A Bacterioferritin from the Strict Anaerobe *Desulfovibrio desulfuricans* ATCC 27774[†]

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Received November 1, 1999; Revised Manuscript Received March 15, 2000

ABSTRACT: A bacterioferritin was isolated from the anaerobic bacterium *Desulfovibrio desulfuricans* ATCC 27774, grown with nitrate as the terminal electron acceptor, which is the first example of a bacterioferritin from a strict anaerobic organism. This new bacterioferritin was isolated mainly as a 24-mer of 20 kDa identical subunits, containing 0.5 noncovalently bound heme and 2 iron atoms per monomer. Although its N-terminal sequence is significantly homologous with ferritins from other microorganisms and the ligands to the di-iron ferroxidase center are conserved, it is one of the most divergent bacterioferritins so far characterized. Also, in contrast to all other known bacterioferritins, its heme is not of the B type; its chromatographic behavior is identical to that of iron uroporphyrin. Thus, *D. desulfuricans* bacterioferritin appears to be the second example of a protein unexpectedly containing this heme cofactor, or a closely related porphyrin, after its finding in *Desulfovibrio gigas* rubredoxin:oxygen oxidoreductase [Timkovich, R., Burkhalter, R. S., Xavier, A. V., Chen, L., and Le Gall, J. (1994) *Bioorg. Chem. 22*, 284−293]. The oxidized form of the protein has a visible spectrum characteristic of low-spin ferric hemes, exhibiting a weak absorption band at 715 nm, indicative of bis-methionine heme axial coordination; upon reduction, the α-band appears at 550 nm and a splitting of the Soret band occurs, with two maxima at 410 and 425 nm. The heme center has a reduction potential of 140 ± 10 mV (pH 7.6), a value unusually high compared to that of other bacterioferritins (ca. −200 mV).

As an indispensable cofactor in a multitude of proteins participating in most life-sustaining processes, iron is an essential element for living organisms. However, the advent of an oxygen rich atmosphere, oxidizing the ferrous ion pool to the ferric form, created two problems. On one hand, Fe(III) is highly insoluble, which led to the development of various powerful systems for taking up and storing iron. On the other hand, ferrous ion activates dioxygen, producing reactive oxygen species, which cause serious hazards through oxidative damage processes (Haber−Weiss−Fenton reactions). To overcome these problems, iron homeostasis is strictly controlled and results from a coordinated integration of assimilation, utilization, and storage of this element (*1*−*4*).

Ferritins constitute a broad superfamily of iron storage proteins, widespread in the three domains of life, in aerobic or anaerobic organisms. One of the major branches of this family consists of the so-called mammalian-type ferritins. This type of ferritin has been observed ubiquitously, from lower invertebrates, amphibians, and fish to higher mammalian systems, including humans, as well as in plants (*3*, *5*). Homologous ferritins are also found in bacteria and archaea (*6*−*10*). The second branch of the superfamily is that of bacterioferritins, which differ from the mammalian ferritins in amino acid sequence, immunological cross reactivity, composition of the iron core, and the presence of a heme group (*3*, *11*). The first bacterioferritin (BFR) from a prokaryote was reported from the nitrogen-fixing bacterium *Azotobacter vinelandii* (*12*) and has been detected in different aerobic bacteria (*3*, *11*, *13*−*16*). Quite interestingly, a bacterioferritin was recently isolated from an eukaryote, the fungus *Absidia spinosa* (*17*). The general characteristic of ferritins and bacterioferritins is that, as isolated, they consist of 24 subunits with a molecular mass of 17−19 kDa and contain 8−10 wt % non-heme iron. In the case of BFR, they also have 3−12 noncovalently bound heme groups per 24 subunits, which was shown to be iron protoporphyrin IX in all cases so far studied (*3*, *11*, *18*, *19*), but its function remains elusive. However, it is established that the uptake of iron involves an initial step of oxidation of ferrous ion, through a binuclear iron center (*11*, *20*−*23*). In terms of three-dimensional structure and the presence of this di-iron center, ferritins and bacterioferritins are distantly related to another group of iron proteins, the rubrerythrin family of di-iron proteins (*24*−*26*). These three protein families are oligomers of four-α-helix bundles, a structural motif typical of most binuclear iron proteins (*25*−*27*). Rubrerythrin was first purified from the sulfate-reducing bacteria *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio desulfuricans* ATCC 27774 (*24*, *28*) and shown to contain both a mononuclear rubredoxin-like center and a binuclear metal

[†] This work was supported by PRAXIS XXI grants (to M.T. and J.L.G.) and by an NIH grant (GM56000-03 to J.L.G. and M.-Y.L.). C.V.R. acknowledges a grant from Praxis XXI (BIC14787/96).

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10.1021/bi992525d CCC: $19.00
Published on Web 05/12/2000

center. Whereas the *D. vulgaris* recombinant protein reconstituted with iron contains a di-iron site (*25*), similar to that of hemerythrin (*29*), the wild-type *D. vulgaris* protein contains an Fe−Zn center in place of the di-iron center (*27*). Recently, a large number of rubrerythrins and similar proteins, including some without the C-terminal rubredoxin-like extension (which have been named erythrins), have been shown to be present in other bacteria, anaerobic or microaerophilic, as well as in anaerobic archaea (*9*, *10*, *26*). Using the *D. vulgaris* recombinant protein, rubrerythrin was shown to have a ferroxidase activity comparable to that of ferritins or bacterioferritins (*30*). This result led to the suggestion that these proteins could be the ferroxidase of several anaerobic bacteria, and it was recently proposed that in prokaryotes there could be a mutual exclusion of these three types of proteins, i.e., none will contain all three (*31*).

The mechanisms and proteins for iron storage in sulfate-reducing bacteria remain unknown. As a first step toward their elucidation, the isolation and characterization of a quite distinct type of bacterioferritin from an anaerobic bacterium of the genus *Desulfovibrio* is described. In particular, it is shown that it does not contain protoheme. Instead, it appears to give a second example of a protein having iron uroporphyrin or a closely related porphyrin as a prosthetic group, following its finding in *Desulfovibrio gigas* rubredoxin: oxygen oxidoreductase (*32*−*34*).

EXPERIMENTAL PROCEDURES

Growth of Microorganisms and Preparation of the Cell-Free Extract. D. desulfuricans ATCC 27774 was grown on a lactate/nitrate medium as previously described (*35*). The cell-free extract was prepared under argon to avoid exposure to air, as described in ref *36*. After centrifugation at 100000*g*, the soluble fraction was separated from the membrane pellet.

Purification. All purification procedures were performed at 4 °C and pH 7.6, in a Coy model a-2463 anaerobic chamber, and protein concentrations were performed in a Diaflo apparatus (Amicon Corp., Danvers, MA) with a YM3 membrane. All buffers were at pH 7.6. The anaerobically prepared soluble extract (800 mL) was loaded on a DEAE-52 column (6 cm × 34 cm; 1 mL/min) previously equilibrated with 10 mM Tris-HCl, and a two-step (2 L each) linear gradient (10 to 250 mM Tris-HCl and 250 to 400 mM Tris-HCl) was applied. A fraction containing cytochromes was eluted at ∼250 mM. This fraction was further purified by passing it through a DEAE Biogel column (5 cm × 41 cm; 1 mL/min) and eluted in a continuous gradient (1 L) of 10 to 450 mM Tris-HCl. The fraction containing the bacterioferritin was then applied to a hydroxylapatite column (3 cm × 20 cm; flow rate of 0.4 mL/min), equilibrated with 300 mM Tris-HCl. The column was eluted using a 0.5 L descending linear gradient (250 to 1 mM) of Tris-HCl, followed by a 1 L ascending linear gradient of 1 to 500 mM potassium phosphate buffer. The fraction that eluted at ca. 300 mM phosphate (which throughout the paper will be designated *as isolated*) contained pure bacterioferritin as judged by SDS−PAGE and N-terminal analysis. A total of 60 mg of pure protein was obtained from 570 g of cells (wet weight). Rubrerythrin was also purified anaerobically essentially as described by Le Gall et al. (*24*, *36*). *D. gigas* rubredoxin:oxygen oxidoreductase was purified as described in ref *32*.

Analytical Methods. The protein total molecular mass was determined by gel filtration on a Sephacryl S-300 HR (Pharmacia) column, eluted with 100 mM Tris-HCl buffer (pH 7.6) with 300 mM NaCl, using Pharmacia high-range protein standards for gel filtration (Pharmacia), as well as by Blue Native PAGE electrophoresis (*37*) using the Pharmacia high-molecular weight calibration kit. The subunit molecular mass was determined by SDS−PAGE (12%), using the SDS-discontinuous buffer system performed according to the method described in ref *38*, but with some differences in the resolving gel: 0.5% SDS and 20% glycerol. The sample was prepared in loading buffer for SDS−PAGE with final concentrations of 3 M urea and 5% SDS. Pharmacia low-range protein standards were used as molecular markers. Gels were stained for protein with Commassie-Blue R-250 and for iron, as described in ref *39*. The protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce) as described in ref *40*. The N-terminal sequence was obtained by Edman degradation (*41*) using an Applied Biosystem model 470A sequencer.

Heme Analysis. The alkaline pyridine hemochromogen method was used for the estimation of heme content as described previously (*42*), using the molar absortivity $\epsilon_{red-oxi,550-535}$ of 24 mM^{-1} cm^{-1} for heme C. The heme was extracted and analyzed as described in ref *43*, using a Beckman HPLC system Module 167 with a Waters Nova-Pak C18 column. Hemoglobin and *D. gigas* rubredoxin: oxygen oxidoreductase were used to obtain heme B and iron uroporphyrin (*33*) standards, respectively.

Iron Content Determination. Labile iron and total iron contents were respectively determined by the 2,4,6-tripyridyl-*s*-triazine (TPTZ) method (*44*) and by atomic absorption on a graphite chamber at Laboratório de Análises, Instituto Superior Técnico, Lisbon, Portugal.

Iron Removal from Bfr. Apo-BFR was prepared according to the method described in ref *21*. The iron content was checked by the TPTZ method.

Iron Uptake Assays. Oxidation of iron(II) to iron(III) was monitored at 310 nm with oxygen as the terminal electron acceptor, using a molar absortivity ϵ_{310} of 2475 M^{-1} cm^{-1} to estimate the amount of iron that was oxidized (*45*).

Kinetic measurements of changes in absorption after the addition of iron(II) freshly prepared prior to each experiment by dissolving weighed amounts of ferrous ammonium in deoxygenated AnalaR-grade water to apo-BFR were taken using a UV−visible spectrophotometer in 100 mM MES at pH 6.5 and 25 °C (*20*, *21*). A protein concentration of 0.5 μM (in 24-mer) was used in all assays; the measurements were obtained in a time range of ∼5−240 s. The rate constants were determined by fitting the experimental data to a single-exponential curve. Ferroxidase activity was measured similarly. To a spectrophotometric cuvette (total volume of 0.5 mL) containing 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonate] (pH 7.0) and 200 mM Na_2SO_4 was added 0.5 mM ferrous ammonium sulfate from the freshly prepared, anaerobic 10 mM stock solution in buffer (*30*). Appropriate blanks were performed for each assay.

Spectroscopic Methods. UV−visible spectra were recorded on a Shimadzu UV 3100 spectrophotometer or on an Olis DW2 instrument, equipped with a liquid nitrogen system.

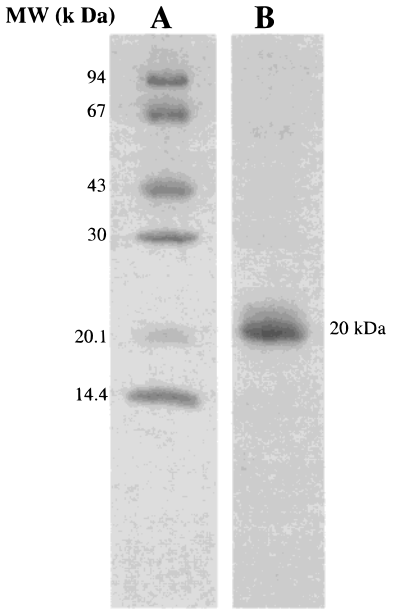

FIGURE 1: SDS−PAGE for *D. desulfuricans* bacterioferritin: (A) molecular mass markers and (B) bacterioferritin.

EPR spectra were obtained and analyzed as described in ref *46*, using a Bruker ESR 900 spectrometer, equipped with Oxford Instruments continuous flow liquid helium and nitrogen cryostats. Spin quantitation was performed by theoretically simulating the experimental spectra, as described in ref *47*.

Redox Titrations. Anaerobic potentiometric titrations, using ~5 mV reduction steps, were performed and analyzed as previously described (*48*). A total of ~60 visible spectra from 350 to 700 nm were recorded for each titration. The data sets were analyzed by following the absolute and differential absorptions at the Soret and α-bands, using the MATLAB software. Five titrations were performed, using samples from two independent purifications, at low and high ionic strengths (50 and 300 mM potassium phosphate buffer, respectively). The reduction potentials are quoted in relation to the standard hydrogen electrode.

RESULTS

General Biochemical Properties. A novel heme protein was purified to homogeneity from *D. desulfuricans*, as judged from SDS−PAGE (Figure 1). Although the protein was purified under anaerobic conditions, there is no evidence that it is sensitive to oxygen. A single band was detected, corresponding to an apparent molecular mass of 20 kDa. By gel filtration analysis, it was found that the protein exists as a 24−26-mer (500 ± 20 kDa); however, at low ionic strength (50 mM phosphate buffer), it exists mainly as a dimer, although larger oligomers are also detected (up to ~24-mer). Identical results were obtained by blue native gel electrophoresis (data not shown). The subunit molecular mass is similar to that of most ferritins (18−19 kDa) (*3*, *11*). The solution quaternary structure is heterogeneous, as has been reported for other ferritins (*49*).

The total iron content determined by atomic absorption and chemical analysis gives 2.0 ± 0.1 Fe/monomer. The heme content was estimated by pyridine hemochrome, assuming that the molar absortivity was identical to that of type B or C hemes (see below); a value of 0.4 ± 0.1 heme per monomer was obtained. These data indicate that the protein was isolated without the iron core, that it contains a heme moiety per two subunits, and that it may harbor one di-iron center per subunit. Preliminary crystallographic data for *D. desulfuricans* BFR show that it is composed of 24 identical subunits, each containing one di-iron site (P. M. Matias and M. A. Carrondo, personal communication).

The protein was shown to load iron, by gel electrophoresis, stained with 3,5-diaminobenzoic acid dihydrochloride (data not shown). A ferroxidase activity of 6 ± 1 mol of Fe^{3+} min^{-1} (mol of monomer)$^{-1}$ (T = 25 °C) was determined for bacterioferritin, using the standard ferroxidase assay (*30*). The activity of wild-type *D. desulfuricans* rubrerythrin was also measured, and is similar to that of the *D. vulgaris* recombinant protein (0.3 ± 0.03 unit). In contrast to the wild-type *D. vulgaris* protein, which contains a heterometallic Fe−Zn center, the *D. desulfuricans* protein does not contain zinc (as determined by atomic absorption, our unpublished data).

Kinetic measurements of the as-isolated and apo form were taken by adding different amounts of an anaerobic solution of ferrous ammonium sulfate; on the time scale that was used, only a single phase was observed, with a rate constant of (6 ± 0.002) × 10^{-3} s^{-1} (T = 25 °C), similar to that obtained for *Escherichia coli* bacterioferritin (*20*, *21*), in which it was assigned to core formation (*20*, *21*).

The N-terminal sequence of *D. desulfuricans* bacterioferritin is significantly homologous to other bacterioferritins, with degrees of identity (using only the equivalent N-terminal sequences) ranging from 20 to 35% and degrees of similarity in the range of 35−52% (Figure 2). As for the other bacterioferritins, the first 80 amino acids are predicted to fold as two α-helices. Most important, the amino acid residues from these two first helices binding the ferroxidase center are all conserved in this protein (stars in Figure 2), as well as the putative methionine ligand to the heme center (*19*, *50*) (arrow in Figure 2). Despite these general similarities, the *D. desulfuricans* protein is among the most divergent from the presently sequenced bacterioferritins, together with those from *Thiobacillus ferrooxidans* and *Chlorobium tepidum*, as can be seen in the phylogenetic tree built using the N-terminal sequences of bacterioferritins, ferritins, and rubrerythrins (ca. 100 residues, Figure 3). This tree was previously reported to consist of three distantly related groups corresponding to the ferritin, bacterioferritin, and rubrerythrin families, which led to the proposal that there could be a mutual exclusion of ferritin and rubrerythrin in the same organism (*31*). *D. desulfuricans* is now shown to contain, besides rubrerythrin (*28*), a bacterioferritin. However, as more sequences become available, new subgroups are apparent, such as those represented by the ferritin from *Listeria innocua*, which clusters together with the family of DNA-binding proteins [named Dps (*11*, *51*)], and by the *D. desulfuricans* and *C. tepidum* bacterioferritins. Clearly, more sequence data are necessary to clarify the evolutionary relationship among all these proteins.

Spectral Properties. As isolated, *D. desulfuricans* bacterioferritin (oxidized form) exhibits a major absorption band at 410 nm, with a shoulder at 390 nm, and broad bands around 540, 615, and 715 nm (Figure 4a). This spectrum is characteristic of low-spin ferric hemes, with a minor contribution of a high-spin ferric form (absorptions at 390

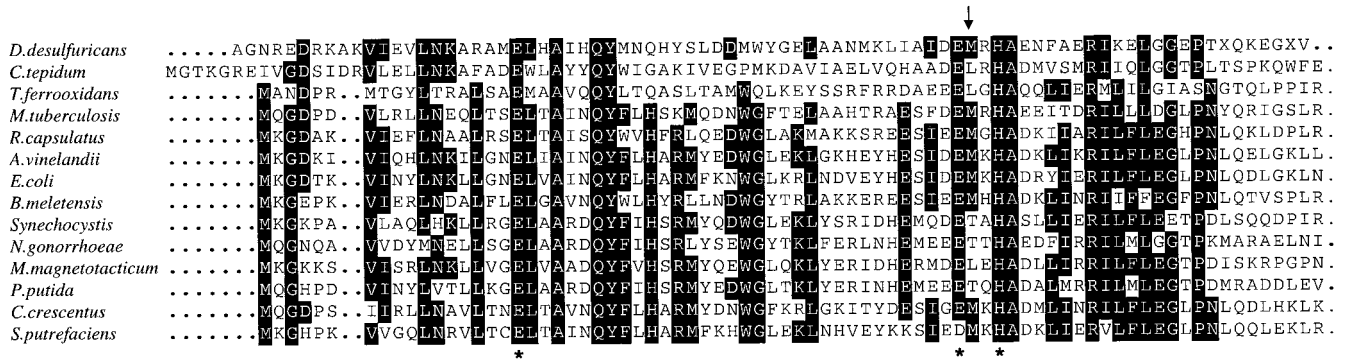

FIGURE 2: N-Terminal amino acid sequences of bacterioferritins from *D. desulfuricans* ATCC 27774 and from several bacteria (% identity, NCB accession number). From a Blast search of the genome: *C. tepidum* (28%), *Thiobacillus ferrooxidans* (20%), *Caulobacter crescentus* (26%), *Shewanella putrefaciens* (25%), *Mycobacterium tuberculosis* (28%, 2225984), *Rhodobacter capsulatus* (28%, 2493294), *Azotobacter vinelandii* (26%, 114931), *E. coli* (33%, 114932), *Brucella meletensis* (32%, 1705456), *Synechocystis* sp. PC6803 (22%, 1653726), *Neisseiria gonorrhoeae* (24%, 2493291), and *Magnetospirillum magnetotacticum* (25%, 2688836). Black boxes include the most conserved residues; stars denote the ligands to the di-iron center, and the arrow denotes the heme methionine ligand.

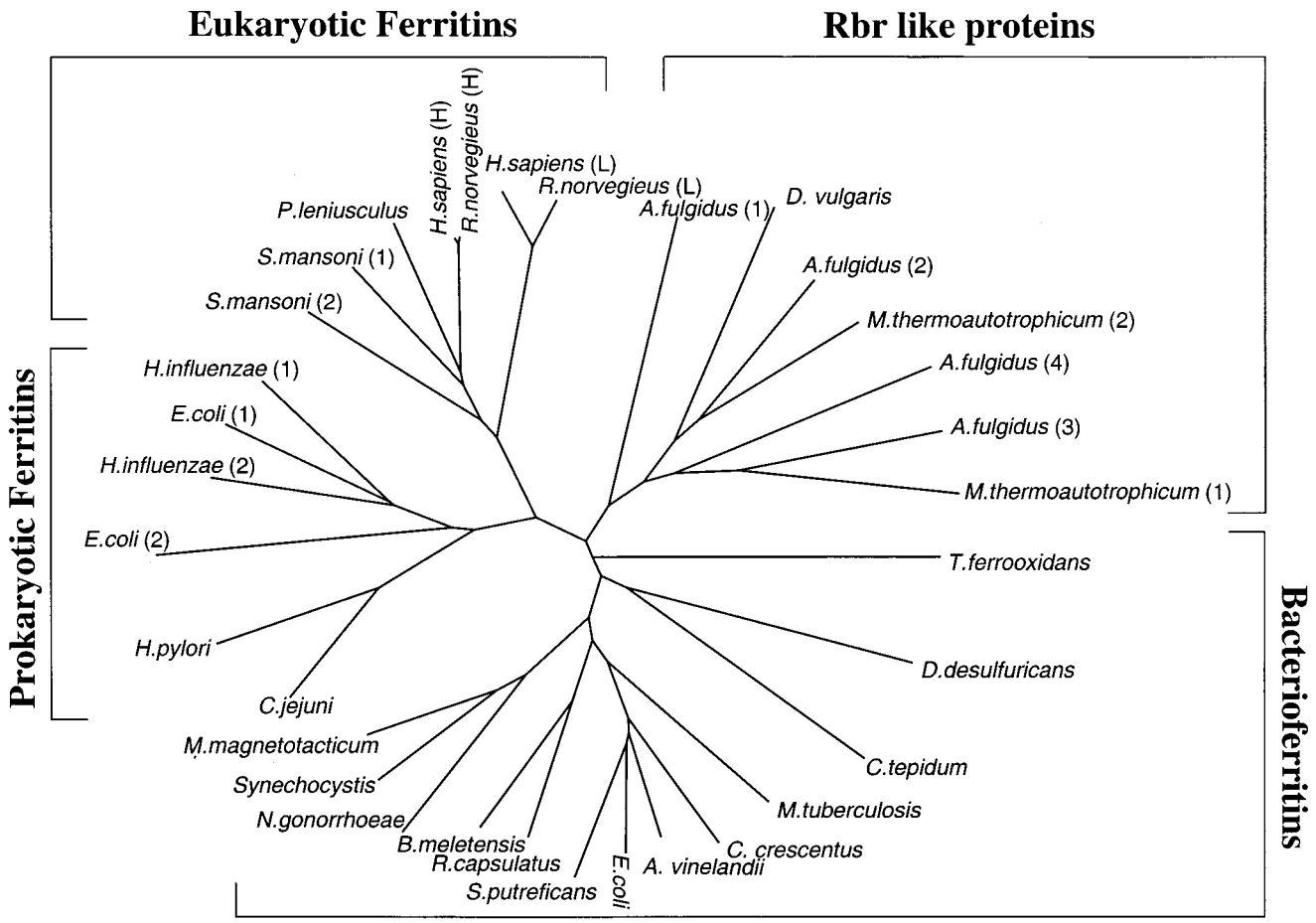

FIGURE 3: Phylogenetic tree constructed using the N-terminal sequences of ferritins, bacterial ferritins, bacterioferritins, and rubrerythrins. Data from the data banks (NCB accession number), including a Blast search of in-progress genomes. The alignment and tree were constructed using Clustal W. Prokaryotic ferritins: *Archaeoglobus fulgidus* (2649772), *Methanobacterium thermoautotrophicum* (2621200), *E. coli* (types 1 and 2, 134036 and 1736565, respectively), *Campylobacter jejuni* (1483147), *Helicobacter pylori* (2506399), and *Haemophilus influenzae* (types 1 and 2, 1173293 and 1173294, respectively). Eukaryotic ferritins: *Schistosoma mansoni* (types 1 and 2, 120501 and 120502, respectively), *Homo sapiens* (L and H forms, 120523 and 120516, respectively), *Rattus norvegieus* (L and H forms, 120527 and 120519, respectively), and *Pacifastacus leniusculus* (945013). Rubrerythrin-like proteins: *D. vulgaris* Hildenborough (134119), *A. fulgidus* (types 1−4, 2649783, 2649773, 2648916, and 2648207, respectively), and *M. thermoautotrophicum* (types 1 and 2, 2621913 and 2621845, respectively). Fpr bacterioferritins, see the legend of Figure 2.

and 615 nm). The band at 715 nm is most probably a result of the bis-methionine ligation to the heme center (*52*), as expected from the comparison of the amino acid sequences (Figure 2), which shows that the methionine heme ligand is conserved in *D. desulfuricans* bacterioferritin. At liquid nitrogen temperature, the heme center becomes 100% low-spin, as evidenced by the total bleaching of the 615 nm band (data not shown). Quite unexpectedly, when the buffer ionic strength is decreased (to ca. 50 mM potassium phosphate), the heme center becomes almost fully high-spin (Figure 4c)

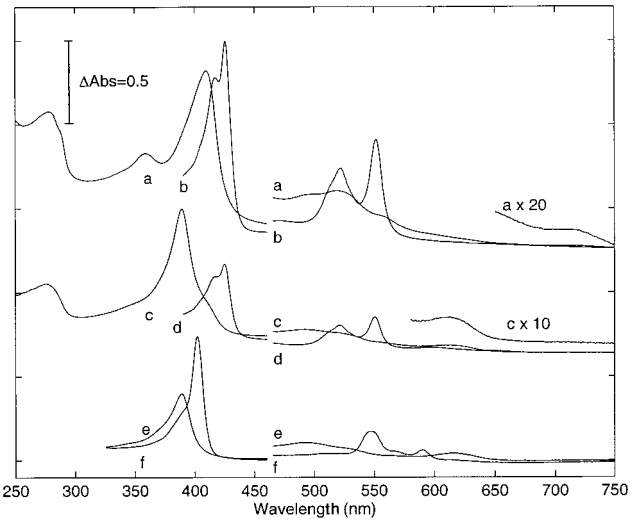

FIGURE 4: Visible spectra of oxidized (a and c) and dithionite-reduced (b and d) *D. desulfuricans* bacterioferritin: (a and b) 300 mM potassium phosphate buffer with a protein concentration of 27 μM and (c and d) 50 mM potassium phosphate buffer with a protein concentration of 12 μM. Visible spectra of oxidized (e) and dithionite-reduced (f) *D. desulfuricans* bacterioferritin heme after extraction with 8% TCA. The α-region is multiplied 3-fold. The cell path length was 10 mm.

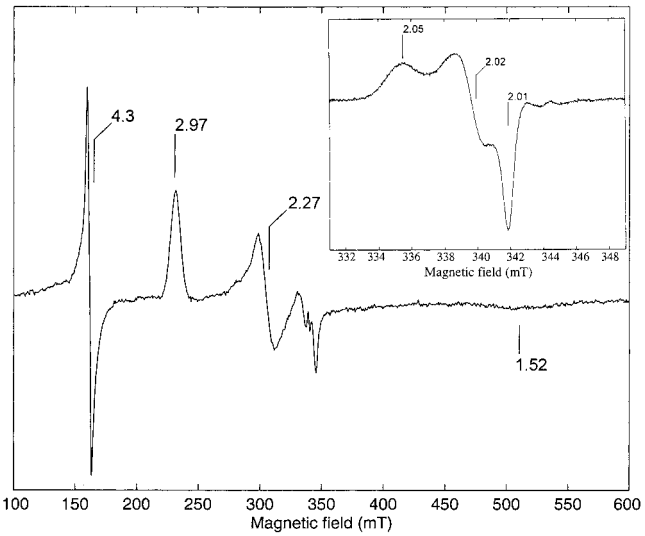

FIGURE 5: EPR spectrum of oxidized *D. desulfuricans* bacterioferritin. The inset shows the EPR spectrum of ascorbate-reduced BFR, upon exposure to NO for 5 min: protein concentration of 250 μM, in 300 mM potassium phosphate buffer; temperatures of 11 and 55 K (inset); microwave power of 0.24 mW; microwave frequency of 9.63 GHz; and modulation amplitudes of 1 and 0.4 mT (inset).

at room temperature, while at liquid nitrogen temperature, it is converted into the low-spin form. This process is reversible; i.e., increasing the ionic strength (to ca. 350 mM phosphate) restores the low-spin form (not shown). This behavior parallels that observed for the protein assembly state, suggesting that the high-spin to low-spin equilibrium may be related to the oligomerization state, a fact not surprising as the hemes in BFRs are located between two subunits (*19*).

In agreement with the visible spectroscopy data, the low-temperature EPR spectrum of the oxidized protein exhibits a major set of resonances at $g = 2.97$, 2.27, and 1.52 (Figure 5) typical of low-spin ferric hemes, as well as a minor intensity resonance at $g = 4.3$, due to high-spin non-heme Fe(III), which accounts for less than 5% of the total iron detected. The heme EPR g values are remarkably different from those of bis-methionine ferric hemes [in general, $g =$ 2.88, 2.30, and 1.45 (*52*)]. Although the heme structure is distinct from that of a B-type heme (see below), the porphyrin type does not affect in general the EPR g values; this appears as another example of how the **g** tensor and crystal field parameters are a very poor indication of the heme axial coordination (*53*−*55*). No EPR signature for a di-iron center could be detected in the protein as isolated (anaerobically) or upon exposure to air, as expected for a spin-coupled center. To probe its oxidation state, air-oxidized, as-isolated, and ascorbate- or dithionite-reduced protein samples were reacted with nitric oxide. Only upon reduction, with either reductant, were resonances characteristic of high-spin ferrous iron bound to NO detected (*21*); a high-intensity signal due to an $S = {}^1/_2$ spin ground state at $g = 2.05$, 2.02, and 2.01 was observed (Figure 5, inset), identical to that attributed to the diferrous site in *E. coli* BFR (*21*). These data strongly suggest that in the as-isolated state the di-iron center is in the ferric form.

Two sharp bands develop in the Soret region following reduction by dithionite, at 417 and 425 nm, and the α- and β-bands appear at 550 and 520 nm, respectively (Figure 4b,d); the spectrum of the reduced protein is essentially independent of the buffer ionic strength. The apparent split of the Soret band, which is also observed at liquid nitrogen temperature, is rather unusual and contrasts with the absence of any structure on the α-band. The redox titration data (see below) show that the appearance of both features at 417 and 425 nm as well as the α-band at 550 nm occur simultaneously and concomitantly with the decrease in intensity of the bands at 410 and 390 nm of the oxidized form, thus strongly indicating that the two Soret peaks are not due to sample heterogeneity. As will be shown below, the protein contains a single type of heme which, upon extraction into an aquous solution, yields a spectrum with a single Soret peak. Soret bands split in heme proteins have been first observed in horse cytochrome undecapeptides (*56*) and later in the tetrahemic split-Soret cytochrome *c* from *D. desulfuricans* (*57*). Recently, a similar splitting was observed in the multihemic menaquinol:HiPIP oxidoreductase from the aerobic bacterium *Rhodothermus marinus* (*48*). For the first two cases, this splitting was clearly assigned to the interaction of the transition dipole moments of heme centers in van der Waals contact (*59*). In *D. desulfuricans* bacterioferritin, this heme arrangement is improbable, due to the low heme content (1 heme per dimer). Furthermore, if the overall three-dimensional structure is similar to that of other ferritins, the inter-heme distance will be too long to cause any electrostatic interaction (*19*). Hence, at present, no explanation for this unique feature is forthcoming. It may, however, be suggested that it is due to an as-yet-unidentified chromophore in rather close spatial proximity of the heme center. In this context, it should be mentioned that these types of interactions have been proposed to occur by interaction with aromatic residues, namely, tyrosinyl (*59*).

The pyridine ferrohemochrome of the protein exhibits an absorption peak at 545 nm, which is quite distinct from *c*-type (549−554 nm) or *b*-type (554−563 nm) cytochromes (data

not shown). The heme is not covalently bound to the protein, since it can easily be extracted from the protein. Following precipitation of the protein with 8% trichloroacetic acid, the heme is solubilized in aqueous solution, yielding a visible spectrum (Figure 4e,f) characteristic of high-spin ferric heme (Soret band at 390 nm and broad bands at 492, 530, and 620 nm); upon reduction by sodium dithionite, the spectrum has maxima at 402 nm (Soret) and at 547 nm. The Soret band is no longer split, which indicates that this effect is not due to the macrocycle structure or to the presence of more than one type of heme. The heme was also extracted by addition of acetone and HCl, followed by solvent exchange to ethyl acetate. The extracted heme was analyzed by reverse-phase HPLC and had an elution pattern rather different from those of A, B, D, O, and siro hemes (ref *43* and our unpublished data). Quite remarkably, *D. desulfuricans* BFR heme has exactly the same retention time as that of iron uroporphyrin (data not shown), obtained from *D. gigas* rubredoxin:oxygen oxidoreductase (*33*). This chromatographic method has proven to be quite sensitive to even very minor modifications of porphyrin side chains, such as the presence of just an extra isopentenyl unit in O- or A-type hemes (ref *43* and our unpublished data). Hence, it may be concluded with a high degree of confidence that *D. desulfuricans* BFR heme is iron uroporphyrin, or a closely related porphyrin. Furthermore, the pyridine hemochrome of iron uroporphyrin has an α-band at 548 nm (*33*) close to that of *D. desulfuricans* BFR. The fact that the molar absortivity of iron uroporphyrin is identical to that of a *c*-type cytochrome (*33*) supports the use of this value in determining the heme content of *D. desulfuricans* BFR.

Redox Behavior. The reduction potential for *D. desulfuricans* bacterioferritin was determined by redox titrations monitored by visible spectroscopy, following the changes in absorbance at 390 and 410 nm (oxidized form) and at 417, 425, and 550 nm (reduced form). Well-defined isosbestic points are observed at 413 and 437 nm (Soret region) and at 506 and 571 nm (α-region) (Figure 6A). A single monoelectronic redox transition was detected (Figure 6B), corresponding to a reduction potential of 140 ± 10 mV (result of five titrations of two different protein batches). Identical behaviors and reduction potentials were obtained with the protein in 50 or 300 mM phosphate buffer, conditions under which it is in the high-spin or low-spin form, respectively. The redox titration data strongly suggest that only one type of heme is present, since the data at all wavelengths follow the same redox behavior. The heme redox potential is also drastically different from those of the other bacterioferritins; it has a value of 140 mV, compared to −475 and −225 mV for loaded or unloaded *Azotobacter vinelandii* bacterioferritin, respectively (*60*), or −204 mV for the protein from *Rhodopseudomonas sphaeroides* (*61*). Preliminary experiments performed with in vitro iron loading of the protein indicate that the heme reduction potential is not affected. The high reduction potential of *D. desulfuricans* BFR cannot be assigned to the porphyrin type, as the heme centers of *D. gigas* rubredoxin:oxygen oxidoreductase [iron uroporhyrin (*34*)] have a quite negative reduction potential of −350 mV (*34*).

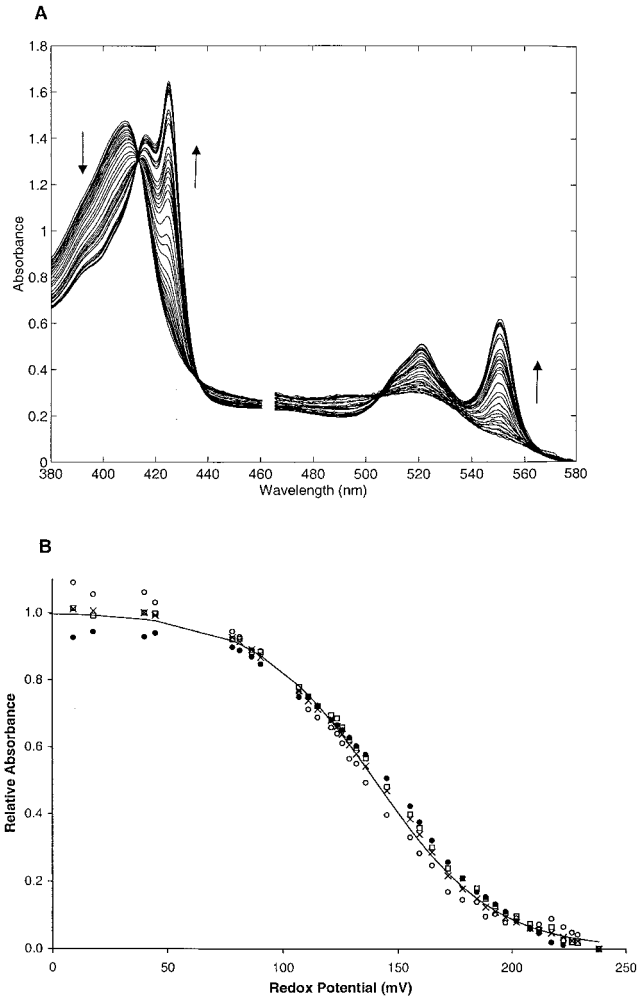

FIGURE 6: Redox titration of *D. desulfuricans* bacterioferritin. (A) Visible spectra of bacterioferritin upon decreasing the redox potential, from 235 to −60 mV (arrows indicate spectral changes upon potential decrease); the α-region is multiplied 2.5-fold. (B) Titration curve, using normalized absorbances at 406 nm (●, oxidized form) and at 417 (○), 425 (×), and 550 (□) nm (reduced form). The solid curve was calculated from a monoelectronic Nernst equation, with a reduction potential of 140 mV. The protein concentration was 30 μM, in 300 mM potassium phosphate buffer. The cell path length was 10 mm.

DISCUSSION

A new type of bacterioferritin was isolated from the anaerobic bacterium *D. desulfuricans* ATCC 27774. It is the first example of a bacterioferritin from a strict anaerobe as well as of an iron storage protein from a sulfate-reducing bacterium; until now, only ferritins were found to be present in anaerobic bacteria or archaea (*7*, *9*, *10*). Interestingly, the bacterioferritin from *D. desulfuricans* has a ferroxidase activity much higher than that of recombinant (*30*) or wild-type rubrerythrin, suggesting that the real physiological role of the latter is still to be determined. Also, the *D. desulfuricans* bacterioferritin is so far unique in that it was isolated without an iron core, but with a stable di-iron site.

This new bacterioferritin has the usual subunit molecular mass and multimeric state. But, unexpectedly, both for a protein from a strict anaerobe and in comparison to other bacterioferritins, the heme center has a quite positive reduction potential of 140 mV; however, many examples of proteins isolated from sulfate-reducing bacteria with even

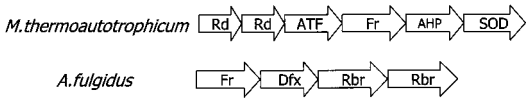

FIGURE 7: Examples of genomic organization of ferritins from the archaea *M. thermoautotrophicum* and *A. fulgidus* (*9*, *10*): Rd, rubredoxin; ATF, A-type flavoprotein; Fr, ferritin; AHP, alkyl hydroperoxide; SOD, superoxide dismutase; Dfx, desulfoferrodoxin; Rbr, rubrerythrin.

higher reduction potentials (up to 250 mV) have been identified, such as neelaredoxin, desulfoferrodoxin, and rubrerythrin (*24*, *62*, *63*), which may be involved in oxygen defense mechanisms (see below). Remarkably, the heme is not of the B type, as in all other known cases and the data presented in this article strongly suggest that it is iron uroporphyrin, although a definitive assignment of the heme structure remains to be fully established. If indeed it will be proven that it is iron uroporhyrin, *D. desulfuricans* BFR will be the second example of an active protein containing this unusual heme, after the finding of uroporphyrin I in rubredoxin:oxygen oxidoreductase from *D. gigas* (*32*, *33*). It is probable that in *D. desulfuricans* bacterioferritin the porphyrin will also be the type I isomer, which may be related to the distinct heme biosynthetic pathway recently identified in sulfate-reducing bacteria (*64*, *65*; see below also). In fact, a closely related strain (*66*), *D. desulfuricans* Multispirans (NCBI 12078), has been shown to accumulate large amounts of uroporphyrinogen I when grown on a lactate/nitrate medium (*67*). It was also noted in the same article that, when sulfate-reducing bacteria grow using the dissimilatory reduction of nitrate, the $Fe^{3+}-Fe^{2+}$ equilibrium is very much dependent on the relative concentrations of NO_3^-, NO_2^-, and NH_4^+, a condition totally different from that encountered when these bacteria use sulfate as the terminal electron acceptor. In this case, as already noted by Emery (*68*) because of the high concentrations of sulfides, all the iron is in the Fe^{2+} form and the bacteria grown under these conditions may not need a special storage protein.

The synthesis and utilization of iron uroporphyrin by *Desulfovibrio* deserve some comment. It has been shown (*64*, *65*) that these bacteria do not form coproporphyrinogen III directly from uroporphyrinogen III via a decarboxylase as eukaryotes and other prokaryotes do. Instead, they use *S*-adenosylmethionine to form precorrin-2 which is decarboxylated to form a didecarboxy precorrin-2 which itself is the precursor of coproporphyrinogen III. This may well be the reason *Desulfovibrio* normally synthesizes, and uses, the uroporphyrinogen I isomer. If the eukaryotic-type decarboxylase were present, the accumulation of uroporphyrinogen I would not be possible as it is well-known that the enzyme is not stereospecific and can use both type I and III isomers (*69*).

The function of ferritins as well as rubrerythrins in anaerobic bacteria and archaea is still unclear. In fact, the in vivo electron acceptor for the ferrous ion oxidation has never been elucidated. Interestingly, data from the fully sequenced genomes (Figure 7) show that in several species these proteins appear to be coded in gene clusters (possibly operons), together with proteins involved in oxygen detoxification (superoxide dismutase and alkyl hydroperoxide reductase) as well as other iron proteins, such as rubredoxin, desulfoferrodoxin (Dfx), and the flavoproteins [named A-type flavoprotein (*70*)] analogous to *D. gigas* rubredoxin:oxygen oxidoreductase. The recent observations that Dfx and the closely related protein neelaredoxin have superoxide dismutase activity (*71*−*74*) supports the general belief that iron metabolism is strongly coordinated with oxygen metabolism and response to oxidative stress conditions. In this context, it is worth mentioning that sulfate-reducing bacteria were until recently considered strict anaerobes. However, it has been shown that *D. gigas* is capable of surviving in the presence of oxygen, as well as benefiting from it, by producing ATP through substrate level phosphorylation, regenerating NAD^+, and reducing dioxygen to water, a process in which rubredoxin:oxygen oxidoreductase is the terminal oxygen reductase (*32*, *34*, *75*, *76*). Very importantly, the A-type flavoproteins are present in most anaerobic archaea for which genomic data are available, as well as in several anaerobic and facultative bacteria, suggesting that similar mechanisms may be operative in a wide range of prokaryotes. In this respect, it should be noted that *D. desulfuricans* is capable of surviving, even if it is not able to grow, after prolonged (up to at least one month) exposure to oxygen (*77*). Thus, it is tempting to speculate that in anaerobic organisms ferritins may act in a concerted manner with oxygen defense systems under oxygen stress conditions, to avoid the highly deleterious effect of a noncontrolled oxidation of ferrous iron. The presence of both iron storage and oxygen-detoxifying enzymes, in strict anaerobes, may have contributed to their survival, after the formation of an oxygen-rich atmosphere ca. 2.5 billion years ago (*78*), making them capable of sustaining transient contact with oxygen in the ever-changing natural environment.

ACKNOWLEDGMENT

We are especially grateful to P. M. Matias and M. A. Carrondo (ITQB) for communication of the crystallographic data prior to publication and thank the skillful technical help of Isabel Pacheco in the protein purification. We thank the UGA fermentation plant for bacterial growth.

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BI992525D