E.p.r. and magnetic circular dichroism spectroscopic characterization of bacterioferritin from *Pseudomonas aeruginosa* and *Azotobacter vinelandii*

Myles R. CHEESMAN,* Fahmi H. A. KADIR,* Jamal AL-BASSEET,* Fareeda AL-MASSAD,* Jaqui FARRAR,* Colin GREENWOOD,† Andrew J. THOMSON*‡ and Geoffrey R. MOORE*

Centre for Metalloprotein Spectroscopy and Biology, Schools of *Chemical and †Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The e.p.r. and magnetic circular dichroism (m.c.d.) spectra of bacterioferritin (BFR) extracted from *Pseudomonas aeruginosa* and *Azotobacter vinelandii* have been studied over a wide temperature range down to liquid-helium temperature. The e.p.r. spectra show the presence of low-spin Fe³⁺ haem with *g* values of 2.86, 2.32, 1.48 (*P. aeruginosa*) and 2.88, 2.31, 1.46 (*A. vinelandii*), in both the presence and absence of the BFR core. Together with evidence from the porphyrin-to-Fe³⁺ charge-transfer band at 2240 and 2270 nm the axial haem ligands are identified as two methionines. The low-temperature m.c.d. spectra in the region 300–1000 nm of *P. aeruginosa* and *A. vinelandii* BFR are identical with one another and unaffected by removal of the iron core. Hence it can be concluded that the presence of the iron core has no detectable effect on the electronic states and on the stereochemistry of the haem group. This was unexpected, in view of the observations by Watt, Frankel, Papaefthymiou, Spartalian & Stiefel [(1986) *Biochemistry* **25**, 4330–4336] that the redox potential of the haem group in *A. vinelandii* BFR shifts from –475 mV to –225 mV on removal of the core. The e.p.r. spectra of holoBFR show a broad symmetrical derivative-shaped band centred at *g* = 2.0 which decreases in bandwidth as the temperature is raised. This signal is assigned to the uncompensated electron spins of the iron core.

INTRODUCTION

Ferritin, the iron-storage protein of animals, has been extensively studied with a wide range of techniques (Crichton, 1990; Harrison *et al.*, 1991). The X-ray structure of the protein from horse spleen is known, together with amino acid and gene sequences of the protein from a variety of sources (Ford *et al.*, 1984; Harrison *et al.*, 1991, and references therein). In addition, many aspects of its biological regulation, including features of its genetic control, are known. However, despite this large body of data, there remain many unanswered questions concerning the mechanisms by which the protein acquires and releases iron, and the nature of the stored iron. One approach to gaining further insight into these aspects of ferritin is by a comparative study of ferritin with its bacterial counterpart, bacterioferritin (BFR) (Stiefel & Watt, 1979). Previous work has shown that there are both marked similarities and major differences between horse and human ferritins and BFR (Stiefel & Watt, 1979; Moore *et al.*, 1986; St. Pierre *et al.*, 1989; Harrison *et al.*, 1991). For example, although all these proteins consist of 24 subunits, of molecular mass approx. 18–23 kDa, that pack together to form a protein coat surrounding the iron core, the bacterial proteins contain haem in the coat, whereas the animal ferritins, as isolated, do not. Also, the morphology of the bacterial iron cores differs considerably from that of the animal ferritins, and there are also differences between the cores of BFRs from different species (St. Pierre *et al.*, 1986, 1989).

Spectroscopic techniques provide an important approach for the study of the iron centres of BFR and ferritin. The main spectroscopic method used previously for the analysis of the iron centres in ferritin has been Mössbauer spectroscopy (Watt *et al.*, 1985; St. Pierre *et al.*, 1986, 1989), although some limited studies have been made by e.p.r. spectroscopy (Boas & Troup, 1971;

Weir *et al.*, 1984). We are engaged on a study of the iron centres of both animal ferritin and BFR using a combination of magnetic circular dichroism (m.c.d.) and e.p.r. spectroscopies. In the present paper we report the u.v.–visible absorption, e.p.r. and m.c.d. spectra of BFRs isolated from *Pseudomonas aeruginosa* and *Azotobacter vinelandii* in order to assign features in the different spectra to the various paramagnetic forms of iron which can be detected. Spectra have been recorded from BFR as isolated (holoBFR) and after partial or total removal of the iron core (apoBFR) by chemical means, and features identified for both the haem iron and non-haem-iron core. The significance of these spectral features to the structure and properties of BFR is discussed.

EXPERIMENTAL

P. aeruginosa (N.C.T.C. 6750) cells were grown anaerobically and with a high-nitrate medium as described by Parr *et al.* (1976). *A. vinelandii* cells were gifts from Professor B. E. Smith (IPSR, Brighton, Sussex, U.K.). BFR was isolated by a procedure adapted from that described by Moore *et al.* (1986). This procedure employs heat-treatment, ammonium sulphate precipitation, ultracentrifugation and chromatography on DEAE-cellulose media to purify the BFR. It is described by Al-Massad *et al.* (1992). Protein concentrations were determined by the method of Lowry (Peterson, 1977), by using a Sigma protein assay kit (P5656) with BSA as the standard. Haem contents were determined by using the absorption coefficient reported by Yariv *et al.* (1981) for the 417 nm band of the ferrihaem of *Escherichia coli* BFR ($1.37 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) or by e.p.r. integration of the ferrihaem electron spins. Iron concentrations were determined by atomic absorption spectroscopy with a Varian Spectra-AA20 instrument equipped with a PSC-56 programmable sample

Abbreviations used: m.c.d., magnetic circular dichroism; BFR, bacterioferritin.

‡ To whom correspondence should be addressed.

changer and a GTA-96 graphite-tube atomizer. Non-haem-iron-free BFR was prepared by exhaustive dialysis of holoBFR against 0.12 M-thioglycolic acid/NaOH at pH 5.0 and 4 °C, followed by dialysis against 25 mM-sodium phosphate buffer (pH 7.4). It took 5–7 days to remove all the core iron, judged by atomic absorption spectroscopic analysis or by constant 278 nm/417 nm absorbance ratios for the oxidized protein.

Spectroscopic measurements

Electronic absorption spectra were recorded on a Hitachi U-2000 or Hitachi 557 spectrophotometer.

M.c.d. spectra were recorded on a Jasco J-500 D spectropolarimeter, for the wavelength range 300–1100 nm, and on a home-built circular dichrograph in the range 800–3000 nm. The sample was loaded into a split-coil superconducting solenoid (SM-4; Oxford Instruments, Osney Mead, Oxford, U.K.) capable of generating a maximum magnetic field of 5 T and giving temperature control at the sample between 1.5 K and 300 K. In order to collect m.c.d. data in the near-i.r. region, buffers were made with $^2\text{H}_2\text{O}$ in place of water. Protein samples were prepared in 100 mM-Hepes solutions at pD 7.4 (Perrin & Dempsey, 1974). To ensure that good optical-quality glasses were formed on freezing, glycerol was added to samples to 50% (v/v). These additions left the absorption and e.p.r. spectra unchanged.

E.p.r. spectra were recorded on an X-band spectrometer (Bruker ER200D with an ESP1600 computer system) fitted with a liquid-helium flow cryostat (Oxford Instruments; ESR9). Concentrations of the low-spin ferric haem of ferritin were obtained by double integration of the e.p.r. spectrum and comparison with the integrated e.p.r. spectrum of a known concentration of an aqueous copper(II)-EDTA complex by the method of Aasa & Vänngård (1975).

RESULTS

Characteristics of the isolated BFRs

The sample of *P. aeruginosa* holoBFR was analysed to have 9.1–9.2% (w/w) (approx. 700 atoms) of iron, whereas the apoBFR contained no iron core detectable by atomic absorption spectroscopy. The phosphate content of the *P. aeruginosa* holoBFR was 1.67 Fe:1 phosphate. These values of Fe and phosphate contents are comparable with those found in the original report of *P. aeruginosa* BFR [8.7% (w/w) Fe and 1.7:1 Fe/phosphate] (Moore *et al.*, 1986). The sample as prepared contains approx. 6 haem groups per 24 subunits.

The sample of *A. vinelandii* holoBFR contained approx. 12 haem groups per 24 subunits. It was not subjected to chemical analysis for non-haem iron and phosphate. However, the u.v.-visible spectral parameters reported below indicate that the holoBFR resembled previously reported preparations of *A. vinelandii* BFR containing approx. 15% (w/w) (approx. 1200 atoms) of iron per molecule (Bulen *et al.*, 1973; Stiefel & Watt, 1979; Jiudi *et al.*, 1980).

U.v.-visible spectroscopy

The absorption spectra of the oxidized and reduced forms of *P. aeruginosa* apo- and holo-BFR are shown over the range 350–850 nm in Fig. 1. These show the presence of low-spin haem in both the oxidized and reduced states. The Soret-band maxima at 417 nm and 422 nm in the oxidized and reduced states of the holoBFR agree well with those previously reported for *P. aeruginosa* BFR (Moore *et al.*, 1986). Treatment of the samples with excess $\text{Na}_2\text{S}_2\text{O}_4$ caused the spectral region below approx. 390 nm to be obscured by absorption due to the reductant. However, a comparison between the ratios $R = A_{278}/A_{417}$ for the

oxidized form of the holo- and apo-BFR shows that the iron core contributes a steadily rising background of absorption below approx. 500 nm. $R = 4.33$ for holoBFR and 1.33 for apoBFR.

The absorption spectra of oxidized *A. vinelandii* holoBFR and apoBFR are given for comparison in Fig. 1. The wavelength maximum of the Soret and visible bands agree well with those of *P. aeruginosa* BFR.

The absorption spectra of BFR from both sources contain a broad band at 690–800 nm which is readily observable for both holo- and apo-BFR of *P. aeruginosa* and *A. vinelandii*, and has also been observed in the spectrum of *E. coli* BFR (M. R. Cheesman, N. Le Brun, S. C. Andrews, P. M. Harrison, J. R. Guest, A. J. Thomson & G. R. Moore, unpublished work). This band, which may be diagnostic of the bis-methionine ligation of the haem in BFR (Cheesman *et al.*, 1990), has not previously been reported for BFR.

E.p.r. spectroscopy

There have been no previous detailed reports or analyses of the e.p.r. spectra of BFR from any source. The study reported here has been carried out over a temperature range between approx. 4 K and 130 K in order to seek paramagnetic centres of differing electron-spin relaxation rates, and on samples of different BFRs, holo and apo, to assign the various signals detected. The spectra of both holo BFRs show at least three distinct paramagnetic metal centres (Figs. 2 and 3). The presence of low-spin Fe^{3+} haem in *P. aeruginosa* and *A. vinelandii* BFRs is shown by a set of three g values at 2.86, 2.32, 1.48 and 2.88, 2.31, 1.46, as previously reported (Cheesman *et al.*, 1990). These g values, and the corresponding integrated spin intensities, are unchanged between holo- and apo-proteins.

The e.p.r. spectra also show evidence of high-spin Fe^{3+} haem as judged by the signals in the region $g \approx 6$. For example, in *P. aeruginosa* BFR the g values at 6.55 and 5.38 are typical of a rhombically distorted high-spin haem. High-spin haem signals are also detected in the spectrum of *A. vinelandii* BFR. However, these signals can be ascribed to a minor quantity (< 5%) of high-spin haem.

The e.p.r. spectra also contain signals in the region of $g \approx 4.3$. The spectra of most iron-containing proteins invariably contain traces of a sharp derivative-shaped signal at $g = 4.3$ which comes from the presence of non-haem Fe^{3+} adventitiously bound in a protein site of low symmetry. Although the spectra of all the BFR samples shown here undoubtedly contain some of this signal, there is additional structure which points to the presence of monomeric non-haem high-spin Fe^{3+} different from adventitious iron. This can be seen, for example, in the e.p.r. spectrum of *P. aeruginosa* BFR by the signal at $g = 3.97$. High-spin Fe^{3+} may also show signals in the region of $g \approx 8$ –9 and indeed traces of such signals are found in the spectrum of *P. aeruginosa* BFR.

Another type of e.p.r. signal can be seen in the spectrum of *P. aeruginosa* holoBFR as the temperature of the sample is raised from approx. 4 K to 130 K (Fig. 4). A broad derivative-shaped signal centred at $g = 2$ is observed in the spectrum at 10 K and this intensifies and narrows in linewidth as the temperature is raised, until at a sample temperature of 130 K this signal dominates the e.p.r. spectrum. Although some traces of the e.p.r. signals of other centres remain, the majority have been broadened by an increasing relaxation rate as the temperature has risen. The broad signal is absent from the e.p.r. spectrum of *P. aeruginosa* apoBFR (Figs. 2 and 3) up to 77 K, and it can therefore be assigned to the iron core. This core signal is also present in the e.p.r. spectrum of *A. vinelandii* holoBFR (Fig. 3b) but only becomes detectable at temperatures of 30 K or above.

In addition to the Fe^{3+} signals, the e.p.r. spectra show features close to $g \approx 2.0$, some of which may be assigned to Cu(II) ions,

Fig. 1. Electronic absorption spectra of bacterioferritins

(a) *A. vinelandii* holoBFR (127 μM) oxidized; (b) *A. vinelandii* apoBFR (54 μM) oxidized; (c) *P. aeruginosa* holoBFR (70 μM) oxidized (—), reduced (---); (d) *P. aeruginosa* apoBFR (200 μM) oxidized (—), reduced (---). Samples are in a mixture of equal volumes of buffer and glycerol. Spectra are referenced to a buffer/glycerol baseline. The concentrations given above in parentheses are those of low-spin ferric haem as determined by integration of the e.p.r. spectrum, and these values have been used to estimate ϵ values.

Fig. 2. X-band e.p.r. spectra of bacterioferritins recorded at a temperature of 10 K

(a) *A. vinelandii* apoBFR (54 μM); (b) *A. vinelandii* holoBFR (127 μM); (c) *P. aeruginosa* apoBFR (200 μM); (d) *P. aeruginosa* holoBFR (280 μM). Other details as in legend to Fig. 1.

Fig. 3. X-band e.p.r. spectra of bacterioferritins recorded at a temperature of 30 K

All other sample and instrumental details are in the legend to Fig. 2.

Fig. 4. X-band e.p.r. spectrum of *P. aeruginosa* holoBFR recorded at a temperature of 10 K

All other details are as in the legends to Figs. 2(d) and 3(d), except for the modulation amplitude, which was 20 G.

and a sharp signal centred at $g = 2.0$ attributable to an organic radical. The copper signals are probably due to adventitiously bound metal ions. The radical signal may be related to the quinone species detected in BFR (Al-Massad *et al.*, 1992).

M.c.d. spectroscopy

The m.c.d. spectra have been recorded at several temperatures between 1.6 and 20 K over the wavelength range 300–3000 nm (Figs. 5 and 6). Optical transitions can be detected throughout

this region in all three samples of BFR examined. Between 300 and 600 nm the spectra have the shape typical of low-spin Fe^{3+} haem co-ordinated by a pair of strong-field ligands (Cheesman *et al.*, 1991). The transitions in this region are primarily from the $\pi \rightarrow \pi^*$ levels of the porphyrin ring and, as a consequence, the m.c.d. spectrum is not much affected by changes in axial ligation provided that the Fe^{3+} remains low-spin. By contrast, the bands at 710 nm in Fig. 5 and at 2240 nm for the *P. aeruginosa* holoBFR in Fig. 6 are characteristic both of the type of the porphyrin ring and of the axial ligands to Fe^{3+} . A preliminary near-i.r. m.c.d. spectrum of *A. vinelandii* (not shown) reveals an almost identical band at 2270 nm. Detailed studies by McKnight *et al.* (1991) of simple model complexes of bis-co-ordinated Fe^{3+} octaethyl- and tetraphenyl-porphyrins have given an assignment of the transition peaking at 2240 nm as porphyrin-to- Fe^{3+} charge-transfer (CT). The energy of the band is dependent on the chemical nature of the axial ligand. The model studies of McKnight *et al.* (1991) show that the energies of the CT band are diagnostic of bis-thioether co-ordination. Hence the haem group in both *P. aeruginosa* and *A. vinelandii* BFRs has bis-methionine ligation. The m.c.d. spectra of the haem group in both *P. aeruginosa* and *A. vinelandii* BFR are virtually indistinguishable over the complete wavelength range, indicating that the haem-binding sites are closely similar.

The optical spectra of all low-spin ferriprotoporphyrin IX species are expected to show two one-electron CT transitions from the highest filled porphyrin π -orbitals, of symmetry a_{1u} and a_{2u} , into the partially filled Fe^{3+} d orbital, d_{yz} (Cheesman *et al.*, 1991). The studies of McKnight *et al.* (1991) show that the peak at 2240 nm is the one-electron transition $a_{1u} \rightarrow d_{yz}$ (I). The position of the CT band, $a_{2u} \rightarrow d_{yz}$ (II), has not been unambiguously located in the spectra of any low-spin ferriprotoporphyrin IX derivatives, although it can be clearly identified in low-spin Fe^{3+} -tetraphenylporphyrin complexes lying to high energy of the transition I. The m.c.d. spectra of all oxidized BFR samples show clearly a positive band with a peak at 710 nm

Fig. 5. U.v.-visible m.c.d. spectra of bacterioferritins

(a) *A. vinelandii* holoBFR; (b) *A. vinelandii* apoBFR; (c) *P. aeruginosa* holoBFR. Spectra were recorded with a magnetic field of 5T and at the following temperatures: 1.6 K (---), 4.2 K (—) and 20 K (····). The samples used were as specified in Figs. 1 and 2.

which is the counterpart of the absorption band at 690–800 nm. Since CT transition I lies at the longest wavelength yet discovered in the spectrum of low-spin Fe^{3+} haem, it may be that the band at 710 nm is CT transition II. Its energy is expected to move in

Fig. 6. Near-i.r. m.c.d. spectrum of *P. aeruginosa* holoBFR

The sample was 280 μM in low-spin ferric haem and dissolved in 25 mM-Hepes (pD 7.8)/glycerol (1:1, v/v). Spectra were recorded with a magnetic field of 5T and at the following temperatures: 1.6 K (---), 4.2 K (—) and 20 K (····).

parallel with CT transition I as the axial ligands to Fe^{3+} are varied. In the spectra of haems with different ligation, transition II may lie hidden beneath the $\pi \rightarrow \pi^*$ transition which dominates the visible-region spectrum. This assignment would imply an energy separation between CT transitions I and II of 9620 cm^{-1} .

Another possible assignment for this band is to charge-transfer from the axial thioether sulphur atom. The spectrum of histidine-methionine-co-ordinated haem contains a weak band called the '695' nm band which can lie between 720 and 660 nm, depending on the nature of the protein [Moore & Pettigrew (1990) and references therein]. The assignment of this band is controversial, but one possibility is charge-transfer from a sulphur p orbital to the $\text{Fe}^{3+} d_{yz}$ orbital. This transition is known to have appreciable out-of-plane polarization (Makinen & Churg, 1983). Hence CT from both methionine ligands to give a more intense band consisting of a single broad envelope is a possible alternative assignment. These questions of assignment are not resolved and therefore are not considered further in this work. However, although the presence of this band appears to indicate bis-methionine ligation, there is scope for confusion with the classic '695 nm' band of methionine-histidine ligation. The CT band at 2240 nm is the only absorption band unambiguously diagnostic of bis-methionine co-ordination of low-spin Fe^{3+} protohaem but this transition can only be detected by near-i.r. m.c.d. spectroscopy.

The m.c.d. spectra of apo- and holo-BFR are identical to within experimental limits. This shows first, and most significantly, that the frequencies of the CT transitions of the haem moiety are unaffected by the presence of the iron core. Secondly there is no detectable contribution to the m.c.d. spectrum from the core in its oxidized state over the temperature range 4.5–20 K. Hence the background absorption below 400 nm (Fig. 1) arising from the core gives no temperature-dependent m.c.d. signals between 1.6 and 20 K. Indeed, we have been unable to detect any m.c.d. signals below 20 K attributable to the core iron.

DISCUSSION

The results show that by means of a combination of absorption, m.c.d. and e.p.r. spectroscopies three distinct types of metal centre in BFR can be detected. They are the haem group, the iron core and monomeric non-haem iron.

The haem of BFR

The e.p.r. and m.c.d. spectra of the low-spin haem in BFR as isolated from *P. aeruginosa* and *A. vinelandii* are virtually identical with one another. This is consistent with both proteins having bis-methionine ligation of the haem group. It is perhaps significant that the e.p.r. g values are so similar, since proteins from different species having a common haem ligation usually show some variation in g values, even when they have a common tertiary structure. For example, in the His-Met-co-ordinated cytochromes c_2 , g_z varies from 3.02 to 3.24 (Moore, 1985). The similarity in BFR g values indicates not only that the haem ligation is the same but also that ligand orientations relative to the haem plane must be very similar.

It is surprising that the presence of the core does not change either the haem e.p.r. or the m.c.d. spectra. This is an important result in view of the measurements of Watt *et al.* (1986) showing that the midpoint reduction potential of the haem of *A. vinelandii* BFR depends strongly on the presence of the core, changing from –225 mV for apoBFR to –475 mV for holoBFR. We interpret these spectroscopic and potentiometric data as indicating that the haem of BFR is not directly affected by short-range bonding interactions with the core. Hence it may be located towards the outer edge of the protein shell, even exposed to bulk solvent. Removal of the core does not cause any conformational change in the protein shell affecting the haem environment, and hence the haem spectroscopic signals are unaffected by the core.

The change in the redox potential of the haem group when the core is removed (Watt *et al.*, 1986) shows that there is a perturbation of the electrostatic properties of the protein caused by the core. This could be either a change in the net charge of the protein, or a change in the local charge distribution, such that the haem iron experiences a more negative electric field in the presence of the core than in its absence. This would lead to a decrease in the haem redox potential. Analogous effects have been observed for a variety of metalloproteins, and relatively small changes in charge can cause substantial redox potential differences. For example, the ionization of a carboxylic acid group of some monohaem cytochromes causes a decrease in redox potential of approx. 65 mV (Leitch *et al.*, 1984).

It was somewhat unexpected to find a bis-methionine-co-ordinated haem with such a low redox potential. One explanation is that the haem is located in a region of the protein with a relatively high local negative charge density arising from, for example, carboxylic acid groups.

The binding site of the haem within BFR is not known. However, the unusual bis-methionine co-ordination does place limitations on the possible sites. Although the three-dimensional structure of BFR has not yet been determined, secondary-structure predictions based on the sequence of *E. coli* BFR

indicate a high α -helical content consistent with the presence of a ferritin-like 4- α -helical bundle (Andrews *et al.*, 1989). Preliminary modelling (Le Brun *et al.*, 1992) points to two sites with potential for haem binding. One site, involving methionine residues 31 and 86, lies within one subunit near the external molecular surface. The other site lies between two subunits with ligands Met-52 from two subunits (Andrews *et al.*, 1991). A complete sequence has been determined only for *E. coli* BFR (Andrews *et al.*, 1989) but partial sequences are available for *A. vinelandii* BFR (Andrews *et al.*, 1991), for *Nitrobacter winogradskyi* cytochrome *b559* (Kurokawa *et al.*, 1989), for *P. aeruginosa* BFR (J. Keen, G. R. Moore & J. B. C. Findlay, unpublished work) and for *Synechocystis* PCC 6803 BFR (Laulhere *et al.*, 1992). Met-31 is not conserved in the *N. winogradskyi* protein, nor is Met-52 in the *Pseudomonas* or *Synechocystis* proteins. However, Met31 is present in *A. vinelandii*, *P. aeruginosa* and *E. coli* BFRs. Hence the haem-binding site is not established with certainty.

The core of BFR

The chemical natures of the cores of animal ferritin and bacterioferritin are different (Stiefel & Watt, 1979; Ford *et al.*, 1984; Moore *et al.*, 1986; St. Pierre *et al.*, 1989). Although all show an electron-opaque core at low resolution in the electron microscope, at high resolution differences are apparent (Mann *et al.*, 1986). These differences are borne out by the iron and phosphorus analyses and by the magnetic properties of the cores as detected by ^{57}Fe Mössbauer spectroscopy. The iron cores of ferritin appear to contain crystalline domains similar to the mineral ferrihydrite $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$ (Towe & Bradley, 1967). However, ferritin cores may also contain a substantial but variable amount of inorganic phosphate. The cores of *A. vinelandii* and *P. aeruginosa* BFR contain higher Fe/P ratios, closer to 1–2 (Watt *et al.*, 1986; Moore *et al.*, 1986). The crystallinity of the core is poor or amorphous. The Mössbauer spectra show that the magnetic ordering of the cores in BFR are different from those of ferritin, and that the cores of *A. vinelandii* and *P. aeruginosa* BFR differ from one another (Watt *et al.*, 1986; St. Pierre *et al.*, 1986).

The iron cores are proposed to consist of antiferromagnetically ordered Fe^{3+} ions. In the case of perfect crystals, a transition to a state of ordered spins, with zero resultant magnetic moment, occurs below the ordering temperature, T_N , called the Néel point. However, because of the imperfect shape of the core particles the two sub-lattices of Fe^{3+} ions do not completely cancel and each core crystallite carries a small net magnetic moment, arising from the so-called uncompensated electron spins. Because the crystallites are anisotropic, the uncompensated spins lie along a preferred crystalline axis (the easy axis). At temperatures at which the thermal energy, kT , is greater than the anisotropy energy, the net magnetic moment can fluctuate in direction between various easy axes and hence give rise to a form of paramagnetism known as superparamagnetism. At T_b , the blocking temperature, these thermal fluctuations are frozen out. T_b depends on the volume of the core crystallites, the magnitude of the anisotropy energy and the relaxation or observation time of the experimental techniques used to measure it. Hence the chemical composition and the crystallinity determine the magnitude of T_b . In the presence of an external applied magnetic field which generates a Zeeman energy greater than the blocking energy, kT_b , the superparamagnetic relaxation takes the form of fluctuations of the magnetization vector about the field direction with characteristic relaxation time.

The ordering temperature, T_N , and the blocking temperature, T_b , of the cores of *A. vinelandii* and *P. aeruginosa* BFR have been determined by Mössbauer spectroscopy to be higher than 20 K

and approx. 3 K respectively for T_N and approx. 18 K and above 3 K for T_B . These values are in marked contrast with those reported for horse and human ferritin of T_B approx. 35–40 K and T_N higher than > 40–50 K [see St. Pierre *et al.* (1989) and references therein].

The e.p.r. spectra of horse spleen ferritin show no broad core signals at 5 K, but, on raising the temperature towards 100 K, a derivative-shaped signal, hundreds of gauss in width and centred at $g \approx 4.0$, is observed (Weir *et al.*, 1984). On raising the temperature further, this signal passes over into a narrower derivative band centred at $g = 2.0$. These properties have been interpreted in terms of the resonance of a spin vector arising from the uncompensated spins of antiferromagnetically ordered crystallites with dimensions of the core cavity size (approx. 8 nm). At very low temperature no superparamagnetic relaxation takes place and all the spins are ordered. No e.p.r. signal is possible since there is no net moment. On warming towards 100 K, paramagnetic relaxation increases but it is highly anisotropic. This gives rise to a signal at $g \approx 4.0$ which is one component of an anisotropic g tensor. As the temperature is raised further, the relaxation of the superparamagnetic moment increases and the time average of the anisotropy decreases. The e.p.r. signal consequently becomes more isotropic centred at $g = 2.0$.

The e.p.r. spectra of the cores of BFR samples from *P. aeruginosa* and *A. vinelandii* make an interesting contrast with the e.p.r. properties of the horse spleen ferritin core. At liquid-helium temperatures the signal is absent, as is the case in ferritin. This suggests that the core has become ordered. In the case of the *P. aeruginosa* core this is not consistent with the Mössbauer proposal of an ordering temperature, T_N , of approx. 3 K. On warming the core, a broad e.p.r. signal appears, which narrows with increasing temperature but is always centred at $g \approx 2.0$. An anisotropic core signal centred at $g = 4.00$ has not been detected in these BFR samples. This suggests that the magnetic anisotropy may be much lower in magnitude in BFR cores compared with the ferritin core.

Clearly the e.p.r. signals of the BFR core contain much valuable information about the relaxation states of the uncompensated spins and, indeed, may permit particle-size analysis to be made. The present work reports for the first time the e.p.r. signals of BFR cores. It remains for future studies of the temperature variation of both Mössbauer and e.p.r. spectra, together with high-resolution electron-microscope studies, using identical samples with chemically well-defined cores, to determine if core size and morphology can be obtained by e.p.r.

The lack of intense m.c.d. signals from the iron core at temperatures below 20 K is due to the antiferromagnetic coupling between the electron spins. The Mössbauer spectra show a high internal magnetic field with BFR cores below the superparamagnetic ordering temperature. Individual iron atoms are therefore subject to a high local internal field. However, the magnetic hyperfine splitting detected by Mössbauer spectroscopy is not sensitive to the direction of the local field, since the sign of the magnetic splitting is not detected by the Mössbauer effect unless circularly polarized γ -rays are used. However, the sign of an m.c.d. signal arising from an internal magnetic field depends on the direction of that field relative to that of the light beam. Since the iron core lattice is antiferromagnetically coupled there must be equal, but oppositely signed, internal magnetic fields acting in each sub-lattice. Hence there will be equal numbers of iron atoms giving rise to m.c.d. signals of opposite sign and therefore a resultant of zero.

We thank the Wellcome Trust for their support of our work on

for their support of the UEA Centre for Metalloprotein Spectroscopy and Biology. We also thank Professor G. D. Watt and Professor E. I. Stiefel for interesting discussions. These workers have independently carried out e.p.r. studies of *A. vinelandii* BFR with broadly similar results and conclusions to ours.

REFERENCES

- Aasa, R. & Vänngård, T. (1975) *J. Magn. Reson.* **19**, 308–315
- Al-Massad, F. K., Kadir, F. H. A. & Moore, G. R. (1992) *Biochem. J.* **283**, 177–180
- Andrews, S. C., Smith, J. M. A., Guest, J. R. & Harrison, P. M. (1989) *Biochem. Biophys. Res. Commun.* **158**, 489–496
- Andrews, S. C., Smith, J. M. A., Yewdall, S. J., Guest, J. R. & Harrison, P. M. (1991) *FEBS Lett.* **293**, 164–168
- Boas, J. F. & Troup, G. J. (1971) *Biochim. Biophys. Acta* **229**, 68–74
- Bulen, W. A., LeCompte, J. R. & Lough, S. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1274–1281
- Cheesman, M. R., Thomson, A. J., Greenwood, C., Moore, G. R. & Kadir, F. H. A. (1990) *Nature (London)* **346**, 771–773
- Cheesman, M. R., Greenwood, C. & Thomson, A. J. (1991) *Adv. Inorg. Chem.* **36**, 201–255
- Crichton, R. R. (1990) *Adv. Protein Chem.* **40**, 281–363
- Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L. & Yariv, J. (1984) *Philos. Trans. R. Soc. London* **304**, 551–565
- Harrison, P. M., Andrews, S. C., Artymiuk, P. J., Ford, G. C., Guest, J. R., Hirzmann, J., Lawson, D. M., Livingstone, J. C., Smith, J. M. A., Treffry, A. & Yewdall, S. J. (1991) *Adv. Inorg. Chem.* **36**, 449–486
- Jiudi, L., Jiwen, W., Zepu, Z., Ya, T. & Bei, D. (1980) *Sci. Sin. Engl. Ed.* **23**, 897–904
- Kurokawa, T., Fukumori, Y. & Yamanaka, T. (1989) *Biochim. Biophys. Acta* **976**, 135–139
- Laulhere, J. P., Labouré, A.-M., Van Wuytswinkel, O., Gagnon, J. & Briat, J.-F. (1992) *Biochem. J.* **281**, 785–793
- Le Brun, N. E., Cheesman, M. R., Kadir, F. H. A., Thomson, A. J., Moore, G. R., Andrews, S. C., Smith, J. M. A., Guest, J. R. & Harrison, P. M. (1992) *J. Inorg. Biochem.* **47**, in the press
- Leitch, F. A., Moore, G. R. & Pettigrew, G. W. (1984) *Biochemistry* **23**, 1831–1838
- Makinen, M. W. & Churg, A. K. (1983) in *Iron Porphyrins* (Lever, A. B. P. & Gray, H. B., eds.), vol. 1, chapter 3, pp. 141–235, Addison-Wesley Publishing Co., Reading, MA
- Mann, S., Bannister, J. V. & Williams, R. J. P. (1986) *J. Mol. Biol.* **188**, 225–232
- McKnight, J., Cheesman, M. R., Reed, C. A., Orosz, R. D. & Thomson, A. J. (1991) *J. Chem. Soc., Dalton Trans.* 1887–1894
- Moore, G. R. (1985) *Biochim. Biophys. Acta* **829**, 425–429
- Moore, G. R. & Pettigrew, G. W. (1990) *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects*, Springer-Verlag, New York and Heidelberg
- Moore, G. R., Mann, S. & Bannister, J. V. (1986) *J. Inorg. Biochem.* **28**, 329–336
- Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W. & Melling, J. (1976) *Biochem. J.* **157**, 423–430
- Perrin, D. D. & Dempsey, B. (1974) *Buffers for pH and Metal Ion Control*, p. 81, John Wiley and Sons, New York
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Stiefel, E. I. & Watt, G. D. (1979) *Nature (London)* **279**, 81–83
- St. Pierre, T. G., Bell, S. H., Dickson, D. P. E., Mann, S., Webb, J., Moore, G. R. & Williams, R. J. P. (1986) *Biochim. Biophys. Acta* **870**, 127–134
- St. Pierre, T. G., Webb, J. & Mann, S. (1989) in *Biomimetalisation* (Mann, S., Webb, J. & Williams, R. J. P., eds.), pp. 295–344, VCH, Weinheim
- Towe, K. M. & Bradley, W. F. (1967) *J. Colloid Interface Sci.* **24**, 384–392
- Watt, G. D., Frankel, R. B. & Papaefthymiou, G. C. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3640–3643
- Watt, G. D., Frankel, R. B., Papaefthymiou, G. C., Spartalian, K. & Stiefel, E. I. (1986) *Biochemistry* **25**, 4330–4336
- Weir, M. P., Gibson, J. F. & Peters, T. J. (1984) *Biochem. Soc. Trans.* **12**, 316–317
- Yariv, J., Kalb, A. J., Sperling, R., Bauminger, E. R., Cohen, S. G. & Ofer, S. (1981) *Biochem. J.* **197**, 171–175