Interaction of nitric oxide with non-haem iron sites of *Escherichia coli* bacterioferritin: reduction of nitric oxide to nitrous oxide and oxidation of iron(II) to iron(III)

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The bacterioferritin (BFR) of *Escherichia coli* consists of 24 identical subunits, each containing a dinuclear metal-binding site consisting of two histidines and four carboxylic acid residues. Earlier studies showed that the characterization of iron binding to BFR could be aided by EPR analysis of iron–nitrosyl species resulting from the addition of NO to the protein [Le Brun, Cheesman, Andrews, Harrison, Guest, Moore and Thomson (1993) FEBS Lett. **323**, 261–266]. We now report data from gas chromatographic head space analysis combined with EPR spectroscopy to show that NO is not an inert probe: iron(II)–BFR catalyses the reduction of NO to N_2O, resulting in oxidation of iron(II) at the dinuclear centre and the subsequent detection of mononuclear iron(III). In the presence of excess reductant (sodium ascorbate), iron(II)–BFR also catalyses the reduction of NO to N_2O, giving rise to three mononuclear iron–nitrosyl species which are detectable by EPR. One of these, a dinitrosyl–iron complex of $S = \frac{1}{2}$, present at a maximum of one per subunit, is shown by EPR studies of site-directed variants of BFR not to be located at the dinuclear centre. This is consistent with a proposal that the diferric form of the centre is unstable and breaks down to form mononuclear iron species.

INTRODUCTION

Bacterioferritin (BFR) from *Escherichia coli* consists of 24 identical subunits (18.5 kDa each) packed together to form a roughly spherical molecule with a hollow interior. The protein as isolated contains large amounts of iron in the form of a ferric-oxy-hydroxide-phosphate mineral, stored in the central cavity [1–3]. Apart from its iron core, *E. coli* BFR contains two other types of metal centre: up to 12 *b*-type haem groups situated between subunits and ligated by two methionines (Met-52, Met-52′), a co-ordination set which is thus far unique [4,5], and 24 dinuclear metal ion centres located at identical intrasubunit sites [5–8].

Sequence-comparison studies together with a three-dimensional structure of the dimanganese(II) form of the dinuclear centre show each metal ion ligated by one histidine, one terminal glutamate and two bridging glutamates, and that it is similar to the dinuclear iron centres of the R2 protein of ribonucleotide reductase (R2 RNR), the hydroxylase subunit of methane monooxygenase (MMOH) and the dimanganese centre of arginase [8,9]. Studies of iron uptake into BFR show that two iron(II) ions are bound per subunit and, in the presence of oxygen, are rapidly oxidized to iron(III). Subsequent studies of BFR site-directed variants indicated that residues serving as ligands to the dinuclear metal centre are essential for the catalysis of rapid iron oxidation (termed the ferroxidase activity), thus identifying the dinuclear centre as the ferroxidase centre of BFR [7,8]. Binding of other transition metal ions, including cobalt(II) and zinc(II), to BFR has also been studied by magnetic, optical and potentiometric methods [10,11]. These all indicate that metal ion binding occurs at the dinuclear centre of BFR. Additional metal-ion-binding sites have been detected in the protein, but with significantly lower affinities [11]. Thus, although there is a lack of direct structural evidence for an iron-containing form of the dinuclear centre in BFR, these studies have led to the classification of BFR as a dinuclear iron protein [2,8,12].

NO has often been used as a spin probe for transition metal centres in proteins because it is paramagnetic and readily forms EPR-active metal–nitrosyl species. Indeed, we have previously reported studies of BFR employing NO as a spin probe in which a number of iron–nitrosyl species were detected by EPR spectroscopy [13]. Recently, the interaction of NO with R2 RNR was reported showing that each iron of the dinuclear centre is able to bind a single NO, giving rise to an EPR-silent complex which is unstable to oxidation of iron(II) to iron(III) and reduction of NO to N_2O [14]. In the light of the increased structural and mechanistic knowledge of non-haem iron centres in BFR, the availability of BFR site-directed variants, and studies of NO reaction with another dinuclear iron protein, we have examined further the interaction of NO with non-haem iron sites in BFR. We report data from EPR spectroscopy and gas chromatography head space (GCHS) analysis that indicate the reduction of NO to N_2O by iron(II) at the dinuclear centre of BFR, resulting in oxidation of iron(II) to iron(III). In the presence of excess reductant, such as sodium ascorbate, the reduction of NO to N_2O becomes catalytic. After exhaustion of one component, stable iron(II)–nitrosyl species are formed. The implications of these findings for the mechanism of iron uptake by BFR and the use of NO as a spin probe are discussed.

MATERIALS AND METHODS

BFR purification and iron, protein and haem assays

BFR and BFR variants were overexpressed and purified as previously described [8]. Iron was assayed by the method of

Abbreviations used: BFR, bacterioferritin; R2 RNR, R2 subunit of ribonucleotide reductase; MMOH, hydroxylase subunit of methane monooxygenase; DEAN, diethylamine nonoate; DNIC, dinitrosyl–iron complex; GCHS, gas chromatography head space; PMS, phenazine methosulphate.

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Drysdale and Munro [15] with 1 % ferrozine in place of 0.5 % 2,2′-bipyridyl. Protein concentrations were measured using the bicinchoninic acid method [16], employing BSA as a standard. Haem was determined using the pyridine haemochromogen method of Falk [17]. Non-haem iron was removed from BFR and BFR variants by reduction with sodium dithionite, as previously described [18].

Additions of ascorbate, iron(II) and NO to apo-BFR, apo-BFR variants and buffer solutions

Ascorbate was added to samples by the addition of microlitre quantities of freshly prepared 2.62 M sodium ascorbate solutions. Solutions of ferrous ammonium sulphate were freshly prepared before each experiment by dissolving weighed amounts of the salt in AnalaR-grade water, previously deoxygenated by bubbling with nitrogen gas for at least 1 h. Additions of the iron(II) solution to protein or buffer solutions were made using a microlitre syringe (Hamilton). Additions of NO to solutions of protein, iron(II) or ascorbate under anaerobic conditions (to avoid side reactions between NO and O_2) were made by one of two methods: (a) addition of NO gas, as previously described [13] or (b) addition by microlitre syringe (Hamilton) of a solution of the NO-releasing reagent diethylamine nonoate (DEAN) (Cayman Chemical Co.). Solutions of DEAN of known concentration ($\epsilon_{250\,nm} = 6500\ M^{-1}\cdot cm^{-1}$) were freshly prepared in 0.01 M KOH before each experiment [19]. Microlitre additions of DEAN to solutions at neutral pH initiate the decomposition of the nonoate, yielding NO and the secondary amine. The average number of NO molecules released per DEAN molecule at pH 6.5 was found to be 1.45 ± 0.05, as determined by the anaerobic titration of excess reduced myoglobin in 0.1 M Mes, pH 6.5, with microlitre aliquots of a DEAN solution of known concentration. From measurements of changes in the optical spectrum caused by complexation of the released NO with the reduced haem of myoglobin, the concentration of the myoglobin–NO complex was calculated. Owing to the extremely high affinity of NO for reduced haem, this was taken to represent the concentration of NO released by DEAN.

The sequence of additions was ascorbate followed by iron(II) and finally NO or NO-releasing agent. Addition and mixing of each component took less than 5 min. Control experiments in which the order of addition was varied showed that NO reduction was not affected by adding iron before ascorbate, but that NO reduction was decreased if NO was added first. This is due, at least in part, to the relatively low solubility of NO in solution.

Gas chromatography

A gas chromatograph (610 series, ATI-Unicam) equipped with a ^{63}Ni electron-capture detector heated to 350 °C was operated in the constant-current pulse-modulated mode. The chromatographic analysis column was a stainless-steel tube (2 m × 0.3175 cm outer diameter) packed with Haye Sep N 80/100 mesh and operated at 50 °C. Nitrogen (99.9 % pure; BOC) was employed as the carrier gas, maintained at a flow rate of 35 ml/min. Data analysis was achieved using a Unicam 4880 data-handling system installed on an interfaced PC.

Standard solutions of N_2O employed for the calibration of the head space analysis system were prepared in 8 ml Wheaton vials under a nitrogen atmosphere provided by a glove box (Faircrest Engineering) in which the atmosphere was always maintained at less than 1 p.p.m. O_2. Vials containing water or 0.1 M Mes buffer, pH 6.5, were sealed with cut-out screw caps fitted with Teflon-faced styrene butadiene rubber septa (Aldrich). The water or buffer solution was then made up to a volume of 1 ml by injection through the septum of microlitre volumes of a saturated solution of NO, in either water or 0.1 M Mes buffer, pH 6.5. Saturated solutions of NO were prepared by bubbling NO gas (Aldrich), via a trap, through a solution of water or 0.1 M Mes buffer, pH 6.5, maintained at constant temperature in a water bath. The concentration of N_2O in saturated water solutions was determined by reference to data tables [20]. The concentration of N_2O in saturated buffer solutions was determined by directly comparing GCHS analysis calibration curves for water and buffer solutions prepared under the same conditions.

Samples for GCHS analysis (1 ml volume) were prepared under nitrogen in 8 ml Wheaton vials fitted with cut-out screw caps and rubber septa. Variable additions of NO in the form of a standard solution of DEAN were made by microlitre injection. Analysis was achieved by injecting head space gas through the injection port septum using a gas-tight microlitre syringe, fitted with an anti-block needle (point-style 5; Hamilton). After the addition of NO, samples were left for at least 2 h before N_2O measurements were made. A series of three measurements was made on each sample with at least 30 min elapsing between measurements. The variation over the three measurements for each of the iron(II)-containing solutions was less than 5 %, except for the iron(II)-free solutions containing ascorbate, in which there was a gradual increase in N_2O with time (see the Results section).

GCHS analysis of a standard solution of N_2O in 0.1 M Mes buffer, pH 6.5, resulted in a chromatogram containing a single peak with a retention time of approx. 5 min due to NO. Similar analyses of a set of standard N_2O solutions, prepared by diluting a solution of 0.1 M Mes, pH 6.5, previously saturated with N_2O gas, as described above, gave a calibration curve for the instrument.

Spectroscopy

U.V.–visible spectra were recorded using an Hitachi U4001 spectrophotometer interfaced to a 486 PC. EPR spectra were measured with an X-band spectrometer (Bruker ER200D with an EPS 1600 computer system) fitted with an ESR9 liquid-helium flow cryostat (Oxford Instruments). Concentrations of the $S = \frac{1}{2}$ iron–nitrosyl species were estimated 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 [21].

RESULTS

GCHS and EPR analysis of the interaction of NO with iron(II)–BFR

Anaerobic *E. coli* BFR solutions (2.0 μM in 0.1 M Mes, pH 6.5) containing 48 iron(II) ions per protein molecule were prepared, and variable quantities (385–575 μM) of the NO-releasing reagent, DEAN, were added. GCHS analysis (results not shown) indicated the presence in each of a significant quantity of N_2O (approx. 10–15 μM). In analogous experiments with Mes buffer alone, apo-BFR in Mes buffer and iron(II) in Mes buffer, detectable quantities of N_2O were not produced.

These data suggest that iron(II) bound to BFR may serve as a reductant, resulting in the oxidation of iron(II) to iron(III). Assuming that two electrons are required for the production of each N_2O molecule (i.e. $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$), the quantities of N_2O detected correspond to the oxidation of between a quarter and a third of the iron(II) present. Oxidation of iron(II) could not be monitored by UV–visible spectroscopy over the range 300–400 nm, which is generally used to monitor the production of iron(III) in ferritins, because iron(II)–nitrosyl

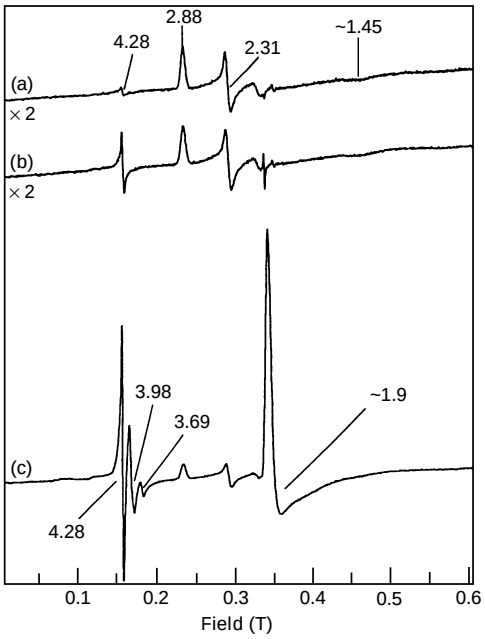

Figure 1 EPR spectroscopic detection of iron(II) oxidation by NO

Spectrum a, 10 K X-band EPR spectrum of *E. coli* wild-type apo-BFR; spectrum b, spectrum remeasured after the anaerobic addition of 48 iron(II) ions per BFR molecule; spectrum c, spectrum re-measured after the addition of excess NO. BFR (6 μM) was in 0.1 M Mes buffer, pH 6.5. Measurement conditions: microwave frequency, 9.39 GHz; microwave power, 2.01 mW; modulation amplitude, 10 G; temperature, 10 K.

complexes have an absorbance in this region [22]. Therefore we employed EPR spectroscopy to investigate whether iron(II) is oxidized by NO.

Figure 1 (spectrum a) shows the X-band EPR spectrum of iron-free (apo) wild-type BFR at 10 K. Signals at $g = 2.88$, $g = 2.31$ and $g \approx 1.45$ arise from the low-spin ferric haem groups of BFR [4], and the low-intensity feature at $g = 4.28$ can be assigned to adventitious iron(III). The EPR spectrum after the anaerobic addition of 48 iron(II) ions per BFR molecule (Figure 1, spectrum b) contains an increased signal at $g = 4.28$, probably due to oxidation of a small amount ($< 5\%$) of iron(II) by residual dixoygen. In addition, a low-intensity radical signal of unknown origin is observed at $g = 2.0$. Addition of NO gas, which was in contact with BFR for 20 min before freezing, resulted in spectrum c of Figure 1. The signal at $g \approx 1.9$ is due to excess NO in solution, and those at $g = 3.98$ and $g = 3.69$ are due to two mononuclear iron–mononitrosyl species [L_xFe(II)NO] $S = \frac{3}{2}$ which have been discussed previously [13] and are considered further below. In addition, the spectrum contains a substantially increased $g = 4.28$ signal, indicating significant oxidation of iron(II) to iron(III) on exposure to NO. Absolute quantification of this signal is uncertain because the zero-field splitting parameters of the iron species giving rise to this signal are not known. Control experiments under similar conditions with iron(II) in solution gave spectra showing that significant amounts of iron(II)–mononitrosyl species are formed under these conditions, but that iron(III) is not formed (not shown).

Taken with the GC data, the simplest interpretation is that in samples of iron(II)–BFR reduction of NO occurs in concert with oxidation of iron(II), i.e.

$$2NO + 2Fe(II) + 2H^+ \rightarrow N_2O + 2Fe(III) + H_2O$$

Table 1 Summary of data on the reduction of NO to N_2O

All the reactions were carried out in 0.1 M Mes, pH 6.5, with an incubation period of 2 h before measurement. Except for the ascorbate-only reaction (see the text) the level of N_2O was constant over the period of measurement.

Reactants	Percentage NO converted into N_2O	Comments
Apo-BFR	~ 0	
Apo-BFR + 48 Fe(II) per BFR	~ 5–10	Corresponds to oxidation of 25–33% of iron in BFR
Apo-BFR + 48 Fe(II) per BFR + ascorbate	63–90	
Apo-BFR + 48 Fe(II) per BFR + NADH/PMS	~ 20	NO reduction proceeds but NADH/PMS is a less efficient reductant than ascorbate
Fe(II)	~ 0	
Fe(II) + ascorbate	94	
Apo-BFR + 48 Fe(II) per BFR + EDTA + ascorbate	~ 10	
Fe(II)–EDTA + ascorbate	~ 10	
Fe(II) + NADH/PMS	~ 40	NO reduction proceeds but NADH/PMS is a less efficient reductant than ascorbate
Ascorbate	40–50	
Buffer	~ 0	

This reaction does not go to completion; the iron(III) generated is detected at $g \approx 4.3$ and the remaining iron(II) stays complexed as mononitrosyl species, two forms of which are detected by EPR, at $g = 3.98$ and $g = 3.69$ respectively.

GCHS and EPR analysis of the interaction of NO with iron(II)–BFR in the presence of additional reductant

Since the conversion of NO into N_2O requires electrons, we investigated the effect of adding ascorbate to BFR solutions. GCHS analysis of anaerobic *E. coli* BFR solutions (0.5 μM in 0.1 M Mes, pH 6.5) containing 26 mM sodium ascorbate, 48 iron(II) ions per protein and variable quantities of DEAN (407–815 μM) resulted in a set of chromatograms containing a single peak due to N_2O. A plot of N_2O peak area as a function of DEAN concentration (not shown) was linear, indicating that NO released by DEAN was reduced in the sample to give N_2O in an apparently quantitative reaction. Calculation of the N_2O concentration present in each BFR indicated that at high concentrations of DEAN, approx. 90% of the NO in the sample vial was converted into N_2O. However, at lower concentrations (i.e. < 300 μM), only 63% of NO was detected as N_2O (Table 1).

In order to determine whether the reaction requires the presence of BFR, experiments were carried out with BFR-free iron(II) solutions. A plot of GCHS N_2O peak area against DEAN concentration (not shown) was similar to that observed for the equivalent BFR samples except that the N_2O peak areas for the BFR-free samples were somewhat higher. Calculation of the N_2O concentrations indicated that $94 \pm 2.5\%$ of released NO was detected as N_2O.

Thus, in the absence of ascorbate, NO reduction was observed only when BFR was present, whereas, in the presence of ascorbate, BFR is not essential for the conversion of NO into N_2O, consistent with an earlier report [23].

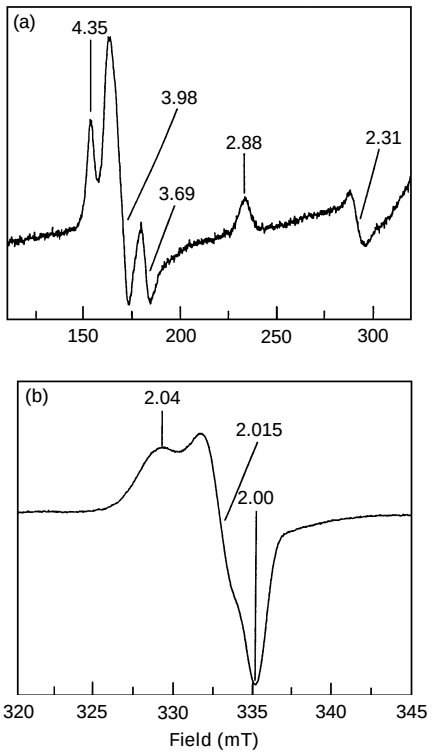

Figure 2 EPR analysis of iron–nitrosyl species formed in wild-type BFR (110–345 mT)

Deoxygenated wild-type BFR (3.6 μM) with excess sodium ascorbate was in 0.1 M Mes, pH 6.5. Then 48 iron(II) ions per BFR molecule were added followed by excess NO. Measurement conditions: microwave frequency, 9.39 GHz; microwave power, 2.01 mW; modulation amplitude, 10 G; temperature, 15 K.

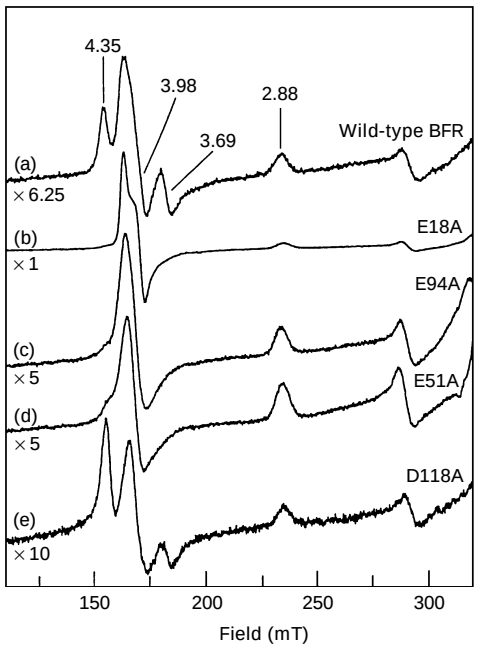

Figure 3 EPR analysis of iron–nitrosyl species formed in wild-type BFR and BFR variants (110–320 mT)

Deoxygenated BFR samples with excess sodium ascorbate were in 0.1 M Mes, pH 6.5. Then 48 iron(II) ions per BFR molecule were added to each sample, followed by excess NO. Spectrum a, wild-type BFR (3.6 μM); spectrum b, E18A BFR (4.7 μM); spectrum c, E94A BFR (5 μM); spectrum d, E51A BFR (5 μM); spectrum e, D118A BFR (5 μM). Measurement conditions were as in Figure 2.

The effect of replacing sodium ascorbate with excess NADH with phenazine methosulphate (PMS) as a mediator (1.3 μM) was investigated. GCHS analysis revealed the presence of N_2O, but at significantly lower concentrations (Table 1). Hence it appears that, although NADH/PMS can function as a reductant, it is less efficient than ascorbate.

In the presence of an additional reductant, GCHS analysis showed few differences between reactions with and without BFR. Thus we investigated, by EPR spectroscopy, whether iron remains associated with BFR in the presence of excess ascorbate.

Excess NO (as a gas or as DEAN) was added to a BFR–ascorbate (or BFR–NADH/PMS) solution containing 48 iron(II) ions per protein. Excess NO could not be detected by EPR, indicating that all free NO had been removed either by turnover to N_2O or by complexation. The 110–320 mT region of the EPR spectrum (Figure 2a) contains signals belonging to two distinct $S = \frac{3}{2}$ species: one with $g = 4.35$ and $g = 3.69$, and the other at $g = 3.98$, as previously reported [13]. The 320–345 mT region of the spectrum (Figure 2b), contains an intense signal at $g \approx 2$ due to a $S = \frac{1}{2}$ species, present at a level of approx. 22 per BFR molecule (i.e. one per subunit [7]) as determined by integration.

An equivalent BFR-free solution gave rise to an EPR spectrum containing low-intensity signals in the $g = 4$ and $g = 2$ regions due to $S = \frac{3}{2}$ iron(II)–mononitrosyl ($g = 3.96$, $g \approx 2$) and $S = \frac{1}{2}$ iron–nitrosyl ($g = 2.03$, $g = 2.01$ and $g = 2.00$) species (not shown). The intensity of the $S = \frac{1}{2}$ species was found to be about 3.5 % of that of the $S = \frac{1}{2}$ species of Figure 2(b), indicating that significant iron–nitrosyl species are not formed under these conditions. Thus, in the presence of ascorbate, iron clearly remains bound to BFR.

In our previous paper [7], the $S = \frac{1}{2}$ signal was tentatively assigned to the dinuclear iron–nitrosyl species $[L_xFe(II)_2NO]$, but recently similar EPR signals have been reported in mammalian ferritins and assigned to mononuclear iron–dinitrosyl species $[L_xFe(I)(NO)_2]$ [24]. The literature contains several examples of $S = \frac{1}{2}$ species, which give EPR spectra similar to that of Figure 2(b) [25–31], and we now concur that the $S = \frac{1}{2}$ species (g values refined to 2.04, 2.015 and 2.00) most probably arises from a mononuclear iron–dinitrosyl species, known as a dinitrosyl–iron complex (DNIC). This species can be formulated with iron in the -1 oxidation state (d^9) bound by two NO^+ ligands, or in the $+1$ oxidation state (d^7) bound by two NO ligands [30].

Role of iron in the catalysis of NO reduction

In order to determine whether the presence of an iron–chelator affects the catalytic reduction of NO to N_2O in either BFR or BFR-free solutions, experiments analogous to those described above were carried out in the presence of 500 μM EDTA. Addition of DEAN (280–830 μM) and subsequent GCHS analysis showed that, in both BFR and BFR-free solutions, approx. 10 % of NO was detectable as N_2O. Hence the addition of EDTA significantly reduced the catalytic activity of the iron(II)–BFR and iron(II) solutions.

Similar GCHS analyses were performed using equivalent solutions of ascorbate alone. N_2O was detected (not shown), but at significantly lower concentrations and, in addition, the reaction

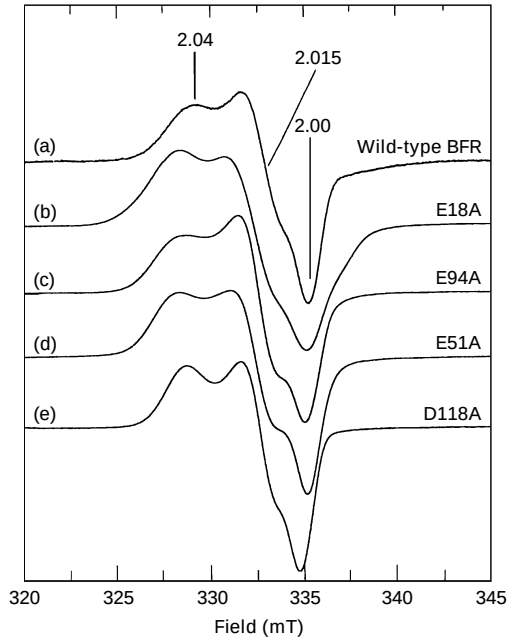

Figure 4 EPR analysis of iron–nitrosyl species formed in wild-type BFR and BFR variants (320–345 mT)

Samples and measurement conditions were as in Figure 3.

was considerably slower (N_2O concentration increased by approx. 10% during the period 2–4 h after addition of DEAN).

Iron–nitrosyl species formed in the presence of EDTA were investigated by EPR spectroscopy. Spectra containing signals at $g = 4.10$, $g = 3.92$ and $g = 1.99$ were observed for both the BFR and BFR-free solutions (results not shown). These g values are consistent with literature values for the $S = \frac{3}{2}$ iron(II)–EDTA–mononitrosyl species [22], indicating that EDTA has a higher affinity than BFR for iron(II) in the presence of NO. An intense signal due to excess NO in solution was also observed.

EPR investigation of the location of iron–nitrosyl species in BFR

The observation of mononuclear iron–nitrosyl species was surprising because previous studies of iron uptake into BFR and of the binding of bivalent metal ions other than iron(II) [5,7,8,10,11] clearly indicated that metal binding occurs at the dinuclear centre. Therefore it was important to establish the location of these species within the protein. EPR studies of the interaction of NO with four site-directed variants of BFR were undertaken. Three of the variants contained substitutions of a single residue at the dinuclear metal centre (Glu-18 → Ala, Glu-94 → Ala and Glu-51 → Ala), and were consequently incapable of catalysing the rapid oxidation of iron(II) to iron(III), or of binding two cobalt(II) ions as wild-type BFR does [8,10]. The fourth variant contained the substitution of a single residue located within the threefold channels of BFR (Asp-118 → Ala), and had the iron-uptake characteristics of wild-type. Addition of 48 iron(II) ions per apo-BFR molecule to each BFR variant sample containing 26 mM sodium ascorbate, followed by excess NO, again either as a gas or as DEAN, yielded the EPR spectra of Figures 3 and 4 (wild-type BFR spectra are included for comparison).

In our earlier work, the two $S = \frac{3}{2}$ species, with $g = 4.35$ and $g = 3.69$, and $g = 3.98$, were assigned to mononuclear iron–nitrosyl species originating from iron bound at partially filled dinuclear centres. This conclusion is supported by the spectra of the variants (Figure 3, spectra b–e). For each of the dinuclear centre variants, signals originating from what appears to be a single species are observed. In the spectrum of Glu-18 → Ala BFR (Figure 3, spectrum b), signals with $g = 4.10$ and $g = 3.94$ are of markedly higher intensity than those of other BFR samples. The reason for this is not clear, but it indicates that some of the iron in wild-type BFR and the other BFR variants may be EPR-silent. In the threefold channel variant, both wild-type $s = \frac{3}{2}$ species are observed. Quantification of the $S = \frac{3}{2}$ species could not be achieved because of the complexity of the spin ground state.

The $S = \frac{1}{2}$ species is present in each spectrum (Figure 4, spectra b–e). The small variations in g values and signal shape observed for the dinuclear centre variants are not substantial enough to indicate that significantly different iron–nitrosyl species are formed and we conclude that the $S = \frac{1}{2}$ iron–nitrosyl species is not located at the dinuclear centre of BFR. Furthermore the similarity between the spectrum of the threefold channel variant, Asp-118 → Ala BFR, and those of the other variants indicates that this centre is not ligated to Asp-118. Thus the data strongly suggest that a previously unrecognized non-haem iron-binding site exists in BFR. Integration showed that, as in wild-type BFR, the $S = \frac{1}{2}$ species was present in each variant at a maximum of 21 ± 2 spins per protein (one per subunit).

DISCUSSION

Iron(II) binding to BFR

Previous iron-uptake studies have shown that the process of iron uptake into iron-free BFR consists of at least three distinct kinetic phases corresponding to: the binding of two iron(II) ions per subunit (phase 1); fast oxidation of each iron(II) to iron(III) (phase 2); and a subsequent slow core-formation phase which is only observed when more than 48 iron(II) ions per BFR are added (phase 3) [7]. Subsequent studies of site-directed variants of BFR showed that a number of residues are essential for the iron-uptake characteristics of wild-type BFR. These residues are located at an intrasubunit site proposed to form a dinuclear iron centre [8]. The crystal structure of BFR [5] showed that this centre was indeed capable of binding two metal ions (Mn^{2+} ions from the crystallization buffer), and confirmed what had been apparent from sequence-comparison studies, that the dinuclear centre of BFR is closely related to those of R2 RNR and MMOH [8]. A detailed magneto-optical study of cobalt(II) binding to wild-type BFR and site-directed variants further confirmed that the dinuclear site of BFR is the only high-affinity bivalent metal-binding site in the protein, with an overall K_d of approx. 1×10^{-5} M [10]. Since the ionic radii or iron(II) and cobalt(II) are similar, it is likely that their K_d values are similar. Hence we base the interpretation of the data presented here on the assertion that the addition of two iron(II) ions per BFR subunit results in the formation of a dinuclear iron(II) complex within each subunit of the protein.

Oxidation of iron(II) and reduction of NO

NO undergoes a number of redox reactions and, in thermodynamic terms, may be regarded as a potential oxidizing agent, with reduction of NO to N_2 occurring in a four-electron process or to N_2O in a two-electron process. The two-electron process can be written as:

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (1)$$

At neutral pH the standard potential (E_0) for the

$2NO,2H^+/H_2O,N_2O$ couple is approx. +1170 mV versus the normal hydrogen electrode. Thus NO is oxidizing with respect to the Fe^{3+}/Fe^{2+} couple ($E_0 = +770$ mV versus normal hydrogen electrode), and the oxidation of iron(II) to iron(III) in aqueous solution by NO has been reported under some conditions [32].

This work establishes that the addition of excess NO to iron(II)–BFR in the absence of additional reductant results in oxidation of up to a third of the iron(II) to iron(III) and the concomitant reduction of NO to N_2O. Such a reaction is not observed for iron(II) in solution under the conditions of the equivalent experiment with BFR. Although it has yet to be accurately determined, the redox potential of iron at the ferroxidase centre of BFR certainly lies considerably below that of the Fe^{3+}/Fe^{2+} couple [13], and it might be expected, at least on thermodynamic grounds, that NO is able to oxidize iron(II) to iron(III) at the ferroxidase centre. However, since the equivalent reaction does not occur for iron in solution under these conditions, kinetic factors are also important.

Recently, studies of the interaction of NO with the diferrous form of the dinuclear iron centre of R2 RNR were reported [14]. The majority species was found to be an anti-ferromagnetically coupled $S = 0$ $[L_xFeNO]_2$ species, which decomposed slowly to give the μ-oxo-bridged diferric form of the centre and a molecule of N_2O. Hence the oxidation of dinuclear iron(II) by NO leading to iron(III) and N_2O is probably a common feature of the interaction of this class of di-iron protein with NO. The X-ray structure of a carboxylate-bridged non-haem di-iron–dinitrosyl complex was recently published. Each iron was found to bind a single NO, as in R2 RNR, and was also found to be anti-ferromagnetically coupled [33]. This complex was found to be stable to decomposition to the diferric form and N_2O, but it is noted that the complex was not in an aqueous solvent.

EPR analysis of iron(II)–BFR after the addition of excess NO indicates that at least some of the iron(III) is present as mononuclear species, with a characteristic signal at $g \approx 4.3$. This is consistent with previous studies of iron uptake into BFR which showed that mononuclear iron(III) signals could be detected shortly after the aerobic oxidation of two iron(II) ions per BFR subunit. This is in contrast with other dinuclear iron proteins, such as R2 RNR and MMOH, in which a stable EPR-silent diferric centre is formed [12]. This observation led to the proposal that, on oxidation, the di-iron centre of BFR becomes unstable and breaks down to mononuclear species. Mobility of iron might be an expected feature of a protein capable of storing a large number of iron atoms. However, the nature of the proposed mononuclear species has yet to be determined.

In the presence of an additional reductant (sodium ascorbate) iron(II)–BFR catalyses the reduction of NO to N_2O, a reaction that is also observed for solutions of iron(II) in excess ascorbate. Without measurements of the relative rates of NO reduction by iron(II)/ascorbate and iron(II)–BFR/ascorbate mixtures it is not possible to define the relative activities of the two systems. However, one major difference between the reactions is that, in iron(II)–BFR/ascorbate samples, stoichiometric quantities (i.e. one per subunit) of a mononuclear iron–dinitrosyl complex are formed along with significant but undetermined quantities of two mononuclear iron–mononitrosyl species, indicating that iron remains associated with BFR in the presence of ascorbate, and that the similarities of the two reactions most likely reflect a similar catalytic mechanism in each. These observations also account for the different amounts of N_2O detected in iron(II) samples with and without BFR. In BFR samples, a significant proportion of NO remains bound to BFR in the form of mono and dinitrosyl species, whereas in the absence of BFR, significant iron–nitrosyl species are not formed, or at least are not stable. Hence in BFR samples, at high concentrations of NO, the ratio of free to bound NO is high. At lower NO concentrations, this ratio decreases and the proportion of NO reduced decreases.

Location of BFR–iron–nitrosyl species

After demonstrating that iron remains associated with BFR in the presence of ascorbate and NO, and that it can be detected as at least three mononuclear species, it is important to determine the location of such species within BFR. The EPR study of a series of site-directed variants of BFR was only partially successful in this aim. The $g \approx 4$ region of the EPR spectra of the three dinuclear centre variants are all significantly altered from that of the wild-type, whereas that of the threefold channel variant is very similar to that of the wild-type (Figure 3). We conclude that at least one of the two mononuclear iron–mononitrosyl species is located at the dinuclear iron centre. The location of the second mononitrosyl species is uncertain because the axial $g = 4$ type signal is rather common and is therefore not discriminatory. The EPR data indicate clearly that the $S = \frac{1}{2}$ mononuclear iron–dinitrosyl species, present at a stoichiometry of one per subunit, is not located at the dinuclear centre or in the threefold channels (Figure 4). Further studies will be required to determine its location. Nevertheless, this is a significant observation.

In order to interpret these data fully, it is important to establish the chemistry that occurs when NO is added to iron(II)–BFR in the presence of a reductant. The reaction of NO with iron(II)–BFR in the absence of a reductant provides some indication. After this reaction, iron is observed as a mononuclear species because of oxidation of iron(II) at the dinuclear centre by NO. Thus we propose that, in the presence of an additional reductant, oxidation of iron(II) at the dinuclear centre also occurs and the resulting mononuclear iron(II)–nitrosyl species are observed as a consequence of reduction of the mononuclear iron(III) species formed during oxidation. These data are therefore consistent with the proposal that the ferric form of the dinuclear iron centre is unstable and breaks down to form mononuclear species. We propose that one of the irons remains at the dinuclear centre and the other is lost from it, to an as yet unidentified site. Hence these data show that, rather than being an inert probe of non-haem iron sites in the protein, NO mimics the interaction of iron(II) at the dinuclear centre with oxygen, and therefore may be of considerable mechanistic significance.

The existence of a mononuclear iron-binding site in addition to a dinuclear iron centre in a ferritin is not without precedent: such a site has been observed in the crystal structures of H-chain ferritin [34] and more recently of FTN, a bacterial ferritin isolated from *E. coli* [35]. In both of these proteins, the site is located close to the ferroxidase centre and is thought to be important in the mechanism of core formation. Future studies will be directed towards identifying the location of the mononuclear iron–dinitrosyl species.

Pathways of NO reduction

The data presented here do not allow us to distingusih between the possible mechanisms of NO reduction. However, they provide evidence for two distinct reduction pathways, corresponding to the reaction of NO with iron at two different centres in BFR: the dinuclear iron centre and the mononuclear site.

Reaction of NO with the dinuclear iron(II) centre most likely results in binding of one NO at each iron [14,33]. Oxidation may occur via the formation of an N–N bond between the two NO molecules [14], but could also result from electrophilic attack of

free NO on one of the bound NO molecules, leading to oxidation of both irons via an intermediate Fe–N_2O_2 species [33].

Reaction of NO with a mononuclear iron(II) site could lead to NO reduction by a number of mechanisms. One such mechanism involves the binding of two NO molecules in a *cis* conformation so that they are sufficiently close to form the N–N bond. An external reductant is required to supply electrons for the reduction, most likely via the iron. Alternatively, as in the mechanism for the dinuclear centre, free NO may bind NO already bound to the iron site. Whatever the mechanism of reduction, we have observed that, in solutions of iron(II)–BFR plus reductant and iron(II) plus reductant, this process is catalytic. Because the reaction is observed with and without the presence of BFR, we cannot discount the possibility that iron in solution is the catalytic species in both cases. However, we have also shown that significant amounts of iron remain bound to BFR in the presence of reductant, and furthermore that it binds NO, so it would be a surprise if this iron was not involved in the reduction process. Reduction of NO is known to occur at mononuclear iron sites. Studies of aqueous nitrosyl–iron(II) chemistry by Pearsall and Bonner [36] showed that DNIC-type species play a principal role in the reduction of NO to N_2O by iron(II). Furthermore it appears that the catalytic centre must have at least two readily exchangeable co-ordination sites. Iron(II)–EDTA, which displays a greatly reduced capacity to catalyse the reduction of NO, most likely has only one exchangeable co-ordination site available to external ligands [37,38] and NO binds to form the mononitrosyl species exclusively. Thus the proposed mechanism in which two NO molecules bind iron in a *cis* conformation is the more likely.

NO has been employed widely as a spectroscopic spin probe for the studies of many non-haem iron-containing proteins. Here we report on an example in which NO is not an inert probe of such centres, and it is important to establish in such studies whether redox chemistry occurs. This finding appears to be critical in the case of proteins in which iron is labile, such as the ferritins.

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