 nm to 429 nm as this haem derivative binds to BFR (Figure 6A). In contrast with the effect of Fe-haem binding to BFR on the kinetics of iron core formation, Co-haem has no influence on the Phase 3 kinetics of BFR (Figures 6B and 6C). This result provides evidence that the central metal of the haem bound to BFR plays a key role in the mechanism by which haem facilitates iron core formation.

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which exogenous haem could enter this binding site, consistent with the observation that addition of haem to the BFR 24-mer that is deficient in bound haem affords little or no reconstitution with haem (Figure 2A). On the other hand, the variant that can form only dimers, and for which exogenous haem should have relatively unrestricted access to the crystallographically defined haem-binding site, does readily bind exogenous haem [28,32]. The mechanism by which the reconstitution method that we have developed in the present study occurs has not been investigated, but a few possibilities merit consideration. For example, as the temperature is increased, the 24-mer could partially dissociate to smaller assemblies, thereby allowing haem entry to the haem-binding site. In fact, just an increase in the rate constant for dissociation of subunits to permit transient access of haem to the central cavity without formation of an appreciable fraction of smaller oligomers could be sufficient to permit haem binding. At low or moderate ionic strength, heating in this manner leads to denaturation of the dissociating protein, but high ionic strength stabilizes the smaller assemblies, regardless of their lifetimes, to permit restoration of the native structure upon cooling.

An alternative mechanism that does not require dissociation of the 24-mer involves structural changes of BFR that would allow haem to move through newly created openings in the protein shell. One region in which such an opening might be created is through the two coils, one from each paired subunit, that are located above the haem-binding site. These coils normally block access of haem to the haem-binding site, but it is possible that, at higher temperatures, the coils located above the haem-binding site split apart and present a route for haem to the haem-binding site. Normally, these two coils interact through hydrogen bonds involving Gln72 of one subunit with the backbone carbonyl of Gly75 and the backbone amine of Leu77 from the other subunit. A hydrogen bond also exists between Asn23 and the backbone carbonyl of Leu77 from a paired subunit. In future studies, it would be interesting to test whether substitution of Gln72 and/or Asn23 would allow the coils to adopt a conformation that permits haem binding at ambient temperature.

**Mechanism of haem enhancement of iron core formation**

Although haem has been shown to play a role in iron release, the effects of haem on iron core formation are unclear. Andrews et al. [12] observed that Phase 2 iron oxidation for the haem-free M52L and M52H variants of BFR is faster than that exhibited by wild-type BFR with haem bound. In addition, Phase 3 was reported to be slightly slower and faster in the haem-free M52L and M52H respectively, than for wild-type BFR with haem bound [12]. It is possible that these observations could result from structural perturbations caused by the substitution of Met52 with leucine or histidine rather than from the absence of haem. The side chain of Glu51, which is adjacent to Met52, participates as one of the bridging ligands in the dinuclear iron site. Consequently, a change in the position of Glu51 resulting from substitution of Met52 might have consequences for the kinetics of BFR ferroxidase activity [12]. Such effects were observed for the W35F/W133F variant of *E. coli* BFR, where the reduced rate of Phase 2 presumably resulted from structural consequences of combining two substitutions that individually had no effect [33]. Preparation of wild-type BFR with various amounts of bound haem provides a means of evaluating the kinetic effects of haem on Phases 2 and 3 in the presence of Met52.

At least two possible mechanisms by which haem could accelerate the late stages of iron core formation by BFR can be identified. Specifically, the binding of haem between adjacent monomers with the propionate groups orientated on the inner surface of the central cavity could simply provide additional anionic sites that promote nucleation of the nascent iron core. Alternatively, bound haem could act by facilitating electron transfer between neighbouring dinuclear iron sites or between the dinuclear iron sites and the iron core. The kinetic analysis of BFR 24-mer reconstituted with Co-haem provides a means of evaluating this possibility because Co-haem has a much lower
reduction potential than does Fe-haem [34,35], a difference that should have consequences for the kinetics of BFR ferroxidase activity if bound haem participates in this reaction by means of electron transfer. Assuming that Co-haem and Fe-haem bind to BFR similarly, as they do in the cases of myoglobin and haemoglobin [36], and cytochrome c [37], the failure of Co-haem to modify the kinetics of Phase 3 provides strong evidence that Fe-haem bound to BFR participates in some electron-transfer process as a component of BFR ferroxidase activity (Figures 5B and 6B). Notably, an engineered BFR constructed as a model for a component of Photosystem II has previously provided evidence that electron transfer between the zinc-chlorin e6 bound to the haem-binding site and the dinuclear site reconstituted with 2 mol of manganese is possible [38]. Nevertheless, there is currently no evidence of redox cycling for the haem iron during iron core formation.

**Role of haem in BFR function**

Proposed functional roles for haem bound to various species of BFR have generally emphasized a role in facilitating iron release from the iron core. For example, reduction of the haem groups and the iron core in *P. aeruginosa* BFR B is promoted by the presence of the apo-form of BFD (BFR-associated ferredoxin) [31]. On the other hand, the release of iron from *Desulfovibrio desulfuricans* BFR may involve RD2 (rubredoxin-2), which possesses an [Fe–(SCys)4] centre ([37], the failure of Co-haem and Fe-haem bind to BFR similarly, as they do in the cases of myoglobin and haemoglobin [36], and cytochrome c [37], the failure of Co-haem to modify the kinetics of Phase 3 provides strong evidence that Fe-haem bound to BFR participates in some electron-transfer process as a component of BFR ferroxidase activity (Figures 5B and 6B). Notably, an engineered BFR constructed as a model for a component of Photosystem II has previously provided evidence that electron transfer between the zinc-chlorin e6 bound to the haem-binding site and the dinuclear site reconstituted with 2 mol of manganese is possible [38]. Nevertheless, there is currently no evidence of redox cycling for the haem iron during iron core formation.

The dinuclear ferroxidase centre of *E. coli* BFR functions as a catalytic centre during core formation, cycling between di-Fe(II) and bridged di-Fe(III) forms [6,18]. Reduction of the centre is proposed to occur through the transfer of electrons from Fe(III) in the protein cavity and an iron site on the inner surface of the protein, co-ordinated by His36 and Asp90, that is important for electron transfer to the ferroxidase centre has been identified [18]. Loss of this site did not completely abolish core formation, indicating that there may be other routes through which electrons can be transferred from the cavity to the ferroxidase centre and that the haem groups may provide one such route.

Formation of the iron core in BFR may change the environment and properties of the bound haem group. The reduction potential of haem in *Azotobacter vinelandii* BFR changed from −225 mV to −475 mV in the absence and presence of an iron core respectively [44,45]. The iron core itself has a reduction potential of −420 mV, which suggests that the haem groups could reduce the iron core [44]. The ability to reconstitute *E. coli* BFR with various amounts of haem or with metal-substituted haem derivatives reported in the present study, combined with the evidence for an electron-transfer-based role for haem in promoting iron core formation, provides further evidence for a functional role for haem in BFR formation and should enable further studies to better define the chemical and mechanistic nature of haem involvement.

**AUTHOR CONTRIBUTION**

Steve Wong, Nick Le Brun and Grant Mauk designed the research. Steve Wong and Raz Abdullagir performed the research and analysed the results. Steve Wong, Nick Le Brun, Geoffrey Moore and Grant Mauk discussed the results and wrote the paper.

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