A [2Fe-2S] Protein Encoded by an Open Reading Frame Upstream of the *Escherichia coli* Bacterioferritin Gene†

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ABSTRACT: An open reading frame located upstream of the bacterioferritin gene in *Escherichia coli* encodes a hypothetical 64-residue protein [Andrews, S. C., Harrison, P. C., & Guest, J. R. (1989) *J. Bacteriol. 171,* 3940−3947)]. The spacing of the four cysteine residues in this hypothetical protein is identical to that in a region of NIFU, a [2Fe-2S] protein found in nitrogen-fixing bacteria [Fu, W., Jack, R. F., Morgan, T. V., Dean, D. R., & Johnson, M. K. (1994) *Biochemistry 33,* 13455−13463)]. The NIFU-like *E. coli* gene was cloned and overexpressed with a C-terminal "His tag" in *E. coli* using the T7 RNA polymerase/promoter system, and the protein was purified by metal-chelate affinity chromatography. UV−vis absorption and EPR spectra together with iron and amino acid analyses conclusively established that this NIFU-like *E. coli* protein contains one [2Fe-2S] cluster which can exist in at least two oxidation levels: +2 for the as-purified protein, and +1 for dithionite-reduced protein. Size-exclusion chromatography established that this His-tagged [2Fe-2S] protein is monomeric in solution. Affinity chromatography demonstrated specific complex formation between bacterioferritin (Bfr) and this NIFU-like [2Fe-2S] protein, which is dubbed Bfd. An open reading frame encoding a homologous Bfd is located near a Bfr gene in at least one other bacterium. Bfd may, therefore, constitute a general redox and/or regulatory component participating in the iron storage or mobilization functions of Bfr.

Bacterioferritin (Bfr)[1] is an iron storage protein which has been found in *Escherichia coli*, *Azotobacter vinelandii*, and several other bacteria (Grossman et al., 1992; Moore, 1994). Bfr resembles eukaryotic ferritin in its ability to catalyze autoxidation of Fe(II) and to deposit the resulting Fe(III) as an iron oxyhydroxide polymer within the hollow cavity of a 24-subunit protein shell (Frolow et al., 1994). However, Bfr differs from ferritin in containing up to one heme group per two subunits, the role of which is not well understood (Andrews et al., 1995). The in vivo interactions of Bfr with other redox or iron donor/acceptor species are also unknown. An open reading frame, *gen-64*, which encodes a hypothetical protein of 64 amino acid residues, is located 266−74 nucleotides upstream of the *E. coli* Bfr gene, *bfr* (Andrews et al., 1989). The spacing of the four cysteine residues, C-X-C-X_{32}-C-X_2-C, in this hypothetical 64-residue protein is identical to that in a region of NIFU, a [2Fe-2S] protein found in nitrogen-fixing bacteria which, at least in *A. vinelandii*, appears to be required for construction of active metal−sulfur clusters in the component proteins of nitrogenase (Fu et al., 1994). The four cysteine residues having the sequence spacing listed above presumably furnish ligands to the Fe_2S_2 core of the cluster in NIFU. The occurrence of homologous amino acid sequences in some other proteins has led to the suggestion that a four-cysteine residue NIFU-like amino acid sequence constitutes a modular domain for binding [2Fe-2S] clusters (Ouzounis et al., 1994). We report here that the product of *gen-64* (hereinafter referred to as Bfd) is indeed a [2Fe-2S] protein when overexpressed in *E. coli* and that this protein appears to form a specific complex with Bfr.

MATERIALS AND METHODS

Molecular biology procedures generally followed those described in Sambrook et al. (1989) or in *Current Protocols in Molecular Biology* (Ausubel et al., 1990) using supplier-recommended buffers. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. Nucleotide sequencing was carried out in the Molecular Genetics Instrumentation Facility at the University of Georgia.

Cloning of Genes for Bfr and Bfd. Genomic DNA from *E. coli* 71/18 (Messing et al., 1977) was purified from a 100 mL culture grown in LB following a procedure described by Robson et al. (1984). *E. coli Bfr* and *gen-64* were amplified by the PCR using this genomic DNA as template. Design of the primers for PCR was based on the published *bfr* and *gen-64* nucleotide sequences [Andrews et al. (1989), accession no. M27176]. For amplification of *gen-64*, the N-terminal primer, 5′ACGATAGCcatatgTACGTTTGTC-TTTGTAATG-3′, contained an *Nde*I restriction site (shown in lower case) incorporating the start codon (atg) and the N-terminal nucleotide sequence of *gen-64* (underlined). Eight extra nucleotides were added to the 5′ end. The complementary C-terminal primer, 5′-TAATctcgagTGCGGACTC-CTTAAA-3′, omitted the stop codon and incorporated a *Xho*I

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[1] Abbreviations: *bfr*, gene encoding *E. coli* bacterioferritin; Bfr, bacterioferritin; Bfd, *E. coli gen-64* gene product; PCR, polymerase chain reaction; OD_{600}, optical density at 600 nm; LB, Luria−Bertani medium; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance; Ni-IDA, nickel-(II) iminodiacetic acid derivatized; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

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restriction site (lower case) followed by the complementary C-terminal nucleotide sequence of *gen-64* (underlined). The restriction sites in these primers were designed such that the C-terminal "6His-tag" nucleotide sequence and stop codon in vector pET-21b (Novagen, Inc.) would be in frame with *gen-64*. Amplification of *bfr* was carried out using the N-terminal primer, 5′-ACGACAGCcatatgAAAGGTGATAC-TAAAG-3′, and the complementary C-terminal primer, 5′-TAATggatccTCAACCTTCTTCGCGGAT-3′. These primers contained, respectively, *Nde*I and *Bam*HI restriction sites (shown in lower case), and the N-terminal and complementary C-terminal nucleotide sequences of *bfr* (underlined, the latter including TCA, which is complementary to the stop codon). "Hot-start" PCR was carried out according to Promega's recommendations in 50 μL volumes containing 1.5 mM $MgCl_2$ and adding *Taq* DNA polymerase (Promega, Inc.) to the reaction tubes at 94 °C prior to the start of 36 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min in a Thermolyne thermocycler. The PCR products were purified using Promega's Wizard PCR preps. The purified *gen-64* PCR product and plasmid, pET-21b were combined, double-digested with *Nde*I and *Xho*I, and then ligated at 15 °C using T4 ligase (Boehringer Mannheim). Similarly, the purified *bfr* PCR product was combined with vector pT7-7 (Tabor, 1990), double-digested with *Nde*I and *Bam*HI, and ligated. Each of these ligation mixtures was transformed separately into *E. coli* BL21(DE3) (Novagen, Inc.; Studier et al., 1990), and plasmids containing appropriately sized inserts were selected by restriction digestion of alkaline lysis "mini-preps". One of the selected plasmids from each ligation, pGENH for *gen-64* and pCV3 for *bfr*, were purified using tip-100 columns (Qiagen, Inc.) and verified to contain the published *gen-64* and *bfr* nucleotide sequences (Andrews et al., 1989).

Overexpression and Purification of Bfr and Bfd. Centrifugations were carried out at 4 °C, and column chromatographies were carried out at room temperature. One-liter batches of LB containing 300 mg of ampicillin were inoculated with 50 mL "overnight" cultures of either *E. coli* BL21(DE3)[pGENH] or *E. coli* BL21(DE3)[pCV3] and placed in a 37 °C/250 rpm incubator/shaker. When the 1 L cultures reached an $OD_{600} \approx 0.8$, lactose (10 g for BL21-(DE3)[pGENH] or 4 g for BL21(DE3)[pCV3]) was added, and incubation was continued at 37 °C/250 rpm. Induction of protein overexpression in the cultures was monitored by Tricine SDS−PAGE (Schagger & von Jagow, 1987) on portions of 1 mL samples withdrawn at various times. The cells were harvested near the optimal induction time (usually about 2 h after addition of lactose) by centrifugation at 9000*g* for 5 min.

For purification of Bfd, the cell pellet from a 1 L culture of the induced BL21(DE3)[pGENH] was suspended in 15 mL of 20 mM Tris, 0.5 M NaCl, 5 mM imidazole (pH 7.9), 10 mL of 1% Triton X-100 and 1 mL of lysozyme (1 mg/mL), incubated at 37 °C for 1 h, and then sonicated in an ice-water bath for 1 min with 1 s bursts at 0.5 s intervals using a Sonic Dismembrator (Fisher Scientific). The resulting cell lysate was centrifuged at 14 000*g*, yielding an orange-pink supernatant, which was passed directly over a 5 mL column containing approximately 2.5 mL of Ni-IDA Sepharose (His·Bind, Novagen Inc.). After the column was washed with 15 mL of 20 mM Tris, 0.5 M NaCl, 60 mM imidazole (pH 7.9), the C-terminal 6His-tagged Bfd was eluted as an orange-red band with 5 mL of 20 mM Tris, 0.5 M NaCl, 0.5 M imidazole (pH 7.9). The protein was further purified by passage over a 1.6 × 50 Superose 12 column (PharmaciaLKB) equilibrated with 50 mM HEPES and 200 mM NaCl (pH 7.0) and elution with the same buffer. The orange-red fraction eluting from the Superose 12 column was concentrated in an Amicon cell equipped with a YM3 membrane. The purity of the protein at each stage was monitored by Tricine SDS−PAGE.

E. coli Bfr was purified from bright pink cell pellets of induced BL21(DE3)[pCV3] cultures. The cell pellets were suspended in 20 mM Tris-HCl (pH 8.0) and then lysed and centrifuged as described above for BL21(DE3)[pGENH]. The resulting supernatant was heated at 65 °C for 15 min (Andrews et al., 1993), and, after centrifugation to remove precipitated proteins, the supernatant was passed over a small QAE-Sepharose column (PharmaciaLKB) equilibrated with 20 mM Tris-HCl (pH 8.0). The unbound fraction, containing most of the Bfr, was further purified by passage over a 1.6 × 50 Superose 12 column equilibrated with 100 mM HEPES (pH 7.5). The fractions were checked for purity by Tricine SDS−PAGE and then pooled and concentrated in an Amicon cell equipped with a YM-30 membrane. The absorption spectra of this Bfr, including the oxidized and reduced heme absorbances, and subunit molecular weight as determined by SDS−PAGE (data not shown) closely resembled previously published data for overexpressed *E. coli* Bfr (Andrews et al., 1993, 1995).

Characterization of Bfd. Molecular weight of the protein in solution was determined by passage over the 1.6 × 50 Superose 12 column equilibrated with 50 mM HEPES and 200 mM NaCl (pH 7.0) after calibration with carbonic anhydrase (29 000), cytochrome *c* (12 400), and aprotinin (6500). Molecular weight of the Bfd in water-diluted samples (containing ≈9 pmol of protein/μL) was also determined by mass spectrometry using a Sciex API-1 quadrupole mass spectrometer with an electrospray ionization source in the Mass Spectrometry Facility at the University of Georgia. The Bfd sample was further diluted to 48:48:4 vol % acetonitrile:water:acetic acid and introduced into the mass spectrometer via loop injection. UV−vis spectra in 50 mM HEPES and 200 mM NaCl (pH 7.0) were obtained on a Shimadzu UV2101PC spectrophotometer. Reduction of the protein was achieved under an Ar atmosphere by addition of a slight excess of sodium dithionite from a concentrated stock solution. X-band EPR spectra of 200 μL samples of oxidized and dithionite-reduced protein (in the same buffer used for UV−vis spectra) which had been frozen in a liquid N_2 bath were recorded at various temperatures on a Bruker ESP-300E spectrometer fitted with an Oxford Instruments ESR-9 continuous flow liquid helium cryostat. Protein concentration and extinction coefficients were determined from quantitative amino acid analysis. Protein samples (10 μL containing approximately 90 pmol of protein) having known absorbance values were diluted into water and analyzed for amino acid content at the Harvard Microchemistry Facility, Cambridge, MA. The protein concentration was computed from the averaged amounts of 15 different amino acids from the analysis, using the amino acid composition of the protein derived from the nucleotide sequence. The iron content of the protein was quantitated by inductively coupled plasma-atomic emission at the

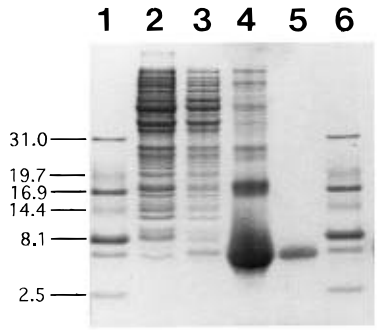

FIGURE 1: Tricine SDS−PAGE monitoring overexpression and purification of *E. coli* Bfd from *E. coli* BL21(DE3)[pGENH], as described in Materials and Methods. Lanes 1 and 6, molecular weight markers (Bio-Rad), as indicated in kDa; lane 2, whole cells before induction of overexpression; lane 3, whole cells approximately 2 h after induction of overexpression; lane 4, eluate from the Ni-IDA column; lane 5, after Superose 12 chromatography. The Bfd protein migrates at an anomalously low molecular weight (≈6000) relative to the markers.

Chemical Analysis Facility, University of Georgia, and the protein concentration was determined from the extinction coefficient at 336 nm.

Complex Formation between Bfr and Bfd. The His-tagged Bfd bound to the Ni-IDA column as described above was used in affinity chromatography. 100 μL of the purified His-tagged Bfd (containing ≈41 μg of protein) solution was passed over a Ni-IDA column consisting of ≈0.5 mL of packed resin equilibrated with binding buffer [20 mM Tris, 0.5 M NaCl, 5 mM imidazole (pH 7.9)] in a 3 mL plastic syringe barrel. Either 100 μL of purified Bfr solution or 500 μL of induced BL21(DE3)[pCV3] cell extract (the supernatant after lysis and centrifugation, as described above) was then applied to the column. Unbound proteins were eluted by passage of 3 mL of binding buffer over the column, and bound proteins were eluted with 3 mL of elution buffer [20 mM Tris, 0.5 M NaCl, 0.25 M imidazole (pH 7.9)]. Bound and unbound protein fractions were analyzed for protein content by Tricine SDS−PAGE using equal volumes of each eluate. Control experiments were performed identically except for omission of His-tagged Bfd.

RESULTS

We found that *E. coli* Bfd could be overexpressed from a vector which adds a "His tag", consisting of the amino acid sequence LEHHHHHH, onto the C-terminus of the *gen-64* product (Andrews et al., 1989). The overexpressed 72-residue "His-tagged" protein could then be purified by metal-chelate affinity chromatography on a Ni-IDA column (Van Dyke et al., 1992; Hochuli et al., 1987). Figure 1 shows the Tricine SDS−PAGE of various fractions during overexpression of the His-tagged *E. coli* Bfd. While overexpression is not readily apparent in whole cells (lanes 2 and 3), Bfd becomes the dominant protein band after Ni-IDA column chromatography (lane 4). The yield of overexpressed, purified Bfd was ≈0.6 mg/liter of culture. The C-terminal His-tag on Bfd is not designed for removal by protease cleavage. Although we attempted to overexpress *E. coli* Bfd without a His-tag by using *gen-64* inserted into pT7-7 (Tabor, 1990), we have so far been unable to isolate and purify significant quantities of the non-His-tagged protein. The molecular weight of the His-tagged Bfd was determined to be 8422 by electrospray ionization mass spectrometry. This value agrees well with the molecular weight of 8428, calculated by assuming that the gene-derived 72-residue polypeptide contains an unblocked N-terminal methionine residue and no iron−sulfur cluster, the latter being lost either in the solvent used for mass spectrometry or during electrospray ionization. The molecular weight determined by gel filtration was 7900; therefore, the His-tagged Bfd is monomeric in solution at pH 7 and 200 mM NaCl.

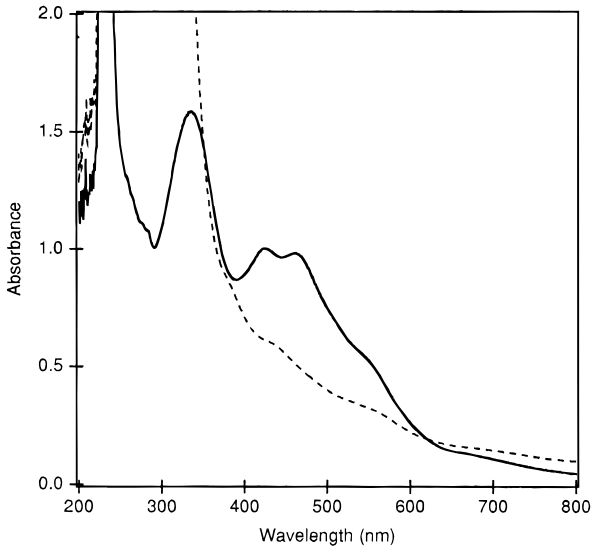

FIGURE 2: UV−vis absorption spectra of as-purified (solid line) and dithionite-reduced (dashed line) *E. coli* Bfd in 50 mM HEPES and 200 mM NaCl (pH 7.0).

Figure 2 shows the UV−vis absorption spectra of as-purified and dithionite-reduced Bfd. The absorbance ratio, A_{280}/A_{336}, is ≈0.7, for as-purified Bfd, and the protein lacks a distinct absorption maximum at 280 nm. These observations are consistent with the absence of tryptophan and the presence of only a single tyrosine in the 72-residue polypeptide together with the relatively high absorptions expected for the iron−sulfur cluster (Johnson, 1994). The absorption spectrum of as-purified Bfd shows maxima at 336 nm (ϵ = 16 800 M^{-1} cm^{-1}), 425 nm ($\epsilon \approx$ 10 600 M^{-1} cm^{-1}), and 465 nm ($\epsilon \approx$ 10 400 M^{-1} cm^{-1}) and a shoulder at ≈545 nm ($\epsilon \approx$ 5900 M^{-1} cm^{-1}). The extinction coefficients are per molar of the 72-residue polypeptide, as determined from quantitative amino acid analyses. The pattern of absorption features and relative intensities for as-purified Bfd is characteristic of a $[2Fe\text{-}2S]^{2+}$ iron−sulfur cluster, and the extinction coefficients, when compared with those of other [2Fe-2S] proteins, indicate one such cluster per 72-residue protein molecule (Fu et al., 1994). This conclusion is supported by the results of iron and amino acid analyses on the same sample, which yielded 1.9 iron/72-residue protein molecule. The UV−vis absorption spectrum of dithionite-reduced Bfd exhibits shoulders at ≈430 and ≈550 nm, and these features are characteristic of $[2Fe\text{-}2S]^{+}$ clusters (Fu et al., 1994).

The conclusions from the UV−vis absorption spectra concerning cluster type and oxidation levels are confirmed by the X-band EPR spectra shown in Figure 3. The EPR spectrum of the dithionite-reduced Bfd exhibits a nearly axial signal with $g_{||} = 2.02$ and $g_{\perp} = 1.94$ (zero-crossing), and the spectrum is observable without detectable broadening up to at least 60 K. In Fe-S proteins, such *g* values and relaxation

Scheme 1

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                10        20        30        40        50        60
Ecoli   MYVCLCNGISDKKIRQAVRQFSPHSFQQLKKFIPVGNQCGKCVRAAREVMEDELMQLPEFKESA
        ||||||:| ::::: ::| : :: : :::     :|::||:| |: |::::
Mlepra MYVCLCAGATNQTVCDVVAR-GATTSKEIAAACGAGSDCGRCRRTLRAIIAASNPTVKPDLTRPAKQYVKLRGGR
                10        20        30        40        50        60        70
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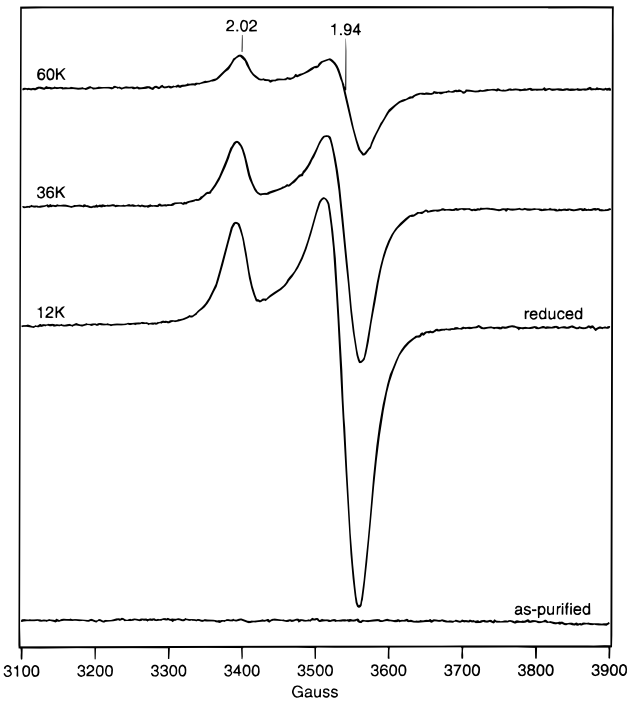

FIGURE 3: X-band first-derivative EPR spectra of as-purified and dithionite-reduced *E. coli* Bfd. The top three spectra are of the same reduced sample. The protein was 10 μM in 50 mM HEPES and 200 mM NaCl (pH 7.0). EPR conditions: microwave frequency, 9.58 GHz; modulation amplitude, 8.1 G; microwave power, 2 mW; temperatures, as indicated.

properties are unique to the $S = {}^1/_2$ ground state of [2Fe-2S]$^+$ clusters (Johnson, 1994). Under the same spectral conditions as used for the reduced protein, the as-purified Bfd shows no detectable EPR spectrum at 12 K. This EPR silence is expected for a [2Fe-2S]$^{2+}$ cluster due to antiferromagnetic exchange coupling of the high-spin Fe(III)'s and shows that the as-purified protein contains no detectable reduced (1+) cluster.

In order to demonstrate complex formation between *E. coli* Bfr and Bfd, an affinity column, consisting of the latter His-tagged protein bound to the Ni-IDA resin, was constructed. Figure 4, lanes 2−5, demonstrate that when purified Bfr (subunit M_r 18 025; Andrews et al., 1989) is added to the Ni-IDA column, the vast majority of the Bfr is bound only when the column is pretreated with the His-tagged Bfd. Lanes 6−8 show that the Bfd-treated Ni-IDA column specifically binds Bfr from a cell extract of induced *E. coli* BL21(DE3)[pCV3], which contains overexpressed Bfr. Separate affinity column experiments of the same type (data not shown) showed that His-tagged Bfd does not form complexes with bovine serum albumin or carbonic anhydrase and that Bfr does not bind to a Ni-IDA column pretreated with a C-terminal His-tagged rubredoxin-like protein from *A. vinelandii* (Chen & Mortenson, 1992).

DISCUSSION

The UV−vis absorption and EPR spectra in Figures 2 and 3 together with the iron and amino acid analyses conclusively

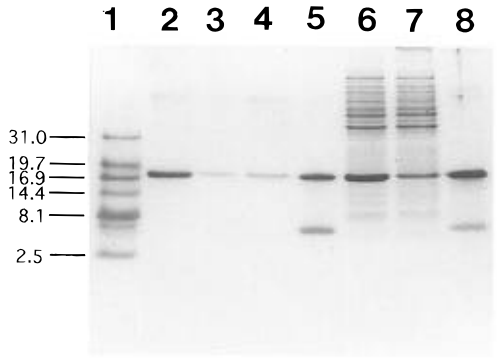

FIGURE 4: Tricine SDS−PAGE of Ni-IDA affinity column eluates (lanes 2−5, 7, 8) demonstrating specific complex formation between *E. coli* Bfr and Bfd. Procedures and buffers are described in Materials and Methods. Lane 1, molecular weight markers; lane 2, purified Bfr passed over Ni-IDA column followed by binding buffer; lane 3, column from lane 2 treated with elution buffer; lanes 4 and 5, same as lanes 2 and 3, respectively, except column was first treated with His-tagged Bfd in binding buffer; lane 6, induced *E. coli* BL21(DE3)[pCV3] cell extract (no column treatment); lane 7, same cell extract as in lane 6 passed over Bfd-treated Ni-IDA column, followed by binding buffer; lane 8, column from lane 7 treated with elution buffer.

establish that the 64-residue NIFU-like protein encoded by *E. coli gen-64* (Andrews et al., 1989), which we dub Bfd, contains a [2Fe-2S] cluster when overexpressed in *E. coli* and that this cluster can exist in at least two oxidation levels: 2+ for the as-purified protein, and 1+ for dithionite-reduced protein. The results of the affinity chromatography shown in Figure 4 demonstrate that a specific complex forms between Bfr and Bfd. Although the details of this complex formation remain to be delineated, the putative attachment of Bfd via its C-terminal His-tag to the Ni-IDA column implies that neither the His-tag nor the carboxyl-terminal portion of Bfd is directly involved in binding to Bfr. This observation together with the placement of the four cysteine residues at positions 4, 6, 39, and 42 in the 64-residue amino acid sequence of Bfd (Andrews et al., 1989; cf. Scheme 1) suggests that one or more of these cysteine residues and, therefore, the [2Fe-2S] cluster of Bfd must be near the binding site on Bfr.

The biological role of Bfd is not known. Under the usual laboratory growth conditions of *E. coli*, Bfd is apparently expressed to a much lower level than is Bfr (Andrews et al., 1989, 1993). Transcriptional regulation constitutes a possible function for Bfd, but the known example of a [2Fe-2S] protein with such a role, soxR, is not of the NIFU type (Hildago et al., 1995; Nunoshiba & Demple, 1994), and, unlike the dimeric soxR, the His-tagged Bfd is apparently monomeric in solution. In *A. vinelandii*, NIFU is presumed to be involved in iron or iron−sulfur donation to the component proteins of nitrogenase (Fu et al., 1994), but the exact role of NIFU in this process is not known. While it is tempting to speculate that Bfd is involved in iron donation to Bfr, NIFU is a much larger protein (≈300 residues per subunit) and is dimeric in solution, making any direct functional analogy to Bfd obscure. The redox requirements

for uptake and donation of iron to and from Bfr, the well-established redox role of biological [2Fe-2S] clusters (Johnson, 1994), and the complex formation between Bfr and Bfd demonstrated in this work[2] point to a redox role for Bfd. However, at least in vitro, Fe(II) oxidation and Fe(III) deposition into the iron oxyhydroxide core of Bfr requires no other proteins or cofactors (not even the heme) and can use O_2 as the only external oxidant (Andrews et al., 1995). One possibility is that the biological redox role of Bfd, perhaps together with the heme group in Bfr, is manifested only under conditions where an electron acceptor other than O_2 is used for iron oxidation. In the photosynthetic bacterium *Rhodobacter capsulatus*, Bfr with an Fe(III) core forms in both aerobically and anaerobically grown cells (Ringeling et al., 1994). The occurrence of NIFU-like four-cysteine domains in bacterial nitrite reductases (Ouzounis et al., 1994), which contain siroheme and FAD prosthetic groups, suggests that a NIFU-like [2Fe-2S] protein in combination with the heme group in Bfr could function in electron transfer to an alternative oxidant leading to iron(III) deposition in the Bfr core. Alternatively, Bfd could catalyze transfer of electrons from an external reducing agent to the iron core of Bfr, thereby facilitating release of iron(II).

The occurrence of a chromosomal Bfd-like open reading frame in the vicinity of a Bfr gene is not limited to *E. coli*. We have identified a similar open reading frame approximately 400 base pairs upstream from the start of and on the opposite strand to the Bfr gene in *Mycobacterium leprae* (Pessolani et al., 1994). This *M. leprae* open reading frame encodes a hypothetical 74-residue protein, the N-terminal 50 residues of which show a 28% identity to that of *E. coli* Bfd, when aligned as shown in Scheme 1. This identity includes the four cysteine residues which presumably furnish ligands to the two iron atoms in *E. coli* Bfd.[3]

Thus, a small NIFU-like [2Fe-2S] protein, dubbed Bfd, may be a general redox and/or regulatory component participating in the iron storage or mobilization functions of Bfr in bacteria. The results reported here provide a basis for delineating the function of Bfd.

NOTE ADDED IN PROOF

We have recently discovered a Bfd-like open reading frame upstream of the *A. vinelandii* Bfr gene (R. P. Garg and D. M. Kurtz, Jr., unpublished).

[2] Evidence for complex formation between Bfr and Bfd in vivo is that one of the contaminating proteins in the Ni-IDA-purified Bfd (Figure 1, lane 4 band at $M_r \approx 18\,000$) appears to have a molecular weight identical to that of *E. coli* Bfr (Figure 4, lanes 2−8). The Ni-IDA-purified Bfd showed ferroxidase activity [assayed according to Andrews et al. (1993, 1995)], but this activity was lost after the subsequent Superose 12 chromatography, which removes the contaminating Bfr-sized protein (Figure 1, lane 5).

[3] Scheme 1 shows that the single histidine residue in *E. coli* Bfd is not conserved in the homologous *M. leprae* open reading frame, indicating that histidine residues do not furnish ligands to the [2Fe-2S] cluster in this protein.

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