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Isolation, characterisation and expression of the bacterioferritin gene of *Rhodobacter capsulatus*

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Abstract

The nucleotide sequence of the *Rhodobacter capsulatus* bacterioferritin gene (*bfr*) was determined and found to encode a protein of 161 amino acids with a predicted molecular mass of 18 174 Da. The molecular mass of the purified protein was estimated to be 18 176.06 ± 0.80 Da by electrospray mass spectrometry. The *bfr* gene was introduced into an expression vector, and bacterioferritin was produced to a high level in *Escherichia coli*. The amino acids which are involved in haem ligation, and those which provide ligands in the binuclear metal centre in bacterioferritin from *E. coli* are conserved in the *R. capsulatus* protein. The sequences of bacterioferritins, ferritin-like proteins, and proteins similar to Dps of *E. coli* are compared, and membership of the bacterioferritin family re-evaluated.

Keywords: Bacterioferritin; Iron metabolism; Ferritin; Dps; *Rhodobacter capsulatus*

1. Introduction

Bacterioferritins are non-haem-iron containing proteins composed of 24 identical subunits, each of molecular mass approximately 18 kDa. There is a marked structural similarity between bacterioferritin (BFR) and animal ferritins. In both cases, the protein subunits form an approximately spherical shell surrounding a central cavity in which the majority of the non-haem-iron is located [1,2]. There is sufficient space in their central cavities for up to 4500 iron atoms per molecule but lower average contents, in the range 600–2400 iron atoms per molecule, are typically found for BFR [2]. In most BFRs the non-haem-iron cores are amorphous and contain a high level of phosphate, usually in the ratio 1 Fe:1.7–2.0 P. These high levels of iron and phosphate have led to the suggestion that BFR functions physiologically as an iron and/or phosphate store, although this has not been proven [2,3]. *Rhodobacter capsulatus* BFR appears to be produced whatever the iron content of the growth medium, with a slightly increased production at elevated iron levels [3]. However, the difference in BFR levels is not as great as might be expected for an iron-inducible storage protein. In all ferritins there are binuclear iron cen-

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tres located within individual subunits that affect the rate of oxidative iron uptake [4]. These centres have been named ferrooxidase centres but their physiological relevance is uncertain.

A major difference between BFR and other ferritin-like molecules is that BFR contains intrinsically bound *b*-type haems whilst other ferritins do not [2]. This gives rise to the alternative names for BFR of non-haem-iron-containing cytochrome b_{557} or cytochrome b_1 [5]. The bound haem is located at highly symmetric intersubunit sites with bis-methionine axial ligation [1,6]. One methionine ligand is provided by each subunit, giving rise to 12 possible such sites per molecule. However, BFRs from different sources have different haem loadings, although the physiological consequences of this have not been established. Indeed the physiological role of the haem of BFR is not known and its presence has given rise to suggestions that BFR functions in a respiratory role, or has some function as an electron store [5]. However, there is no compelling evidence for either of these possibilities.

Under the appropriate growth conditions, *R. capsulatus* is capable of photosynthesis and nitrogen fixation, processes which impose a severe demand on iron acquisition and metabolism. However, little is known about iron homeostasis in this well characterised organism. It has previously been shown that *R. capsulatus* expresses a BFR during both aerobic and photosynthetic growth [3], and the isolation and nucleotide sequence of the *R. capsulatus* gene encoding BFR (*bfr*) are now reported. The sequence is discussed in the context of the recently proposed relationship between BFR and Dps, a DNA binding protein expressed in stationary phase *E. coli* that protects DNA against oxidative damage [7,8].

2. Materials and methods

2.1. Bacterial strains, phage and plasmids

The strain of *R. capsulatus* used was 37b4 which was grown chemoheterotrophically on RCV medium for chromosomal DNA isolations [9]. The strains of *E. coli* used were XL1-Blue MR *ΔmcrA Δ(mcrCB hsdSMR mrr) recA endA gyrA thi supE relA lac* [F′ *proAB* $^{+}$*lacI*^{q}Z ΔM15 Tn*10*] and XLOLR *ΔmcrA Δ(mcrCB hsdSMR mrr) recA endA gyrA thi relA lac* [F′ *proAB* $^{+}$*lacI*^{q}Z ΔM15 Tn*10*] for the propagation of λZAP-Express and pBK-CMV derivatives respectively, and a λDE3 lysogen of BL21 F^{-} *ompT* r_{B-} m_{B-} (Novagen) for expression studies. *E. coli* strains were grown at 37°C on LB medium supplemented with kanamycin (50 mg l^{-1}) and ampicillin (100 mg l^{-1}) as appropriate. λZAP-Express (Stratagene) and its derivatives were propagated according to the manufacturer's instructions. Plasmids used were pUC18 for routine cloning procedures, pET21a (Novagen) for overexpression, and derivatives of the phagemid pBK-CMV (Stratagene) which were excised from λZAP-Express clones in vivo.

2.2. Library construction and immunoscreening

Chromosomal DNA from *R. capsulatus* 37b4 was subjected to partial digestion with *Mbo*I. Digestion products in the range 4–9 kb were purified from an agarose gel and ligated into *Bam*HI-digested and dephosphorylated λZAP-Express arms. The recombinant library was plated on XL1-Blue MR and plaques transferred to nitrocellulose filters according to the protocols provided by Stratagene. The filters were probed with an anti-BFR antiserum as previously described [3]. Immunopositive recombinant λZAP-Express derivatives were purified, and inserts were excised as phagemids by performing a mixed infection with ExAssist (Stratagene).

2.3. DNA manipulations

Plasmid DNAs were purified using Qiagen plasmid kits (Hybaid). Probes for hybridisation experiments were prepared by priming with random hexanucleotides in the presence of [α-^{32}P]dCTP. The *R. capsulatus* library was screened by hybridisation on nitrocellulose filters according to Stratagene protocols. DNAs for sequencing were cloned into pUC18 according to standard procedures. Restriction endonucleases and modifying enzymes (Boehringer) were used as described by the manufacturer. Fluorescent oligonucleotides were synthesised for use as sequencing primers, and sequencing reactions were performed using the Autoread Sequencing Kit and were separated on an Automated Laser Fluorescence sequencer (Pharmacia). DNA sequences were analysed with the Lasergene software (DNAstar, Madison, WI, USA), and the Genetics Computer Group (Wisconsin) package. Non-fluorescent oligonucleotides were used to prime PCR reactions, which were carried out according to standard procedures. The DNA sequence, which was determined on both strands, has been deposited in the EMBL database and assigned the Accession Number Z54247.

2.4. Protein analysis

Electrospray mass spectrometry was carried out with 10 μl aliquots of 1 mg/ml samples dissolved in 20 mM Tris-HCl (pH 7.5) or in double-distilled water. The aliquots were injected into the carrier solvent stream (50% methanol, 1% acetic acid in water; flow rate 4 μl/min) of a VG BIO Q quadrupole mass spectrometer.

Proteins were extracted from intact bacterial cells at 100°C for 5 min, and separated in 12% SDS polyacrylamide gels using a discontinuous buffer system at 120 V for 2–3 h. Gels were stained in Coomassie brilliant blue overnight and then destained.

3. Results

3.1. Determination of the molecular mass of BFR

BFR was purified from *R. capsulatus* as previously described [3] and was subjected to electrospray mass spectrometry. The major species in the mass spectrum (> 95% of total intensity) had a molecular mass of 18176.06 ± 0.80 Da. Holo-BFR containing non-haem iron (as isolated) was used for the mass spectrometry, since studies with mammalian ferritins have shown that removal of the non-haem iron by treatment with sodium dithionite or thioglycollic acid yields a sample that gives multiple species in the electrospray mass spectrum (G.R. Moore, unpublished data). Under the conditions used for holo-BFR the sub-units dissociate in the spectrometer and the non-haem-iron and haem groups dissociate from the protein. These do not fly in the spectrometer and thus the observed mass corresponds to that of the monomeric BFR polypeptide.

3.2. Isolation and characterisation of the bfr gene

A *R. capsulatus* expression library in λZAP-Express was screened with an antiserum raised against the purified BFR. Several immuno-positive clones were isolated, and the first to be characterised was found to contain an in-frame fusion of bacterioferritin-like sequences to the *N* terminus of ß-galactosidase encoded by the vector. Subsequent sequence analysis revealed that this fusion was at the *Mbo*I site (nucleotides 289–292) in the completed *bfr* sequence. A 137 bp *Hin*dIII–*Ava*I restriction fragment from this clone was used to screen the library and a number of hybridisation positive clones were isolated. Each contained an approximately 3 kb *Sac*I–*Kpn*I fragment which was thought likely to contain the full length *bfr* gene. This fragment was cloned into pUC18 (generating a plasmid designated pSAD21), and the sequence of a 531 bp region was determined on both strands using four synthetic

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    1            +                     50+* +
Rca MKGDAKVIEFLNAALRSELTAISQYWVHFRLQEDWGLAKMAKKSREESIEEMGHADKIIARILFLEGHPNLQKLDPLRIGE
Eco MKGDTKVINYLNKLLGNELVAINQYFLHARMFKNWGLKRLNDVEYHESIDEMKHADRYIERILFLEGLPNLQDLGKLNIGE
Avi MKGDKIVIQHLNKILGNELIAINQYFLHARMYEDWGLEKLGKHEYHESIDEMKHADKLIKRILFLEGLPNLQELGKLLIGE
Bme MKGEPKVIERLNDALFLELGAVNQYWLHYRLLNDWGYTRLAKKEREESIEEMHHADKLINRIIFFEGFPNLQTVSPLRIGQ
Mle MQGDPDVLRLLNEQLTSELTAINQYFLHSKMQENWGFTELAERTRVESFDEMRHAEAITDRILLLDGLPNYQRIGSLRVGQ

             +   100                    + +             150
Rca GPRETLECDLAGEHDALKLYREARDYCAEVGDIVSKNIFESLITDEEGHVDFLETQISLYDRLGPQGFALLNAAPMDAAE
Eco DVEEMLRSDLALELDGAKNLREAIGYADSVHDYVSRDMMIEILRDEEGHIDWLETELDLIQKMGLQNY..LQAQIREEG
Avi HTKEMLECDLKLEQAGLPDLKAAIAYCESVGDYASRELLEDILESEEDHIDWLETQLDLIDKIGLENY..LQSQMDE
Bme NVKEVLEADLKGEYDARASYKESREICDKLGDYVSKQLFDELLADEEGHIDFLETQLDLLAKIGEERYGQLNAAPADEAE
Mle TLREQFEADLAIEYEVMSRLKPGIIMCREKQDSTSAVLLEKIVADEEEHIDYLETQLALMGQLGEELYSAQCVSRPPS
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Fig. 1. Alignment of the *R. capsulatus* BFR with bacterioferritins from *E. coli* (Eco), *A. vinelandii* (Avi), *B. mellitensis* (Bme) and *M. leprae* (Mle). The conserved methionine which provides two axial haem ligands in the *E. coli* protein is indicated (*), as are those residues associated with the binuclear metal centre (+).

oligonucleotides as primers. An open reading frame of 486 bp was detected which encodes a protein with a predicted molecular mass of 18 174.3 Da. This is in good agreement with the value of 18 176.06 ± 0.8 Da determined for the purified protein by electrospray mass spectrometry, and confirms that the major species detectable by electrospray mass spectrometry was the full-length, unmodified protein. The first ten amino acids of the gene-derived BFR sequence (Fig. 1) are in perfect agreement with those determined for the purified protein [3]. Thus, there is good evidence that the DNA sequence characterised encodes the BFR protein of *R. capsulatus*.

3.3. Features of the BFR sequence

The BFR sequence was aligned with other known bacterioferritins [10–12] for which full length sequences are available (those from *Azotobacter vinelandii*, *E. coli*, and *Mycobacterium leprae*) and with a protein from *Brucella melitensis* which on the grounds of sequence homology is very likely to be a bacterioferritin [13]. The *R. capsulatus* bacterioferritin shows a high degree of sequence similarity to the other known bacterioferritins (Fig. 1). It has all six of the conserved histidine and glutamate residues which are proposed to act as ligands in the binuclear metal centre: Glu-18, Glu-51, His-54, Glu-127 and His-130 [1]. In the *E. coli* BFR, Met-52 provides two axial ligands for the haem which is located at the interface between two subunits [1] and this residue is also conserved in the *R. capsulatus* BFR, which is consistent with the previous designation of this protein as cytochrome b_{557} and the suggestion that the haem might be bis-methionine ligated [3].

The five BFR sequences were also aligned with proteins which have more distant sequence homology to bacterioferritins: proteins (FTN) from *E. coli* [14] and *Haemophilus influenzae* [15] which are similar to eukaryotic ferritins; DNA binding proteins (Dps) from *E. coli* [7], *Bacillus subtilis* [16] and *Synechococcus* sp. strain PCC7942 [17]; a Dps homologue from *Streptomyces auriofaciens* [18]; a protein of unknown function from *Anabaena variabilis* (GenBank Accession JU0384); a pilin protein from *Helicobacter ducreyi* (GenBank Accession U18769) and proteins from *Treponema pallidum* [19] and *Helicobacter pylori* [8] which are reported to be

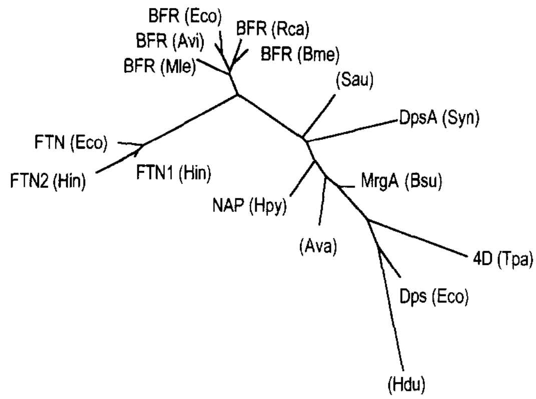

Fig. 2. Dendrogram derived from the sequences of bacterioferritins (details as in Fig. 1); ferritin-like proteins from *E. coli* (Eco) and *Haemophilus infuenzae* (Hin); the Dps protein of *E. coli*; Dps-like proteins from *S. aureofaciens* (Sau), *Synechococcus* PCC (Syn), *Anabaena variabilis* (Ava) and *Helicobacter ducreyi* (Hdu); antigenic proteins 4D from *Treponema pallidum* (Tpa) and NAP from *Helicobacter pylori* (Hpy). Sequences were aligned with the program PILEUP of the Genetics Computer Group (Wisconsin) package. A distance matrix was constructed with PROTDIST, and the tree drawn using NEIGHBOR and DRAWTREE, all from the Phylogeny Inference Package.

major antigens. The sequence alignment was used to construct a dendrogram which reveals that the ferritins and bacterioferritins each form tightly clustered groups, whereas the remaining proteins (some of which have previously been suggested to be bacterioferritins) are much less closely related to one another, and to the ferritins and bacterioferritins (Fig. 2).

3.4. Over-expression of the bfr gene

The *bfr* coding sequence was amplified by the PCR using synthetic oligonucleotide primers designed to introduce an *Nde*I site (underlined) at the start codon (5′-CACATATGAAAGGCGACGCAAAGGTCATC-3), and an *Eco*RI site (underlined) just downstream of the stop codon (3′-GGCTACCTGCGCCGGCTTATTCTTAAGGT-5′). The PCR product was cloned into pUC18, was sequenced to ensure that no mutations had been introduced during amplification, and was subsequently cloned into the expression vector pET21a(+). The recombinant plasmid (designated pSAD23) was introduced into *E. coli* BL21 (λDE3) which contains T7 RNA polymerase (chromosomally encoded and IPTG-inducible) to drive expression. Cultures were grown to mid-exponential growth phase and induced with 1 mM IPTG. Induced cultures containing pSAD23 expressed large quantities of a polypeptide which had the mobility expected for BFR (data not shown).

4. Discussion

Determination of the nucleotide sequence of the *R. capsulatus* bacterioferritin gene (the first *bfr* gene from a phototroph to be characterised), has revealed that its product is likely to be very similar to the well characterised BFR of *E. coli*. All of the features associated with the ferrooxidase centre of BFR are conserved in the *R. capsulatus* protein; though the physiological role of this redox centre remains unclear [3]. Like the *E. coli* protein, *R. capsulatus* BFR contains bis-methionine ligated haem, and the conserved Met-52 is almost certainly the residue which provides two axial ligands for the haem located between BFR subunits. Since photosynthesis requires relatively large amounts of iron, it will be of interest to examine further the physiological role of BFR, and the relationship between iron homeostasis and photosynthesis.

It has recently been pointed out that BFR has distant sequence homology to the Dps protein of *E. coli*, a DNA binding protein which is expressed in stationary phase and protects DNA against oxidative damage [7,8]. BFR- and Dps-like sequences from *H. pylori, A. variabilis, B. subtilis, S. auriofaciens* and *T. pallidum* have been analysed and suggested to be bacterioferritins on the basis of sequence homology [8]. A number of lines of evidence argue that these proteins are not BFR homologues but rather are Dps homologues, or form a distinct sub-family of proteins. Firstly, the *H. pylori, A. variabilis, S. auriofaciens* and *T. pallidum* proteins are more similar to DPS than to BFR (Fig. 2). Secondly, it seems likely that both the *H. pylori* and *T. pallidum* proteins have 10–12 identical subunits, as is the case for Dps, rather than the 24 subunits that make up native BFRs [1,7,8,20]. Thirdly, there is no spectroscopic evidence for the presence of a haem group in the *H. pylori* protein [8]. However, the simple distinction between 24 subunit haem-containing bacterioferritins and 10–12 subunit non haem-containing Dps homologues is complicated by the recent observation that Dps from *Synechococcus* sp. strain PCC7942 contains haem [17]. This protein lacks the conserved methionine which ligates haem in the bacterioferritins, and lacks most of the conserved residues of the ferrooxidase centre. Thus, the relationship between bacterioferritins and Dps-like proteins remains to be fully elucidated. The *B. subtilis mrgA* gene encodes a Dps homologue and is, like *dps* itself, stationary phase inducible [16]. Interestingly, expression of the *mrgA* gene is repressed by the presence of iron in growth media [16]. Clearly it is of some interest to establish the physiological functions of the stationary phase inducible Dps proteins, which are probably not bacterioferritins in the strict sense, yet have an interesting structural relationship to bacterioferritins, and may also have some functional similarities.

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