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An EPR investigation of non-haem iron sites in *Escherichia coli* bacterioferritin and their interaction with phosphate

A study using nitric oxide as a spin probe

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EPR studies of bacterioferritin (BFR), an iron-storage protein of *Escherichia coli* [1993, Biochem. J. 292, 47–56.], have revealed the presence of non-haem iron (III) (NHI) sites within the protein coat which may be involved in iron uptake and release. When nitric oxide was used as an EPR spin probe of the Fe(II) state of the NHI sites, two distinct mononuclear NHI species were found. Under certain conditions, an iron dimer was also observed. The reaction of phosphate with NHI species has been investigated Results point to a function for this anion in core nucleation.

Bacterioferritin (BFR); NHI site; Nitric oxide; Phosphate

1. INTRODUCTION

Bacterioferritin (BFR) is an iron-storage protein found in the bacterium *Escherichia coli*. The protein consists of 24 identical polypeptide chains, each of M_r 18,500, packed to form a highly symmetrical, approximately spherical protein shell surrounding a central 8 nm cavity, in which large amounts of iron can be stored. The *bfr* gene of *E. coli* has recently been cloned, sequenced and overexpressed to give 15% of cell protein as BFR [2,3]. Secondary structure prediction and molecular modelling [1,2] point to a close structural similarity between BFR and ferritin, the eukaryotic iron-storage protein [4,5]. Previous studies of BFR by absorbance, EPR, and MCD spectroscopies revealed the presence of three different types of metal centres: protohaem groups liganded by two methionine residues; an iron core; and some monomeric non-haem iron (III) (NHI) sites [1,6]. Mammalian ferritins are composed of two subunit types, known as H and L. The first step in iron storage is the uptake of Fe(II) and its oxidation to Fe(III) by dioxygen [7,8]. This 'ferroxidase' reaction is catalysed by H-chains [9] and is proposed to take place within the protein envelope at a site in the crystal structure which may be occupied by Tb(III) or Ca(II), which is thought to be capable of binding iron [5]. The effects of site-directed replacement of residues at this site supports this proposal [10,11]. A speculative model in which two adjacent Fe (III) ions are bound at a conserved site, has been proposed [11,12].

E. coli BFR also exhibits ferroxidase activity [13] and modelling studies have identified at least two potential sites for the binding of non-haem iron [1]. One is analogous to the ferroxidase centre of mammalian ferritin. Recent sequence alignments [14,15] show that five of the seven ligands of this centre are conserved in BFR. The other potential sites are located in the eight hydrophilic threefold channels in the protein shell, through which iron may enter and exit the central cavity [4,16].

It is also possible that the iron binding sites exist on the inside or outside of the protein shell. For example, carboxy residues are thought to be involved in iron core nucleation [17,18]. However, since the BFR samples used in this study possessed a small iron core, it is likely that any iron present in the cavity would be part of the core itself and not as isolated mononuclear species on the cavity surface. Furthermore, mononuclear iron is unlikely to be located on the outer surface of the protein, since such sites rarely provide a well defined symmetry environment of the type observed [1].

Nitric oxide has been employed as a spin probe in EPR studies of iron (and copper) centres in proteins. It is a neutral, paramagnetic molecule which readily forms complexes with high spin Fe(II) in non-haem iron proteins, to give either $S = {}^3/_2$ or $S = {}^1/_2$ species. Here, evidence is presented for a reaction between NO and the

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reduced non-haem iron centres of BFR, giving nitrosyl EPR signals, characteristic of both monomeric and dimeric iron sites.

The effect of phosphate on the non-haem iron signals of BFR was also investigated in view of the reports that phosphate plays a significant role in the formation of the BFR iron core [19], and significantly influences the morphology of the ferritin core [20,21].

2. MATERIALS AND METHODS

2.1. *Preparation of* E. coli *BFR*

BFR was isolated from the over-producing strain, *E. coli* JM101 (pGS281), as described previously [22]. Three different preparations were used.

2.2. *EPR measurements*

EPR spectra were measured with an X-band spectrometer (Bruker ER200D with an ESP 1600 computer system) fitted with a liquid helium flow cryostat (Oxford Instruments plc, ESR9). Concentrations of NHI-NO complexes were estimated, in the case of the NHI-NO dimer, by double integration of the EPR spectrum and comparison with the integrated EPR spectrum of a known concentration of an aqueous copper (II) EDTA complex [23]; and for the monomeric NHI-NO complex, by direct comparison with the spectrum of a nitric oxide complex of iron (II) EDTA

2.3. *Reduction of the NHI site*

Selective reduction of the NHI site was achieved by the anaerobic addition of 5 μl of 2.62 M solution of sodium ascorbate to 300 μl BFR in 50 mM HEPES, pH 7 0 (approximately 3 mg/ml), with stirring for 15 min.

2.4. *Addition of gaseous nitric oxide*

Nitric oxide (NO) gas was added to solutions of protein under anaerobic conditions to avoid side reactions between NO and O_2. A glass vessel was filled from a NO gas cylinder (Aldrich) via a vacuum line pumped and flushed with dry argon gas. The vessel was stored in a high integrity anaerobic glove box (Faircrest Engineering) in which the atmosphere was maintained < 1 ppm O_2. The vessel was injected with 1 ml of 5 M NaOH to remove NO_2. Typically, 500 μl protein solutions were placed in the sample vessel and stirred anaerobically for at least 1 h. The NO and sample vessels were coupled together for approximately 20 min, with continuous stirring of the sample solution.

2.5. *Buffer exchange*

Exchange of the buffering system was achieved using an ultrafiltration unit (Amicon) fitted with a PM 30 membrane operating at a pressure of 55 psi.

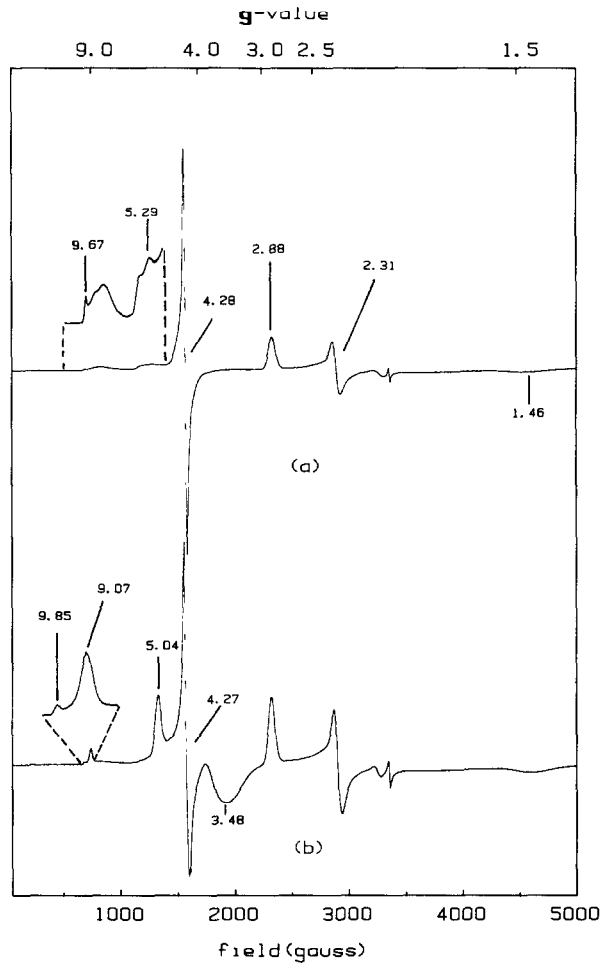


Fig. 1. X-band EPR spectra of *E. coli* BFR. (a) BFR, 400 mM, in 25 mM HEPES in D_2O at pD 7.8. Conditions: Modulation amplitude 10 G, frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW; Temperature 10K Low field region is shown in greater detail. Conditions: Modulation amplitude 10 G, frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW: Temperature 4 K. (b) BFR, 200 μM, in a mixture (1:1, v/v) of 25 mM HEPES and glycerol. Conditions. Modulation amplitude 10 G, frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW; Temperature 10K. Low field region is shown in more detail. Conditions: Modulation amplitude 5 G, at a frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW; Temperature 10K.

3. RESULTS AND DISCUSSION

3.1. *EPR spectroscopy of native BFR*

EPR spectra of native BFR have been reported previously [1]. The X-band EPR spectrum of BFR at 10K, Fig. 1a, contains, in addition to the resonances at $g = 2.88$, 2.31, and 1.46, from the $S = {}^1/_2$ low spin bis-methionine ligated haem groups of the protein [6], features at $g = 4.3$ and low field arising from high spin $S = {}^5/_2$ Fe(III). The addition of glycerol (50% v/v) as a cryogenic glassing agent for low temperature optical studies, causes a 'splitting' of the $g = 4.3$ signal into a trio of signals at $g = 5.04$, 4.27, and 3.48, Fig. 1b. Measurement of the low field spectrum shows a sharpening of the spectral features to reveal two resonances at $g = 9.85$ and $g = 9.07$.

Under the action of low symmetry fields generated by the ligand environment, the ground state of $S = {}^5/_2$ high spin Fe(III) is split into three pairs of Kramers doublets separated in energy by the zero field splitting term. The remaining degeneracy is lifted by the applied field and can give rise to three sets of g-values. A total of nine g-values could, in principle, be observed in the EPR spectrum of monomeric Fe(III). In practice, some transitions lie at very high field and are too broad to detect. The signals of Fig. 1 have been interpreted previously [1], as arising from magnetically isolated high spin non-

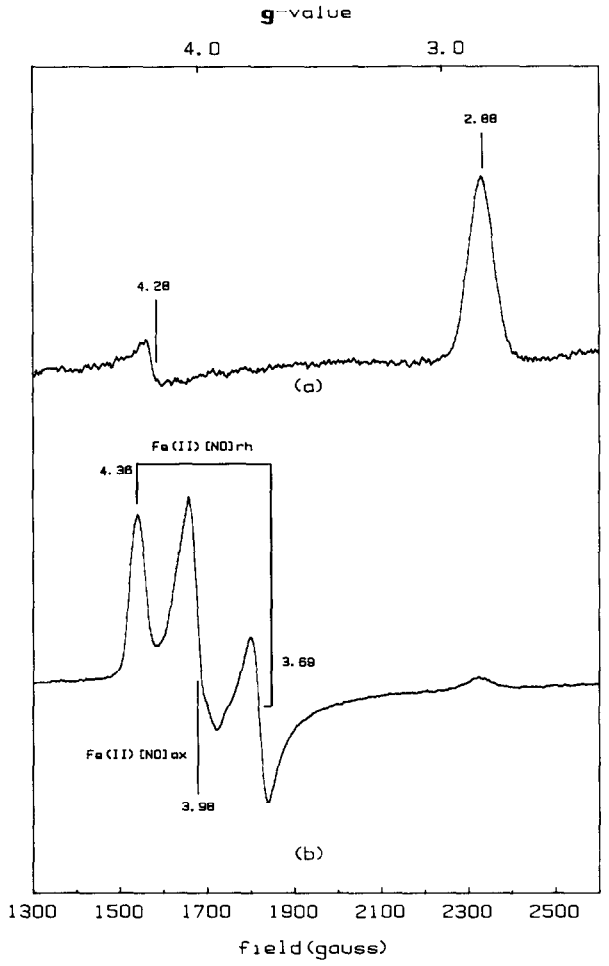


Fig. 2. X-band EPR spectra of (a) ascorbate reduced BFR and (b) the nitric oxide complex of ascorbate reduced BFR. BFR, 6 μM, in 50 mM HEPES pH 7. Conditions: Modulation amplitude 10 G, frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW; Temperature 5K.

haem Fe(III) sites (with some structural variation) which, upon the addition of glycerol, are converted to a single species. More recent studies (Le Brun et al., unpublished results) indicate that there may be more than one Fe(III) species present.

In the EPR spectrum of ascorbate reduced BFR (Fig. 2a) the signal intensity at $g = 4.3$ is reduced to a level which can be assigned to adventitious iron (III), whereas the Fe(III) haem EPR signal intensity is unaffected. Reoxidation could be achieved by aerobic stirring of the sample over several hours.

3.2. *EPR spectroscopy of the reduced BFR-NO adduct*

The 5K EPR spectrum of the NO-BFR adduct produced by adding NO gas to ascorbate-reduced BFR can be interpreted in terms of two distinct $S = {}^3/_2$ species, each derived from antiferromagnetic coupling between NO and iron (Fig. 2b). Each species can be assigned as [Fe(III)NO^-] ($S = {}^3/_2$), though a molecular orbital description may be more appropriate (J.A. Farrar, R. Grinter, D.L. Pountney and A.J. Thompson, unpublished results).

Resonances at $g = 4.36$ and $g = 3.69$ arise from a slightly rhombic [Fe(III)NO^-] species, given the short hand notation Fe(II)$[NO]_{rh}$. The signal at $g = 3.98$ originates from a more axial [Fe(III)NO^-] species, termed Fe(II)$[NO]_{ax}$. Both species also give rise to a feature at about $g = 2$ (not shown). Estimates of the $S = {}^3/_2$ species by spin integration indicate a ratio of total [Fe(III)NO^-] to BFR subunit of approximately 1:1.

Several examples of mononuclear non-haem ferrous nitrosyls are known to give the characteristic axial or near axial $S = {}^3/_2$ type spectrum with g-values at 4 and 2 (see [25] and references therein). Thus the iron is high spin, coordinated by weak field oxygen and/or nitrogen based ligands most likely in a pseudo-octahedral arrangement.

3.3. *EPR spectroscopy of the native BFR-NO adduct*

In the EPR spectrum of the native BFR-NO adduct (not shown) signals at g-values corresponding with those of the two [Fe(III)NO^-] species were observed, with an associated intensity of at least an order of magnitude less than those of the NO complex of ascorbate-reduced BFR, Fig. 2b. It is concluded that little of the non-haem iron of isolated BFR is present as Fe(II). Also, the $g = 4.3$ signal intensity is unaffected, indicating that NO does not bind to the ferric form of the NHI.

3.4. *The effect of phosphate*

The EPR spectrum (not shown) of BFR stirred anaerobically in sodium phosphate buffer for 48 h, was identical to that of ascorbate-reduced BFR (Fig. 2a). The EPR spectrum of its NO adduct (not shown) was also indistinguishable from that of the ascorbate-reduced BFR-NO adduct (Fig. 2b). These observations indicate that ferric NHI has been reduced and NO has been bound giving both of the Fe(II)$[NO]_{ax}$ and Fe(II)$[NO]_{rh}$ species described earlier, without adding an external reductant. The reduction was not reversible upon aerobic stirring, although it did occur slowly after the addition of glycerol.

Repeated aerobic dialysis of BFR against sodium phosphate led to the loss of the monomeric non-haem iron signals from the EPR spectrum (not shown). This may be a consequence either of removal of non-haem iron, or the formation of a spin-coupled EPR silent state. Addition of NO gas to the sample after anaerobic stirring in phosphate for 1 h gave an EPR spectrum lacking signals assigned to the Fe(II)[NO] species (spectrum not shown). However, anaerobic stirring of a similar sample for 48 h followed by the addition of NO gas yielded an EPR spectrum containing signals associated with both $S = {}^3/_2$ Fe(II)[NO] species (Fig. 3a). The intensity of the Fe(II)$[NO]_{ax}$ signal is greater, relative to that of Fe(II)$[NO]_{rh}$, than previously observed. These obser-

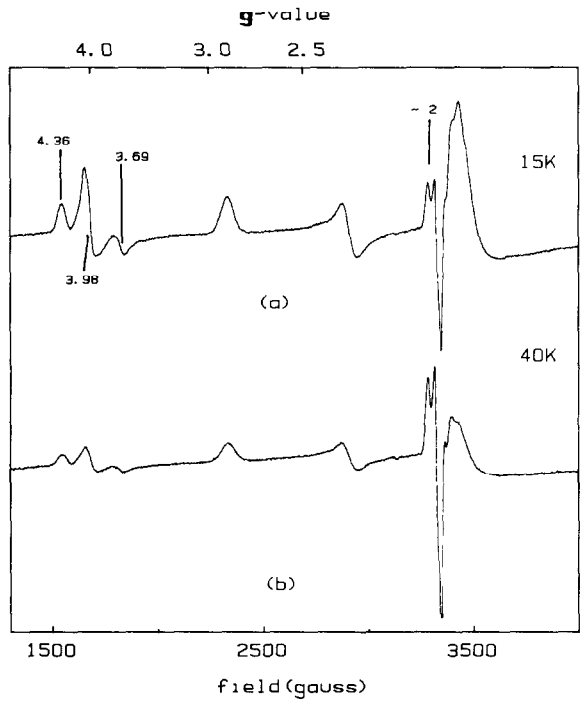


Fig. 3. X-band EPR spectrum of the nitric oxide complex of BFR. The sample was repeatedly dialysed in air against 50 mM sodium phosphate pH 7 and subsequently stirred anaerobically in phosphate for 48 h. (a) BFR, 6 μM, in 50 mM sodium phosphate pH 7. Conditions as in Fig. 2, temperature 15K. The feature below $g = 2$ arises from the presence of excess nitric oxide in solution. (b) Sample and conditions as in (a), except for temperature 40K.

vations suggest that repeated aerobic dialysis of BFR leads to removal of non-haem iron, and that anaerobic stirring of NHI-free BFR, in phosphate, results in the gradual reoccupancy of the non haem iron sites, the 'axial' site being slightly favoured.

The spectrum in Fig. 3a contains another interesting feature in the $g = 2$ region. Persistence of the signal at high temperature is apparent from the 40K EPR spectrum shown in Fig. 3b. The form of the signal is more clearly seen in Fig. 4. The signals at $g = 2.05$, 2.02, and 2.01 can be assigned to a $S = {}^1/_2$ species displaying slightly rhombic g-value anisotropy. Double integration of the rhombic signal versus a Cu(II)EDTA standard, according to the method of Aasa and Vänngård [23], reveals that this is a minority species, present in a concentration equal to about half that of the assembled protein.

It is proposed that the $S = {}^1/_2$ species is due to the binding of NO to an iron dimer. Both [Fe(III)Fe(III)] and [Fe(II)Fe(II)] could reasonably give rise to an exchange coupled species of $S = 0$ and both would be expected to give rise to an $S = {}^1/_2$ species upon binding of NO. Since this species is observed after anaerobic treatment, it is thought more likely that a NO[Fe(II)Fe(II)] species is present.

A similar $S = {}^1/_2$ species has been observed after the addition of NO to the binuclear iron centre of the deoxyhemerythrin of *Phascolopsis gouldii* [26]. The EPR absorption arising from the formally assigned [Fe(II)Fe(III)NO^-] species is slightly more anisotropic than that observed in this instance. However, synthetic models in which this type of coupling is observed have not been reported, and hence, g-value differences are difficult to interpret.

The observation by EPR of an iron dimer during the early stages of core formation in ferritin has been reported [27,28]. However, the dimer in this case is assigned to a mixed valence Fe(III)-Fe(II) state, which gives an EPR spectrum without addition of a spin label. The g-values, all below 2, are indicative of a μ-oxo-bridged dimer. Iron (III) μ-oxo-bridged dimers have been observed by Mössbauer [11,29] and UV-difference [12] spectroscopies, as early species formed after the oxidation of Fe(II) added to both horse spleen and recombinant human H-chain apoferritin.

A summary of the observations presented here is shown in Scheme 1.

4. CONCLUSIONS

(1) Two mononuclear non-haem iron sites have been detected by EPR spectroscopy in *E. coli* BFR, both in the Fe(III) form, and, in the Fe(II) form, as iron-nitrosyl of spin $S = {}^3/_2$.

(2) The EPR g-values identify at least two sites of differing symmetry, both for the Fe(III) and Fe(II)-NO forms. If these two sites are close together they can only be observed by EPR when one or other but not both of

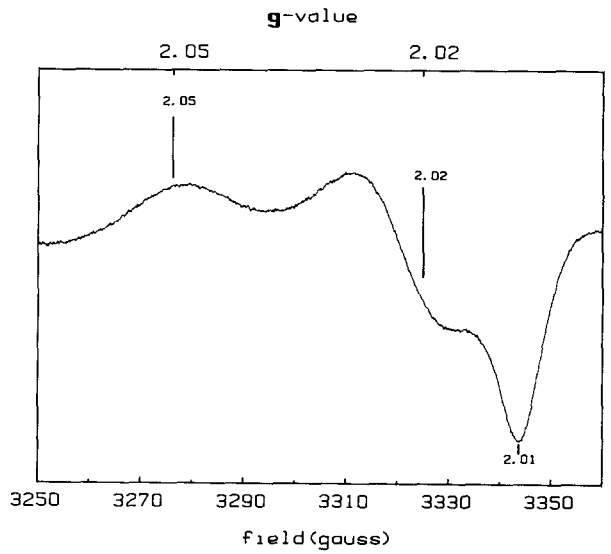
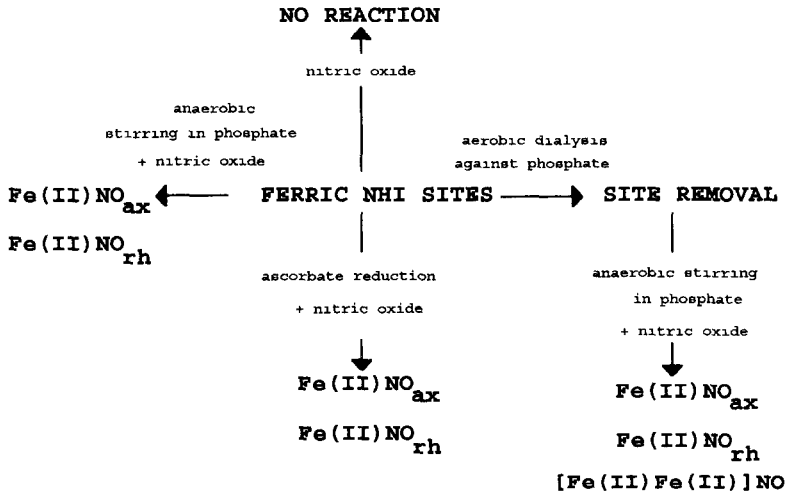


Fig. 4. X-band EPR spectrum of the nitric oxide complex of BFR. Sample was repeatedly dialysed in air against 50 mM sodium phosphate pH 7 and subsequently stirred anaerobically in phosphate for 48 h. BFR, 6 μM, in 50 mM sodium phosphate pH 7. Conditions: Modulation amplitude 5 G, frequency 100 kHz; Microwave frequency 9.39 GHz; Power 2.01 mW, Temperature 40K.



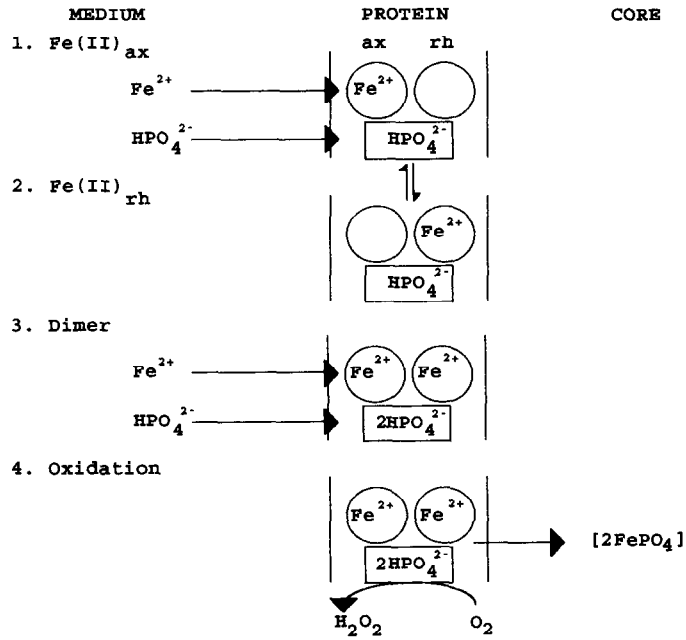
Scheme 1. A summary of species observed employing nitric oxide as a spin probe.

the sites is occupied. A minor trace of a novel nitrosyl signal indicative of the presence of a dimer [Fe(II)Fe(II)NO] has also been detected. This could arise from simultaneous occupancy of the two neighbouring sites.

(3) The hydroxylic solvent glycerol affects the structure of the site. Phosphate does not appear to bind directly to iron in either site, since the *g*-values of EPR signals of the Fe(III) form and the Fe(II)NO are unaffected by the presence of phosphate. However, it is possible that phosphate could remain bound to iron in the sites, irrespective of the buffering system.

(4) Dialysis against phosphate, in the presence of air, leads to the loss of all NHI from the sites. The free NHI presumably forms an insoluble ferric phosphate complex.

(5) In the absence of air, phosphate causes reduction of the Fe(III) site to Fe(II), without its removal from the



Scheme 2. Hypothesis for the loading of iron into BFR. Where 'ax' and 'rh' refer to the respective symmetries of the two Fe(II) sites detected with the spin label NO.

site. On the contrary, anaerobic incubation with phosphate leads to the loading of the Fe(II) sites, both 'axial' and 'rhombic' with iron presumably from the core, and to the formation of an Fe(II) dimer. This suggests that in the absence of air, phosphate promotes the transfer of Fe(II) from the core into the non-haem iron sites in the protein. The presence of air is required to drive the deposition of iron (III) into the core.

(6) The presence of non-haem iron sites within the protein envelope is confirmed, but their precise location remains unknown.

The results presented here lead to a hypothesis for the loading of iron into BFR, Scheme 2.

The formation of hydrogen peroxide has been shown to be associated with ferritin iron oxidation at low iron loading [30], and it is proposed that it is formed here. BFR cores are known to be associated with high levels of phosphate (1 P_i per 1–2 Fe atoms) compared with mammalian ferritins (see [31] and references therein) and it is suggested here that in BFR phosphate plays a role in shepherding iron from the oxidation centre into the cavity. Once iron core nuclei have built up they may provide alternative sites for iron (II) oxidation, as has been suggested for horse spleen ferritin [7].

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