Structural heterogeneity of *Pseudomonas aeruginosa* bacterioferritin

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The subunit composition, amino acid sequence and haem-binding characteristics of bacterioferritin (BFR) from *Pseudomonas aeruginosa* have been studied. Unlike other BFRs, *P. aeruginosa* BFR was found to contain two subunit types, designated α and β, which differed considerably in their amino acid sequences. The N-terminal 69 and 55 amino acids of the α and β subunits respectively were determined. The α subunit differed most from other BFRs. The two subunits were present in variable proportions in different preparations. The maximum stoichiometry of haem binding was found to be sample-dependent and to be different from the previously reported one per subunit [Kadir and Moore (1990) FEBS Lett. **271**,141–143]. This previous haem-binding study was shown to have been carried out with damaged protein, which contained both normal α and β subunits and shorter versions of these that appeared to have been produced by cleavage of the normal subunits. The possibility that aging processes degrade ferritins and affect their haem-binding characteristics is discussed.

INTRODUCTION

The bacterial iron-storage protein bacterioferritin (BFR) has been isolated from *Azotobacter vinelandii* (Stiefel and Watt, 1979), *Escherichia coli* (Yariv et al., 1981), *Pseudomonas aeruginosa* (Moore et al., 1986), *Rhodobacter capsulatus* and *Rb. sphaeroides* (Meyer and Cusanovich, 1985; Cheesman et al., 1990), *Synechocystis* PCC6803 (Laulhère et al., 1992), *Mycobacterium paratuberculosis* and *M. leprae* (Hunter et al., 1990; Brooks et al., 1991) and *Nitrobacter winogradskyi* (Kurokawa et al., 1989). Important characteristics of these proteins, as isolated, are that they consist of 24 subunits of 17–19 kDa molecular mass, contain 8–10% by weight of non-haem iron, and have 3–12 non-covalently bound haem groups per 24 subunits. Their three-dimensional structures appear to bear a strong resemblance to those of animal ferritins, as indicated by low-resolution X-ray diffraction data (Smith et al., 1989; Frolow et al., 1993) and molecular modelling studies (Grossman et al., 1992; Cheesman et al., 1993). However, despite the evident similarities between the BFR and animal ferritin classes of protein, and the marked functional and spectroscopic similarities within the BFR group, there are major differences between different groups of iron-storage proteins. Thus BFRs contain intrinsically bound haem (Stiefel and Watt, 1979) attached to two methionine residues (Cheesman et al., 1990), whereas ferritins do not possess intrinsic haems; and although some BFRs, such as those of *E. coli* and *A. vinelandii* (Andrews et al., 1989; Grossman et al., 1992) appear to consist of only one type of subunit, *P. aeruginosa* BFR consists of two (Moore et al., 1986; Al-Massad et al., 1992) and animal ferritins two or three. There is a further complication with the BFRs: wild-type *E. coli* and *A. vinelandii* BFRs contain 12 haem groups in 24 subunits and do not bind additional haem (Stiefel and Watt, 1979; Yariv et al., 1981; Smith et al., 1988), whereas wild-type *P. aeruginosa* BFR contains 3–9 haem groups in 24 subunits as isolated and can bind additional haem *in vitro* up to a maximum of 24 in 24 subunits (Kadir and Moore, 1990). One possible explanation for this difference is that haem binding to some BFRs is negatively co-operative, with only half the sites able to accommodate haem, whereas in other BFRs all sites can bind haem (Moore et al., 1992). The implication of such a model is that each of the subunits contains an intra-subunit haem-binding site or participates in the formation of two inter-subunit binding sites. The low-resolution structure of *E. coli* BFR proposed by Frolow et al. (1993) has two inter-subunit sites per subunit, to give a maximum of 24 such sites, but because of space limitations only 12 can be occupied. However, the molecular modelling studies of Grossman et al. (1992) and Cheesman et al. (1993) have proposed possible bis-methionine inter-subunit haem-binding sites that are only present to a maximum of 12 per 24 subunits. Since each subunit contributes only one methionine ligand, this is not compatible with the haem-binding studies of *P. aeruginosa* BFR reported by Kadir and Moore (1990). In all these comparisons of BFRs it is the *P. aeruginosa* BFR that appears unusual. Therefore we have re-investigated haem binding to *P. aeruginosa* BFR. The results are reported in the present paper, together with the partial amino acid sequences of the two subunits of *P. aeruginosa* BFR, and chromatographic data showing that preparations of this protein are indeed heterogeneous as far as haem content is concerned. During the course of this work, we have become aware that the published isolation procedure for *P. aeruginosa* BFR is not wholly satisfactory, and therefore we also describe an alternative procedure for purifying this protein.

EXPERIMENTAL

Materials

Bovine pancreas DNAase and RNAase, types DN-25 and 1-AS respectively, were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All other reagents were obtained from Merck (Poole, Dorset, U.K.) or Sigma Chemical Co.

Biochemical manipulations and analysis

Protein concentrations were determined by the method of Lowry et al. (1951). Subunit molecular masses were determined by

Abbreviation used: BFR, bacterioferritin.

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SDS/PAGE (Laemmli, 1970) using 15% polyacrylamide gels containing 0.1% (w/v) SDS. The running buffer was 0.025 M Tris/HCl/0.19 M glycine/0.1% SDS at pH 8.6. BDH 'Electran' molecular-mass markers were used as standards. Haem contents were estimated optically by using the absorption coefficient of *E. coli* oxidized BFR reported by Cheesman et al. (1993): $\epsilon_{418} = 109\ mM^{-1} \cdot cm^{-1}$.

Growth of cells

P. aeruginosa cells (N.C.T.C. 6750) were grown in a 100-litre fermenter either anaerobically with the high-nitrate medium of Parr et al. (1976) or micro-aerobically (2% saturated with O_2) at pH 7.4 on a low-nitrate medium consisting of 4 g of yeast extract, 5 g of KNO_3, 5 g of sodium citrate, 0.5 g of $MgSO_4$ and 3 mg of $FeCl_3$ per litre. The latter cells were grown originally for the production of cytochrome *c* peroxidase, which is enhanced by micro-aerobic growth compared with anaerobic growth. All cells were frozen and stored at −20 °C until used for the extraction of BFR. Usually cells were stored at −20 °C for no more than 2 months, but some cells were stored at −20 °C for up to 12 months.

Isolation of BFR

The frozen cell paste was thawed and homogenized in 2 vol. of 25 mM sodium phosphate buffer (pH 7.4) containing 0.1% Triton X-100. The cell suspension was then passed through a Manton–Gaulin Homogenizer at a flow rate of 2 litres/min and a pressure of 70 MPa (700 bar). Three passes through the homogenizer were sufficient to break most of the cells. The temperature was not controlled during this stage of the procedure, and it reached a maximum of 45 °C. After disruption of the cells, the homogenate was cooled to 4 °C and DNAase (0.5 mg/l), RNAase (0.5 mg/l) and $MgSO_4,7H_2O$ (0.5 mg/l) were added. The mixture was left overnight at 4 °C and then centrifuged at 15000 *g* for 60 min. $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 30% (w/v) and the resulting mixture was centrifuged at 15000 *g* for 60 min. The supernatant was then fractionated with $(NH_4)_2SO_4$ over the concentration range 40–90% (w/v) in 10% (w/v) steps. At each stage the precipitate was collected by centrifugation, redissolved in 25 mM phosphate buffer (pH 7.4), and the absorption spectrum of the resulting solution was measured. BFR appeared in the precipitates resulting from 40–60% $(NH_4)_2SO_4$, and in a typical bulk preparation the precipitate resulting from 1.5 kg of cells would be redissolved in 50 ml of buffer.

The solution obtained from redissolving the $(NH_4)_2SO_4$ precipitate was dialysed exhaustively against 25 mM phosphate buffer (pH 7.4) at 4 °C and then applied to a Whatman DEAE-cellulose DE-52 column which had been equilibrated with the same buffer. After loading, the column was washed for 15 h with the phosphate buffer, and the BFR was eluted with the same buffer containing 0.3 M NaCl. In some preparations this chromatography stage was repeated. The eluate containing BFR was dialysed against 25 mM phosphate buffer (pH 7.4) at 4 °C, concentrated with an Amicon ultrafiltration cell containing a PM-30 membrane, and then applied to a Sephacryl S-300 gel-filtration column equilibrated with 25 mM phosphate buffer (pH 7.4). The eluate was monitored spectrophotometrically at 417 nm for the haem of BFR, and the fractions containing BFR were combined and subjected to ultracentrifugation at 150000 *g* for 16 h. The brown pellet was resuspended in 25 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, and the cycle of ultracentrifugation and resuspension continued until the A_{278}/A_{417} ratio remained constant. This usually required ~ 6 cycles. Protein isolated by this procedure was used for magneto-optical experiments (Cheesman et al., 1990, 1992).

For some experiments, portions of the BFR were further purified by f.p.l.c. using a Superose TM-12 column equilibrated with 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA.

Core-iron-free BFR (apo-BFR) was obtained from holo-BFR by exhaustive dialysis against 0.12 M thioglycollic acid at pH 5.0 and 4 °C, followed by dialysis against 25 mM phosphate buffer (pH 7.4). It generally took 5–7 days to remove all the core iron. For some experiments apo-BFR was prepared from impure holo-BFR obtained after the DE-52 column step, and the apoprotein was purified by the chromatographic procedures described for the holoprotein.

Haem-binding studies

The maximum haem loading of BFR was investigated through a series of haem titration experiments by the procedure used by Kadir and Moore (1990).

N-terminal amino acid sequence determination

Sequencing was performed with an automated solid-phase sequencer essentially as described by Findlay et al. (1989). In summary, freeze-dried protein was dissolved in 0.2 M $NaHCO_3$/0.25% SDS at pH 8.5, and coupled at 56 °C for 60 min under N_2 to di-isothiocyanate-glass (17 nm pore size, 200–400 mesh). After washing, the coupled protein was subjected to automated solid-phase Edman degradation. The derived anilinothiazolinone-amino acids were converted at 70 °C for 20 min in aq. 30% (v/v) trifluoroacetic acid into the corresponding phenylthiohydantoin derivatives and identified by reverse-phase (C_{18}) microbore h.p.l.c. using a gradient of acetonitrile in sodium acetate at pH 4.9. Particular attention was paid to the location of methionine residues and the identity of residue 52, given their possible significance for the identification of haem-binding sites (see the Results and discussion section).

RESULTS AND DISCUSSION

Isolation of *P. aeruginosa* BFR

The isolation of BFR was monitored by u.v.–visible spectroscopy, and the wavelength of the haem Soret band was found to be a sensitive indicator of the purity of the preparation. With increasing purification the band shifted from 412 nm to 417 nm, and this coincided with the removal of contaminating proteins as judged by SDS/PAGE. The A_{278}/A_{417} ratio was found to be a good guide to purity for the non-haem-iron-free BFR. Figure 1(a) shows a typical f.p.l.c. trace for a sample of apo-BFR prepared from holo-BFR after ultracentrifugation. The main band is BFR, but the different fractions comprising this band have different u.v.–visible spectra (Figure 1b). These spectra indicate that the haem content varies for the different fractions, with the fractions being eluted in the order of those with greatest haem content first: fraction 5 contained 3.8 haems per molecule; fraction 6, 2.9 haems per molecule, and fraction 7, 2.4 haems per molecule.

Subunit composition

SDS/PAGE showed that the *Pseudomonas* protein consists of two types of subunit (see, for example, Figure 4 of Al-Massad et al., 1992), with molecular masses of 18 and 19 kDa. The relative proportions of these two appear to vary in different growths, as judged by the relative intensities of the bands on the stained gels (results not shown). It has not been possible to correlate the

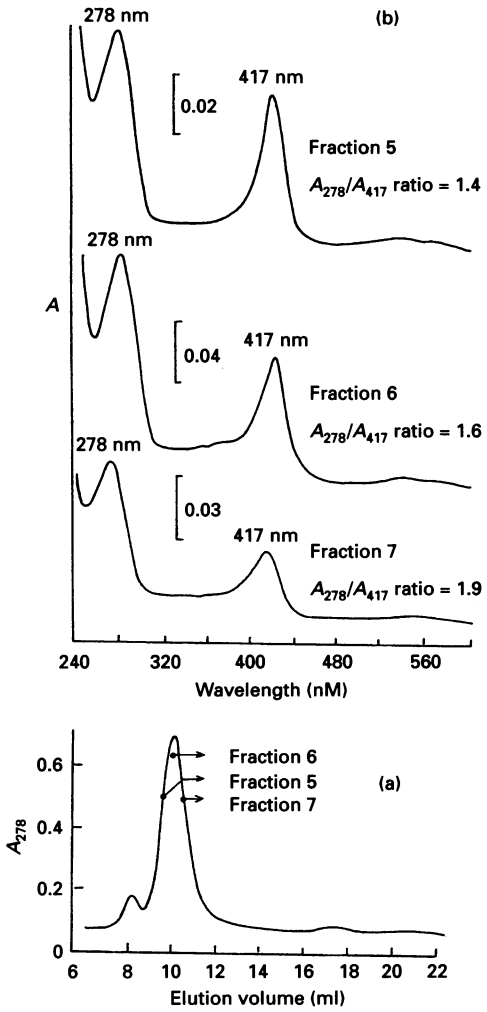

Figure 1 Separation of apo-BFR

(**a**) F.p.l.c. profile for apo-BFR prepared from holo-BFR after six cycles of ultracentrifugation. A Superose-12 column and 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA was used for the f.p.l.c. The main band eluted with a volume of 9–11 ml is apo-BFR. (**b**) u.v.–visible absorption spectra of the f.p.l.c. fractions indicated in (**a**).

subunit composition with growth factors, since complex media and large-scale batch cultures were used to grow the cells. The amino acid sequence studies supported the above observations, with two components being identified in variable proportions in different batches of protein. The major component (designated subunit α) was present at a 4:1 excess over the minor component (designated subunit β) in some preparations, allowing the two sequences to be deduced. However, a ratio of 1:1 was encountered in other preparations. Animal ferritins also consist of two types of subunit, known as heavy and light chains (Arosio et al., 1978; Theil, 1987; Levi et al., 1989; Harrison et al., 1991): and, although most other studies of BFRs have emphasized that they consist of a single type of subunit, Harker and Wullstein (1985) have reported that *A. vinelandii* BFR consists of two types of subunit. Since Grossman et al. (1992) only cloned one gene for this protein, the observations by Harker and Wullstein (1985) need to be re-investigated.

In one sample of *P. aeruginosa* BFR that was prepared from micro-aerobically grown cells, some of which had been stored at −20 °C for up to 12 months, there were four polypeptides detected. N-terminal amino acid sequencing revealed that the sample contained the two normal subunits plus two shorter versions of these which appeared to have undergone cleavage towards their C-terminus. This cleaved sample was not investigated further, though before the discovery of the degradation it was used in a study of haem binding (Kadir and Moore, 1990; Moore et al., 1992).

Amino acid sequence determination

The first 69 residues of the amino acid sequence of the α subunit, and the first 55 residues of the sequence of the β subunit, of *P. aeruginosa* BFR were determined and aligned with the N-terminal sequences of other BFRs (Figure 2). The alignment reveals that there are only 15 identities out of the first 42 amino acids for the six sequences in Figure 2. Pair-wise comparisons (Figure 3) reveal that the *Pseudomonas* α-subunit partial sequence is more different from the *Pseudomonas* β-subunit partial sequence than the β sequence is from any of the other sequences. The comparison of the β sequence with the *E. coli* and *A. vinelandii* sequences is particularly striking for the high degree of identity. If the overall identity remains at about 33% once the complete amino acid sequences are known, then the BFR family will be less similar as a group than the animal H-chain and L-chain ferritins (see compilations by Andrews et al., 1992; Grossman et al., 1992).

Interestingly, both *Pseudomonas* sequences have glutamates at positions 18 and 51, which have been proposed to be important for ferroxidase activity (Grossman et al., 1992; Cheesman et al., 1993). However, not all the proposed ferroxidase residues are invariant; *Synechocystis* BFR has Ala-54 in place of His-54, though perhaps its His-53 plays the role normally taken by His-54 in other BFRs.

The *P. aeruginosa* BFR partial sequences help to identify the two methionine ligands to the haem iron indicated by e.p.r. and magnetic-c.d. studies (Cheesman et al., 1990). Comparison of *E. coli* and *A. vinelandii* BFR sequences reveal that only Met-1, Met-31, Met-52 and Met-86 are conserved in both sequences (Grossman et al., 1992; Cheesman et al., 1993). Met-31 was eliminated as a haem ligand because of its absence from *N. winogradskyi* BFR (Kurokawa et al., 1989), and models were proposed based on haem ligation to Met-1, Met-52 and Met-86. Molecular modelling studies by both Grossman et al. (1992) and Cheesman et al. (1993) suggested an intersubunit site composed of Met-52 residues from adjacent subunits, or intrasubunit sites involving Met-31 and Met-86 (Cheesman et al., 1993) or Met-1 and Met-86 (Grossman et al., 1992). The absence of Met-52 from *Synechocystis* BFR and its replacement by threonine was either not considered or suggested to be an error. An alternative explanation, however, is that Met-48 of *Synechocystis* BFR plays the role that Met-52 of the other BFRs carries out. This would be consistent with the presence of Met-48 and Thr-52, instead of Met-52, in the *P. aeruginosa* BFR α-subunit (Figure 2). According to the molecular modelling studies, both residues 48 and 52 are contained in the second α-helix of the structure and, given the difference in sequence position, side chains of both residues should be on the same side of the helix. Thus proposals for the methionine ligands for the haems of BFR should remain with Met-1, Met-48/-52 and Met-86.

Haem binding to *P. aeruginosa* BFR

Two samples of holo-BFR from anaerobically grown cells containing one and seven haem groups per molecule respectively were treated by addition of portions of a solution of haemin

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                1                  10                  20                  30
E. coli         M K G D T K V I N Y L N K L L G N E L V A I N Q Y F L H A R M F K N W G L K R
A. vinelandii   M K G D K I V I Q H L N K I L G N E L I A I N Q Y F L H A R M Y E D W G L E K
P. aeruginosa β M K G D K K V I Q H L N K I L G N E L I A I N Q Y F L H S R M W N D W G L K R
N. winogradskyi M K G D P K V I D Y L N K A L R H E L T A I N Q Y W L H Y R L L D N W G I K D
M. paratuberculosis M Q G D P E V L R L L N E Q L T S E L T A I N Q Y F L H S K M Q D N W G F T E
Synechocystis   M K G K P A V L A Q L H K L L R G E L A A R D Q Y F I H S R M Y Q D W G L E K
P. aeruginosa α M Q G H P E V I D Y L N T L L T G E L A A R D Q Y F I H S R M Y E D W G F S K

                40                  50                  60                  70
L N D V E Y H E S I D E M K H A D R Y I E R I L F L E G L P N
L G K H E Y H E S I D E M K H A D K L I K R I L F L E G L P N
L Y A H L Y H E S I D E M K X A
L A K K W R A E S I E
L A E
L Y S R I D H E M Q D E T H A
L Y E R L N H E M E E E T Q H A X X L L R R I L X L E G T P
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Figure 2 N-terminal amino acid sequences of the α and β subunits of *P. aeruginosa* BFR and their comparison with the sequences of *E. coli* (Andrews et al., 1992), *A. vinelandii* (Grossman et al., 1992), *N. winogradskyi* (Kurokawa et al., 1989), *M. paratuberculosis* (Brooks et al., 1991) and *Synechocystis* PCC 6803 (Laulhère et al., 1992) BFRs

Residues in **bold** are constant. X indicates residues that have not been unambiguously identified.

	E.c	*A.v*	*P.aβ*	*N.w*	*M.p*	*Syn.*	*P.aα*
E.c	X						
A.v	72	X					
P.aβ	74	82	X				
N.w	60	54	56	X			
M.p	52	50	52	57	X		
Syn.	48	54	52	42	45	X	
P.aα	46	50	50	44	57	66	X

Figure 3 Matrix of pair-wise sequence comparisons for amino acid sequences in Figure 2 expressed as percentage identities

All comparisons are for the N-terminal 50 residues only, except for those involving *M. paratuberculosis* (*M.p*) BFR, which are for the N-terminal 42 residues. Other abbreviations: *E.c, E. coli; A.v, A. vinelandii; P.a, P. aeruginosa; N.w, N. winogradskyi; Syn., Synechocystis.*

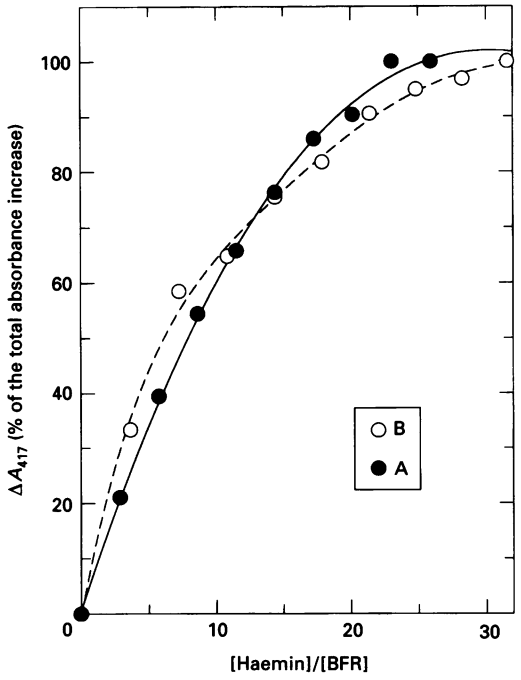

Figure 4 Haem binding to *P. aeruginosa* BFR

Titration curves A and B were obtained from different samples of apo-BFR at a concentration of 0.5 mg/ml, in 0.1 M phosphate (pH 7.0). The ● and ○ symbols are normalized experimental data points. The normalization procedure was as follows: the maximum increase in A_{417} observed in each titration (ΔA_{417} = 0.114 and 0.159 for A and B respectively) was assumed to correspond to 100% haem binding. A_{417} changes measured during the titration were then converted into a percentage of full saturation, and plotted against the appropriate haem:BFR ratio.

chloride in accordance with the procedure described by Kadir and Moore (1990). Difference absorption spectra in the visible region were recorded after each addition of haemin chloride, and binding curves were constructed of the change in A_{417} against the molar ratio of added haem:ferritin. Two typical normalized binding curves are shown in Figure 4. The curves had different maximum absorbance changes, and so they have been normalized to each other for ready comparison. The shapes of the curves are different. In general, we have found with *P. aeruginosa* and *E. coli* BFRs that haem binding follows one of two patterns: one in which a large initial increase in absorption (indicative of binding) is observed, followed by smaller absorption changes; and the other in which a smooth increase in absorption is observed. *E. coli* BFR is invariably found to follow the former pattern (see Cheesman et al., 1993), whereas *P. aeruginosa* BFR has been observed to follow mainly the latter pattern. However, one of the freshly prepared samples of *P. aeruginosa* BFR used in this study has been found to follow a binding pattern similar to that found with *E. coli* BFR. Curve A in Figure 4 is typical of that observed previously for *P. aeruginosa* BFR and analysis by the method used by Kadir and Moore (1990), which was based on normal saturation binding plots assuming no co-operativity, a method previously shown to describe haem binding to albumin satisfactorily (Bearden et al., 1974; Beaven et al., 1974), indicated that ~ 14 haem groups have been added to each molecule of BFR. Since they already contained ~ 1 haem group per molecule, these data indicate that this sample of *P. aeruginosa* BFR can only bind 15 haem groups per molecule. Curve B (Figure 4) is of the type previously found for addition of haemin chloride to *E. coli* recombinant BFR (Cheesman et al., 1993). Such curves are

not open to simple analysis, but, even assuming that the procedure used to analyse curve A was applicable, only ~ 12 haem groups per molecule are required to saturate the binding sites. Together with the 7 intrinsic haem groups per molecule, this gives a maximum haem loading of ~ 19 haems per molecule. However, much of the additional haem bound to this sample of BFR was in the high-spin state, as indicated by absolute u.v.–visible absorption spectra.

In summary, haem binding to two freshly prepared samples of *P. aeruginosa* BFR gave results different from each other and different from those previously reported. Although the shapes of the curves for the two samples studied herein differed, the key point is that in neither case was 24 haems per molecule bound. Thus the haem- binding properties of *P. aeruginosa* BFR are sample-dependent.

One explanation for the haem-binding data is that some aging or degradative process occurs to the BFR molecule which affects haem binding. If BFR contains 24 haem-binding sites, as the X-ray structure of Frolow et al. (1993) indicates, and there is negative co-operativity between some of these sites that prevents their full occupation, then a degradative process that leads to a relaxation of the negative co-operativitiy might allow full occupation of the binding sites. Alternatively a degradative process could open up other binding sites that are not occupied in native BFR. Such explanations would account for the early work on haem binding to *P. aeruginosa* BFR (Kadir and Moore, 1990; Moore et al., 1992) which was carried out with damaged proteins (see discussion above on subunit composition), and might also account for the variable haem-binding characteristics of *E. coli* recombinant BFR (Cheesman et al., 1993). Related studies of haem binding to animal ferritin have shown that this too is sample-dependent. Horse spleen ferritin obtained from Sigma Chemical Co, bound 16 haems per molecule (Kadir et al., 1992) and different recombinant H-chain ferritins bound variable amounts of haem (F. H. A. Kadir, G. R. Moore, A. Treffry and P. M. Harrison, unpublished work), but horse spleen ferritin from Boehringer and freshly prepared ferritin have been found not to bind haem (J. McKnight and G. R. Moore, unpublished work).

In conclusion, then, the studies reported in the present paper clarify the situation with regard to the maximum haem binding by *P. aeruginosa* BFR at its native bis-methionine co-ordination sites: 24 haems per molecule cannot be bound at these. *P. aeruginosa* BFR still appears to be distinct from *E. coli* and *A. vinelandii* BFRs in having two subunit types, and in being isolated with a haem loading of less than 12 per molecule.

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