Spectroscopic Studies of Cobalt(II) Binding to Escherichia coli Bacterioferritin*

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Angus M. Keech‡, Nick E. Le Brun‡, Michael T. Wilson§, Simon C. Andrews¶, Geoffrey R. Moore‡, and Andrew J. Thomson‡‡

From the *Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom, ‡Department of Chemistry and Biological Chemistry, University of Essex, Colchester CO4 3SQ, United Kingdom, and ¶The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, United Kingdom

The iron storage protein bacterioferritin (BFR) consists of 24 identical subunits, each containing a dinuclear metal binding site called the ferroxidase center, which is essential for fast iron core formation. Cobalt(II) binding to wild-type and site-directed variants of Escherichia coli BFR was studied by optical and magnetic techniques. Data from absorption spectroscopy demonstrate the binding of two cobalt(II) ions per subunit of wild-type and heme-free BFR, each with a pseudotetrahedral or pentacoordinate geometry, and EPR studies show that the two cobalt(II) ions are weakly magnetically coupled. Studies of variants of BFR in which a single glutamic acid residue at the ferroxidase center is replaced by alanine confirm that this is the site of cobalt(II) binding, since the altered centers bind only one cobalt(II) ion. This work shows that the electroneutrality of the ferroxidase center is preserved on binding a pair of divalent metal ions. Optical and EPR data show that cobalt(II) binding to BFR exhibits positive cooperativity, with an average $K_c = 1 \times 10^{-5}$ M. The favored filling of the ferroxidase center with pairs of metal ions may have mechanistic implications for the iron(II) binding process. Discrimination against oxidation of single iron(II) ions avoids odd electron reduction products of oxygen.

Bacterioferritin (BFR)1 from Escherichia coli is an iron storage protein consisting of 24 identical subunits of $M_r = 18,500$. The subunits are packed together to form a highly symmetrical, approximately spherical molecule with a hollow center, in which large amounts of iron can be deposited as a ferric-oxy-hydroxide-phosphate mineral (1, 2). In addition to the iron core, BFR contains three other types of metal centers: up to 12 b-type heme groups situated between symmetry-related subunit pairs and ligated by two methionines (Met-52 and Met-52'), a heme co-ordination set, which is thus far unique (3, 4); a dinuclear metal binding site within each subunit called the ferroxidase center (4–6); and mononuclear non-heme iron sites that bind NO to form dinitrosyl complexes.2 Studies of iron uptake by BFR and variant proteins have shown that the ferroxidase center is essential for the catalysis of rapid iron(II) oxidation by BFR (5, 6).

Although the mechanistic details of oxidative iron uptake are complex, the following principles have been established. The process involves at least three kinetically distinguishable phases, corresponding to: (i) binding of iron(II) at the ferroxidase center (phase 1); (ii) fast oxidation of iron(II) to iron(III) at the ferroxidase center (phase 2); and (iii) subsequent formation of the mineral core (phase 3). A schematic representation of the ferroxidase center is shown in Fig. 1a. The heme groups do not cycle their oxidation state during iron uptake, and recent studies of heme-free variants of BFR have demonstrated that iron uptake rates in the heme-free proteins are not significantly different from those of the wild-type protein (8). However, it has been shown that the optical spectrum of the heme responds to the binding of metal ions at the ferroxidase center. This is not surprising, given the structural relationship between the heme binding site and the ferroxidase center (see Fig. 1b).

Studies of the ferrous form of the ferroxidase center are hampered by the lack of easily detectable EPR and optical signals from iron(II) and by the reactivity of the center toward oxygen. Hence, direct spectroscopic evidence of the dinuclear iron center has been elusive. To explore further the divalent metal binding properties of BFR, with a view to characterizing the structural and functional properties of the ferroxidase center, the binding of cobalt(II) to wild-type BFR and four site-directed variants, two heme-free variants (M52H and M52L) and two ferroxidase center variants (E18A and E94A), has been investigated. Cobalt(II) has been used previously for studies of dinuclear iron proteins (9, 10) because it is similar to iron(II) in size and charge and has optical absorbance bands and magnetic properties that are characteristic of site geometry and the nature of the ligands (11). For octahedrally coordinated d7 cobalt(II), such as [Co(H2O)]2+3, extinction coefficients for d-d bands in the 500–650-nm region are $\sim 30 \text{ M}^{-1} \text{cm}^{-1}$. For tetrahedrally coordinated cobalt(II), this figure rises to $>300 \text{ M}^{-1} \text{cm}^{-1}$. Pentacoordinate cobalt(II) species have extinction coefficients that lie at intermediate values. Hence, the binding of cobalt(II) at a protein site of less than octahedral symmetry increases the absorption intensity and thus provides a convenient method of following metal-ion binding. We report data from optical and EPR spectroscopies, which clearly show that two cobalt(II) ions per subunit are bound to BFR and that they are located at the ferroxidase center. The data also indicate that cobalt(II) binding shows positive cooperativity.

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‡To whom correspondence should be addressed. Fax: 44-1603-259-396; E-mail: A.Thomson@uea.ac.uk.

1 The abbreviations used are: BFR, bacterioferritin; MES, 2-(N-morpholino)ethanesulfonic acid.

2 N. Le Brun, S. C. Andrews, G. R. Moore, and A. J. Thomson, submitted for publication.
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K. E. Presta, J. R. Hames, D. A. Frolow, and E. E. Monroy

MATERIALS AND METHODS

Purification, Heme Determination, and Iron Removal from BFR and BFR Variants—Generation of heme-free and ferroxidase center variants of BFR and the overexpression and purification of BFR and BFR variants were achieved by methods described previously (6, 8). Non-heme iron was removed from BFR and BFR variants by reduction with sodium dithionite and complexation with 2,2-bipyridyl (12). Protein concentrations were determined using the bicinchoninic acid method (13), using bovine serum albumin as a standard. Heme contents of wild-type and heme-containing BFR variants were determined by the pyridine hemochromagen method of Falk (14), and found to be (per BFR molecule) approximately five to six for wild-type BFR and approximately eight to nine for E18A and E94A BFRs.

Additions of Cobalt(II) Ions to apoBFR and apoBFR Variants—Solutions of cobalt salts (chloride or perchlorate, AnalaR grade) were freshly prepared prior to each experiment by dissolving weighed amounts of the salts in AnalaR grade water. For optical and EPR titrations microliter additions of cobalt(II) solutions were made using a micro-syringe (Hamilton). Each addition was followed by stirring and an incubation time of approximately 10 min.

Spectroscopy—Optical titrations were performed in one of two ways. In the first, protein solution was present in both sample and reference cuvettes, and cobalt(II) additions were made to the sample only, whereas an equal volume of water was added to the reference. In the second, protein solution was present in the sample cuvette, and buffer was present in the reference cuvette. Cobalt(II) was added to both sample and reference. Whichever method was used, additions were made until no further changes in the optical difference spectra were apparent. Spectra were measured using a Hitachi 4001 spectrophotometer interfaced to a 486 personal computer. The absorbance spectra of cobalt(II) in the presence of wild-type and variant BFRs were unaltered by the presence of oxygen, indicating that the species formed are stable in air. Therefore, no precautions were taken to exclude oxygen from the cobalt(II)-containing samples.

Kinetic measurements were made using a stopped flow apparatus (Applied Photophysics DX17MV).

The EPR titration was performed using a series of protein samples to which increasing aliquots of cobalt(II) were added. EPR spectra were recorded using an X-band spectrometer (Bruker ER200D) with an ESP computer system fitted with an ESR9 liquid helium flow cryostat (Oxford Instruments). Quoted g values are effective g values, and quoted g values are true g values.

RESULTS

Studies of Cobalt(II) Binding to BFR and BFR Variants by Absorbance Spectroscopy—Detection of cobalt(II) d-d transitions in BFR is complicated by the presence of low spin heme groups within the protein, since these have absorption bands in the 500–650-nm region of the spectrum, with extinction coefficients at least an order of magnitude greater than those of cobalt(II). To overcome this problem, two heme-free site-directed variants, M52H and M52L, were used. These corresponding substitutions do not significantly perturb functional characteristics of the ferroxidase center (8).

Changes in the absorbance spectrum of the iron-free form of one variant (apoM52H BFR) were monitored during a titration with cobalt(II) ions (Fig. 2a). Addition of cobalt(II) resulted in an increasingly intense absorption band at 555 nm with weaker bands at 520, 600, and 625 nm. A plot of absorption increase at 555 nm (expressed as fractional saturation) as a function of the concentration of cobalt(II) to M52H BFR is shown in Fig. 2a, inset. Analysis of the data was achieved using Equation 1, which describes the simplest instance of ligand binding to a protein site.

\[ y = \frac{(S + K + L) - \sqrt{(S + K + L)^2 - 4 \times S \times L}}{2 \times S} \]  

where \( y \) is the fractional saturation of sites, \( K \) is the dissociation constant; \( L \) is the total [Co(II)], and \( S \) is the concentration of ligand sites.

A curve was fitted to the data using the software program GraFit (15), using an iterative calculation, which allowed both \( K \) and \( S \) to float (see Fig. 2a, inset). From the fit, a value for \( K_d \) of 9.55 ± 0.46 \( \times 10^{-4} \) M was determined, with a stoichiometry of 48.4 ± 0.3 metal binding sites/BFR. Thus two cobalt(II) ions are bound per BFR subunit. Extinction coefficients at 520, 555, 600, and 625 nm were determined to be 126, 155, 107, and 75 \( \text{M}^{-1} \text{cm}^{-1} \), respectively, for fully loaded cobalt(II)-BFR.

To determine whether the optical data indicate cooperativity of metal binding, the data were first analyzed using the Scatchard equation (16), which yielded the plot shown in Fig. 2b. The data points can be fitted to a straight line, which does not indicate the presence of cooperativity. Further analysis of the data was attempted using the Hill equation (16). The slope of the plot (Fig. 2c) gave a Hill coefficient \( (h) \) of 1.1, which, for a two site model, indicates that cooperativity may be present (a value for \( h \) of 2 would indicate complete cooperativity for such a system). Further efforts to detect the presence of cooperativity involved repeating the cobalt(II) binding titration under conditions in which the \( K_d \) was similar to the concentration of metal binding sites (0.9 \( \mu \text{M} \) in BFR in 100 mM HEPES buffer, pH 7.1; path length, 5 cm). Fitting of the data to Equation 1 did not give a good fit, whereas the Scatchard plot gave a nonlinear curve indicative of positive cooperativity (Fig. 2d). Analysis using the Hill equation gave the plot in Fig. 2e. The curve is sigmoidal in shape, as is commonly observed for systems that exhibit less than complete cooperativity. In such cases, \( h \) is taken to be the slope of the linear part of the curve at a saturation level of 50% and here is found to be 1.6, confirming that cooperativity of binding is present. Reducing the protein concentration further such that \( K_d > \text{metal ion concentration} \) was not practicable, due to the relatively low intensity of the...
observed signals. Cooperativity of binding is considered further in the EPR section.

A cobalt(II) binding titration was carried out using heme-containing wild-type BFR in 100 mM MES, pH 6.5. The titration profile was identical in form (Fig. 3), indicating that the absence of heme from the protein does not significantly affect cobalt(II) binding to the heme-free proteins (8). Fig. 3, inset, shows a plot of absorption increase at 555 nm (again, expressed as fractional saturation) as a function of the ratio of cobalt(II) to wild-type BFR. Analysis of the data revealed a $K_d$ of $1.4 \pm 0.3 \times 10^{-5}$ M with a stoichiometry of 45 ± 0.6 sites/BFR molecule. Again, similar fits were obtained from plots of absorption increases at the other wavelength maxima (not shown).

To determine whether anions bind with the cobalt(II) ions at the protein sites, the cobalt(II) titration was carried out using cobalt(II) perchlorate instead of cobalt(II) chloride. The data (not shown) corresponded to a $K_d$ of $1.1 \pm 0.2 \times 10^{-5}$ M and

49.8 ± 0.8 sites/BFR, indicating a close similarity between the titration profiles for cobalt(II) chloride and perchlorate. Since the perchlorate anion does not readily form complexes with metal ions in aqueous solution and in the present case may also be too bulky to gain access to the BFR binding site, the similar binding data indicate that chloride ions do not bind at the cobalt(II) binding site. This is consistent with the absence of any change in the absorbance spectrum of cobalt(II) bound to BFR when chloride (100 mM final concentration) is added at a 100-fold excess over cobalt(II).

To confirm the location of cobalt(II) binding, absorption titrations were performed using samples of two variants, E18A BFR and E94A BFR. The titration profiles varied considerably relative to those of the heme-free variants and wild-type BFR. With E18A BFR, three bands were observed at 510, 550, and 590 nm (Fig. 3a), and the plot of absorbance increase at 590 nm (expressed as fractional saturation) as a function of cobalt(II) concentration yielded a $K_d$ of $1.05 \pm 0.2 \times 10^{-5}$ M and a stoichiometry of $29 \pm 0.9$ cobalt(II) ions/BFR (Fig. 3b). The corresponding extinction coefficients were 165, 165, and 200 $\text{cm}^{-1} \text{M}^{-1}$ for the bands at 510, 550, and 590 nm, respectively. With E94A BFR, the absorption bands were at 510, 553, 595, and 620 nm (Fig. 3c), and the plot of absorbance increase at 595 nm (expressed as fractional saturation) versus cobalt(II) concentration (Fig. 3d) gave a $K_d$ of $8.8 \pm 2.3 \times 10^{-6}$ M, a stoichiometry of 19.3 ± 3.0 cobalt(II) sites/BFR, and extinction coefficients of 123, 171, 138, and 116 $\text{cm}^{-1} \text{M}^{-1}$ for bands at 510, 553, 595, and 620 nm, respectively.

The results of the cobalt(II) binding titrations are summarized in Table I.

**Kinetic Studies of Cobalt(II) Binding to Wild-type BFR**

Previous studies of wild-type BFR have shown that divalent metal ion binding at the ferroxidase center can be monitored by following a small perturbation of the visible absorbance due to the heme groups of the protein (4, 5). The absorbance at 425 nm has been measured as a function of time after the addition of 48 cobalt(II) ions/BFR molecule, showing that the absorbance change at this wavelength is very similar to that observed for the addition of iron(II) ions and can be similarly assigned to a blue shift of less than 1 nm in the Soret absorbance. Fitting of the data gave a pseudo-first order rate constant of $19 \pm 3 \times 10^{-3}$ s$^{-1}$ (4.7 $\mu$M BFR, 20 °C), which compares with $14 \pm 3 \times 10^{-3}$ s$^{-1}$ for iron(II) and 146 s$^{-1}$ for zinc(II) under similar conditions (data not shown). If it is assumed that the heme perturbation rate is equivalent to the on rate of metal binding, then, together with the average binding constant obtained from optical titration data, we can calculate the off rate. For a total binding site concentration of 120 $\mu$M, a pseudo-first order rate constant of 19
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s$^{-1}$ indicates a second order rate constant of $1.6 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ for the binding process. From this, the off rate is found to be $2 \times 10^{-1}$ s$^{-1}$.

**EPR Spectroscopy of Cobalt(II)-substituted BFR and BFR Variants**—Fig. 5c shows the 10 K EPR spectrum of wild-type BFR after the addition of 48 cobalt(II) ions/protein molecule. In addition to signals at $g = 2.88$, 2.31, and 1.45, due to the low spin ferric hemes of the protein, and a feature at $g = 4.3$, due to adventitious high spin iron(III), the spectrum contains a very broad ($\sim 500–4000$ G), derivative-shaped feature centered at $g = 5.3$ with a crossing point at $g = 5.4$. Hyperfine structure is resolved in the low-field region.

Optical data showed that only 1 cobalt(II) binds per E18A and E94A BFRs. Therefore, the 24 and 48 cobalt(II)-substituted derivatives of these variants were investigated. EPR spectra of the ferroxidase center variant E18A BFR following the addition of 24 and 48 cobalt(II) ions/protein are shown in Fig. 5, a and b, respectively. They are similar to the wild-type spectrum in that they contain resonances at $g = 2.88$, 2.31, and 1.45, due to heme, but differ significantly in the lower field region. Both spectra contain a broad, derivative-shaped signal crossing over at $g = 5.3$. An intensity ratio of approximately 1:2 (24 Co(II):48 Co(II)) at the field indicated ($g = 5.3$) is found.

**Fig. 4. Optical titrations of apoE18A and apoE94A BFRs with a solution of cobalt(II) ions.** a, apoE18A BFR. Conditions were as in Fig. 2a, except protein (8.6 $\mu$M) was in 100 mM MES buffer, pH 6.5. b, plot of absorbance changes at 560 nm (expressed as fractional saturation) as a function of cobalt(II) concentration. Data were fitted as in Fig. 2a, inset, giving a stoichiometry of binding of $29 \pm 0.9$ cobalt(II) ions/BFR and a $K_d$ of 1.05 $\pm 0.2 \times 10^{-5}$ M. A similar fit was obtained from absorbance changes at 553 nm (expressed as fractional saturation) as a function of cobalt(II) concentration. Data were fitted as in Fig. 2a, inset, giving a stoichiometry of binding of 19.3 $\pm 3.0$ cobalt(II) ions/BFR and a $K_d$ of 8.8 $\pm 2.3 \times 10^{-6}$ M. A similar fit was obtained from absorbance changes at 512 nm (not shown).

Spectra of similar samples of cobalt(II)-substituted E94A BFR were recorded (Fig. 6, a and b). These are substantially different from both wild-type and E18A BFR. After addition of 24 cobalt(II) ions, a signal with a peak at $g = 6.8$ is observed with an intensity somewhat less than that of the $g = 4$ signal in the equivalent E18A BFR spectrum. The spectrum of the 48 cobalt(II)-substituted sample (Fig. 6b) appears to be a superposition of the spectrum of Fig. 6a and the broad $g = 4$ signal observed for E18A BFR.

Thus, the cobalt(II)-substituted forms of wild-type, E18A, and E94A BFRs give rise to EPR spectra, which, apart from signals due to heme, are significantly different from one another. For the assignment of EPR signals, it should be recognized that the cobalt(II)-BFR samples contain mixtures of cobalt(II) species, including cobalt(II) not bound to BFR. This is clear from the optical data described above, which show that the $K_d$ for the interaction of cobalt(II) with wild-type and BFR variants has a value on the order of $1 \times 10^{-5}$ M (see Table I).

Using these data it is possible to calculate the free concentration of cobalt(II) ions for any given concentration of cobalt(II)-BFR complex at room temperature. Calculations of this type show the relative proportions of cobalt(II) bound and unbound to the protein (see later).

To aid the assignment of features in the above spectra, the 10 K EPR spectrum of cobalt(II) in buffer solution was recorded (Fig. 6c). The spectrum consists of a rather broad feature with a crossover value at $g = 4$, which is typical of a $S = 3/2$ cobalt(II) system of near axial symmetry with a positive $D$ value such that the $m_s = \pm 1/2$ Kramer doublet is lowest in energy. The predicted $g$ values originating from the $m_s = \pm 1/2$ doublet for a system with an isotropic true $g$ value of 2 and with $D \gg Zeeman$ interaction is $g_a = 4$; $g_2 = 2$.

The 24- and 48-cobalt(II)-substituted E18A BFR spectra resemble the mononuclear cobalt(II) solution spectrum in both signal shape and intensity. Thus we conclude that in E18A BFR, cobalt(II) is mononuclear. Whether cobalt(II) is bound to the protein cannot be determined from the EPR data alone, but taken together with the optical titration data, it is clear that the first 24 cobalt(II) ions form mononuclear species in near axial environments at the altered ferroxidase centers, and any additional cobalt(II) remains in solution or binds nonspecifically to the protein.

The addition of 24 cobalt(II) ions/molecule of E94A BFR gave an EPR spectrum (Fig. 6a) having a sharp feature at $g = 6.8$, which is very different from either cobalt(II) in solution or in E18A BFR. This type of cobalt(II) spectrum is characteristic of mononuclear cobalt(II) in a rhombic environment (17, 18). As the rhombicity of the site increases the $g$ values move away from their values in the axial limit (g = 4, 4, and 2), with an accompanying decrease in intensity. That the observed $g$ value is $>6$ indicates that the true $g$ value is somewhat higher than 2 (17). Temperature dependence data (not shown) indicate that the signal originates from the lowest energy doublet. In addition to the signal at $g = 6.8$, there is a weak shoulder in the $g = 4$ region. This can be assigned to a small component of cobalt-

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TABLE I

| Bacterioferritin          | $K_d$ ($\times 10^5$ M$^{-1}$) | No. of Co(II) bound per BFR | Absorption characteristics $\lambda_{max}$ nm ($e \times 10^3$ cm$^{-1}$) |
|---------------------------|-------------------------------|-----------------------------|------------------------------------------------------------------|
| Wild-type BFR             |                               |                             |                                                                  |
| With CoCl$_2$             | 1.4 $\pm$ 0.3                 | 45.0 $\pm$ 0.6              | 520 (126), 555 (155), 600 (107), 625 (75)                         |
| With Co(ClO$_4$)$_2$      | 1.1 $\pm$ 0.2                 | 49.8 $\pm$ 0.8              | 520 (133), 555 (162), 600 (111), 625 (75)                         |
| M52H BFR (6.34 $\mu$M BFR)| 0.96 $\pm$ 0.3                | 48.4 $\pm$ 0.3              | 520 (120), 555 (155), 600 (111), 625 (74.4)                       |
| E18A BFR                  | 1.05 $\pm$ 0.2                | 29.0 $\pm$ 0.9              | 510 (155), 550 (165), 590 (200)                                  |
| E94A BFR                  | 0.88 $\pm$ 0.2                | 19.3 $\pm$ 3               | 510 (123), 552 (171), 595 (138), 620 (116)                        |
apoBFR, a titration was undertaken. The presence of cobalt(II) ions was studied by measuring the peak to trough height, 1300–2000 G, as a function of cobalt(II) concentration (see Fig. 5). Knowing the EPR spectrum of the free cobalt(II) ion, the EPR intensity at $g = 5.3$ could be calculated and plotted as a function of cobalt(II) concentration (see Fig. 7b), where it is compared with the EPR titration data. The remaining EPR spectrum, after subtracting that due to unbound cobalt(II), is due to bound cobalt(II). The bound species can be one of two types, either a single cobalt ion or a pair of cobalt(II) ions bound at the ferroxidase center. The spectrum does not have the characteristics of a single cobalt(II) ion bound at the ferroxidase center, as exemplified by E18A and E94A (Figs. 5 and 6). This suggests that the residual spectrum is due to occupancy by a pair of cobalt(II) ions. The ability to distinguish between signals for single ions and pairs of ions can be used to test whether the binding at the ferroxidase center is cooperative, anticooperative, or noncooperative. A simple statistical analysis, assuming an infinitely high binding constant, of the relative occupancy by single ions and by pairs shows, as expected, that in the absence of cooperativity the maximum number of singly occupied centers will arise when the binding stoichiometry is 24 cobalt(II) ions/BFR molecule. In the limit of negative cooperativity, a maximum is also observed when the binding stoichiometry is 24 cobalt(II) ions/BFR molecule, whereas in the limit of positive cooperativity, singly occupied centers are not observed. These points are illustrated graphically in Fig. 7c.

If it is assumed that bound mononuclear cobalt(II) gives rise to an axial type EPR signal, then, given the conditions of the titration, it is possible to estimate the EPR intensity that would be expected in the absence of cooperativity (again assuming an infinite binding affinity) and to compare this with the observed EPR intensities (Fig. 7d), which have already been shown to contain a significant contribution from unbound cobalt(II). Clearly, intensity is less than expected for the situation of no cooperativity, and a maximum is not observed. Hence we conclude that positive cooperativity is present, favoring the filling of sites with pairs of metal ions and, furthermore, that the dinuclear pair gives rise to an EPR signal, although of weaker intensity than that of mononuclear cobalt(II). A signal due to a dinuclear center is expected to increase linearly in the presence of strong cooperativity.

The EPR signal of the dinuclear cobalt(II) pair consists of a broad, derivative signal with a crossing at $g = −4.0$. The
presence of low amounts of residual iron(III) ion in the protein (observed at g = 4.3), probably bound within the central BFR cavity, partially obscures the higher field part of the spectrum. The characteristic feature of the spectrum is the pronounced hyperfine splitting, which shows at least 8 lines in the 48-cobalt(II) wild-type BFR spectrum (see Fig. 5a). The splitting is due to the 59Co nucleus, for which I = 7/2. Hence, for a single ion, a maximum of 8 lines should be detectable. However, in the spectrum of the cobalt(II) form of M52L BFR (Fig. 8b), at least 10 lines can be resolved. If two equivalent cobalt(II) ions were interacting, a total of 15 hyperfine lines, with intensity ratios following the binomial distribution, should be observed. The data are not sufficiently well resolved to allow a unique simulation to be carried out at this stage.

The ferroxidase center of BFR is very similar to the di-iron center of ribonucleotide reductase and other class II dinuclear iron proteins (6). Studies of the cobalt(II)-substituted form of ribonucleotide reductase showed that the cobalt(II) ions are not magnetically coupled (10). In BFR, there is evidence of coupling. However, if the cobalt(II) ions were strongly coupled, an EPR signal would not be expected. Since an EPR signal is observed, and because the g value is not much changed from that of a mononuclear complex, we propose that the coupling between the two ions in BFR is very weak and probably dipolar in origin, despite the presence of two carboxylate bridges between the two paramagnetic ions.

In summary, the data indicate that two cobalt(II) ions bind per BFR subunit and that the binding exhibits a form of positive cooperativity. Modeling of the system using an average Kd of 1 \( \times \) 10^{-5} M indicates that a significant (and increasing) proportion of the EPR intensity at g = 5.3 is due to unbound cobalt(II) (Fig. 8b), and the remainder can be assigned to the magnetically coupled dinuclear cobalt(II) species.

The crystal structure of the manganese(II) derivative of wild-type BFR (4) indicates that the two sites of the dinuclear center, when occupied by divalent metal ions, are very similar in terms of ligands and symmetry. Consequently, it is likely that the substitution of a ligating residue at one site of the center affects the symmetry of the metal ion at the other site. This appears to be more easily detectable for E94A BFR than for E18A BFR. In support of this conclusion is the observation that a superposition of the optical spectra of E18A and E94A BFRs, after the addition of 24 cobalt(II) ions to each, is somewhat different from the spectrum of wild-type BFR after the addition of 48 cobalt(II) ions/BFR molecule.

**DISCUSSION**

Previous studies of iron uptake by BFR have shown that apoBFR binds 48 iron(II) ions, and modeling studies indicated that a dinuclear metal center may exist within the four-a helix bundle of each subunit (19). Further studies of BFR variants confirmed that the dinuclear center is the site of iron(II) binding and that it is essential for catalyzing fast core formation by BFR (6). Subsequently, structural evidence of the existence of a dinuclear metal center was provided by the crystal structure of *E. coli* BFR (4), which showed a dinuclear site within each subunit, probably occupied by manganese(II) ions from the crystallization solution. The absorbance titration data from wild-type and heme-free variants show clearly that each BFR crystallization solution. The absorbance titration data from wild-type and heme-free variants show clearly that each BFR
binding to BFR is positively cooperative. Conditions under which the optical titrations were performed were found to be crucial for the detection of cooperativity. Under conditions in which the average $K_d$ was much less than the concentration of cobalt(II) binding sites, cooperativity was largely obscured but became more readily detectable at a lower protein concentration. EPR, in this case, proved a more sensitive probe of positive cooperativity, since it readily distinguishes mononuclear and dinuclear cobalt(II), whereas the optical spectrum apparently does not. The presence of cooperativity in divalent metal binding to the ferroxidase center of BFR is likely to be of mechanistic significance. It implies that on addition of iron(II) to apoBFR, the ferroxidase center preferentially takes up a pair of divalent metal ions. Hence, oxidation takes place at a filled ferroxidase center. The reduction products of dioxygen are likely to be even electron species, such as hydrogen peroxide or water, rather than radicals such as superoxide.

It cannot be ruled out that other modes of cooperativity are also present, including intersubunit interactions mediated by the heme group, which cross-link the subunit interface. Indeed, the heme Soret band does respond to the binding of divalent metal ions at the ferroxidase center. Another mechanism of cooperativity that is not ruled out by the present work is ligand-induced BFR association. Further experiments are needed to distinguish between these possibilities. However, the balance of evidence shows a moderate degree of interaction between cobalt(II) ions as they bind to the ferroxidase center. This clearly has mechanistic advantages when the center is reducing $O_2$.

The substitution of cobalt(II) for manganese(II) is not expected to affect greatly the structure of the center. From Fig. 1a it is clear that the two metal ions have identical coordinating ligands but are in different geometric arrangements. Nevertheless, cobalt(II) at either site should have very similar optical properties to one another. The position and intensity of cobalt(II) d-d absorption bands is in agreement with this. However, from the absorbance data alone it is not possible to distinguish between distorted tetrahedral and pentacoordinate geometries.

Analysis of the optical titration data has afforded an estimate of the overall dissociation constant for cobalt(II) binding at the ferroxidase center. This value is largely independent of the presence or absence of the heme groups, which form a structural unit with the ferroxidase centers (see Fig. 1b). This is at first sight surprising but is consistent with the finding that iron uptake rates into BFR are similarly independent of the presence or absence of heme (8). An indication of the on rate of cobalt(II) binding at the ferroxidase center was obtained from the stopped flow measurement of the perturbation of the BFR heme absorption spectrum. Knowledge of the dissociation constant ($K_d = 1 \times 10^{-5} M$) and the on rate ($1.6 \times 10^5 M^{-1} s^{-1}$) permitted an estimate of a value for the off rate of cobalt(II) binding on the order of $2 s^{-1}$. This indicates that divalent metal ions at the ferroxidase center are in relatively fast exchange with solvated ions. The data also confirm that BFR does not contain any alternative divalent metal binding sites with a comparable binding affinity to that of the ferroxidase center. Thus, binding of divalent metal ions to apoBFR results in an initial metal complex at the ferroxidase center.

Studies of the ferroxidase center variants E18A and E94A BFRs, in addition to providing confirmation of the site of cobalt(II) binding, also illustrate that the metal binding stoichiometry is strictly controlled by the charge of the binding pocket (i.e. ferroxidase center). The replacement of one negatively charged residue at the binding pocket with a neutral residue reduces the binding stoichiometry to one divalent metal ion, with a dissociation constant similar to that of the wild-type binding pocket. It has previously been proposed that the apo form of the wild-type ferroxidase center will contain four protons (6), although the $pK_a$ values were not determined. These protons will be released on binding of two divalent metal ions, thus preserving the charge neutrality of the center. For each of the altered ferroxidase centers it is likely that one cobalt(II) ion binds and one proton remains bound, again preserving the charge neutrality of the binding pocket. The principle of charge neutrality is further illustrated by the absence of anion binding to cobalt(II) at the ferroxidase center.

The effects of substituting the carboxylate residues at each end of the dinuclear center are not identical. Differences in the characteristics of the two variants were observed in both the absorption and, more obviously, the EPR spectrum. This is consistent with studies of the iron uptake properties of these two variants (6), in which E18A BFR was found to form an iron core considerably more slowly than E94A BFR. In previous mechanistic studies of BFR, the lack of spectral features associated with iron(II) meant that a clear conclusion regarding the stoichiometry of binding at the altered ferroxidase centers was not possible. The current data indicate that a single iron(II) binds per altered center, leading to the significantly reduced catalytic activity of the proteins.

That the two sites at the dinuclear center are not equivalent may be an important feature of the mechanism of iron uptake. Cobalt(II) binding to BFR appears to share many common features with iron(II) binding. The on rate for iron(II) binding is very similar to that of cobalt(II), as determined through measurement of the heme perturbation. If we assume that the dissociation constant of iron(II) binding is similar to that of cobalt(II), then we can conclude that iron(II) is also in rapid exchange at the ferroxidase center. Therefore, we suggest that it is the oxidation of iron(II) to iron(III) at the ferroxidase center that “traps” iron at this site. Cobalt(II) does not undergo aerobic oxidation at the ferroxidase center, and consequently, the cobalt(II) derivatives of BFR studied here are all stable under aerobic conditions.

EPR studies of cobalt(II)-substituted wild-type and heme-free BFRs indicate that the two cobalt ions are weakly metal-ligand coupled, resulting in a broad EPR signal with pronounced $3^9$Co hyperfine splitting. Finally, BFR is only one of a number of related proteins belonging to the ferritin family. Ferroxidase centers similar to, but less symmetrical than, that of BFR have been characterized crystallographically in human H chain ferritin and E. coli ferritin, FTRN (7, 20). No spectroscopic studies of metal ion uptake have so far included cobalt(II) substitution, and since BFR is the only member of the family that contains heme, these proteins are ideally suited to an investigation of this type.

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Angus M. Keech, Nick E. Le Brun, Michael T. Wilson, Simon C. Andrews, Geoffrey R. Moore and Andrew J. Thomson

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