Isolation and Characterization of Rubrerythrin, a Non-Heme Iron Protein from *Desulfovibrio vulgaris* That Contains Rubredoxin Centers and a Hemerythrin-like Binuclear Iron Cluster†

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ABSTRACT: A new non-heme iron protein from the periplasmic fraction of *Desulfovibrio vulgaris* (Hildenbourough NCIB 8303) has been purified to homogeneity, and its amino acid composition, molecular weight, redox potential, iron content, and optical, EPR, and Mössbauer spectroscopic properties have been determined. This new protein is composed of two identical subunits with subunit molecular weight of 21 900 and contains four iron atoms per molecule. The as-purified oxidized protein exhibits an optical spectrum with absorption maxima at 492, 365, and 280 nm, and its EPR spectrum shows resonances at $g = 4.3$ and 9.4, characteristic of oxidized rubredoxin. The Mössbauer data indicate the presence of approximately equal amounts of two types of iron; we named them the Rd-like and the Hr-like iron due to their similarity to the iron centers of rubredoxins (Rds) and hemerythrins (Hrs), respectively. For the Rd-like iron, the measured fine and hyperfine parameters ($D = 1.5$ cm^{-1}, $E/D = 0.26$, $\Delta E_Q = -0.55$ mm/s, $\delta = 0.27$ mm/s, $A_{xx}/g_n\beta_n = -16.5$ T, $A_{yy}/g_n\beta_n = -15.6$ T, and $A_{zz}/g_n\beta_n = -17.0$ T) are almost identical with those obtained for the rubredoxin from *Clostridium pasteurianum*. Redox-titration studies monitored by EPR, however, showed that these Rd-like centers have a midpoint redox potential of $+230 \pm 10$ mV, approximately 250 mV more positive than those reported for rubredoxins. Another unusual feature of this protein is the presence of the Hr-like iron atoms. At 4.2 K, the Mössbauer spectrum of these Hr-like iron atoms is a quadrupole doublet with parameters ($\Delta E_Q = 1.47 \pm 0.05$ mm/s and $\delta = 0.52 \pm 0.03$ mm/s) typical of high-spin ferric ions ($S = {}^5/_2$) with oxygenous and/or nitrogenous ligands. Mössbauer spectra recorded with strong magnetic fields up to 8 T indicate that these Hr-like ions are in a diamagnetic ($S = 0$) environment. Consequently, these two Hr-like iron atoms must be strongly antiferromagnetically coupled to form a diamagnetic binuclear center. Similar Mössbauer properties have been observed for the binuclear iron center in the methemerythrin. As for the reduced hemerythrin, the reduced Hr-like center in this new protein exhibits a single quadrupole doublet with parameters ($\Delta E_Q = 3.14 \pm 0.04$ mm/s and $\delta = 1.30 \pm 0.03$ mm/s) typical of high-spin ferrous ions. Also, the EPR spectrum of the native protein exhibits a very weak signal at $g = 1.98$, 1.76, and 1.57, very similar to those signals reported for the binuclear iron centers in the semimet forms of hemerythrin. In conclusion, our spectroscopic data indicate unambiguously that this protein contains two rubredoxin-like FeS_4 centers and a hemerythrin-like binuclear iron cluster. Due to this unusual combination of prosthetic groups, this new protein is named *rubrerythrin*. The physiological function of rubrerythrin is presently unknown and is under investigation.

Rubredoxin (Rd) is an iron–sulfur protein found in many microorganisms and has been studied extensively by a variety of physical techniques (Peisach et al., 1971; Debrunner et al., 1977; Moura et al., 1978, 1979; Shulman et al., 1978; Watenpaugh et al., 1979; LeGall et al., 1984). The Rds isolated from anaerobic microorganisms are generally small proteins with molecular weights on the order of 6000. A much larger Rd ($M_r \simeq 19\,500$) has been found in the aerobic bacterium *Pseudomonas* (*P.*) *oleovorans* (Peterson et al., 1967). Among all of the iron–sulfur proteins, Rds contain the simplest active site, which is composed of a high-spin iron atom coordinated to four cysteinyl sulfur ligands in a tetrahedral arrangement. Rd is redox active, with a redox potential close to zero (−60 to +10 mV), and can function as an electron carrier. In the as-isolated Rds, the iron atom is high-spin ferric, and in the reduced state it is high-spin ferrous. The Rd from *P. oleovorans* was shown to participate in a ω-hydroxylation reaction (Peterson et al., 1967), but the physiological role of the Rds from the anaerobic bacteria remains obscure. A NADH–rubredoxin oxidoreductase has been found in *Desulfovibrio* (*D.*) *gigas* (LeGall, 1968), as well as from some strains of *Clostridia* (Petitdemange et al., 1979; Ballongue et al., 1986), and the *D. gigas* oxidoreductase was reported to be very specific for the Rd of this organism (Odom et al., 1976).

Hemerythrin (Hr) is an oxygen-transport protein found in several marine invertebrate phyla. It has been well characterized, and the structure of the oxygen-binding site is known (Garbett et al., 1971; Kurtz et al., 1977; Sanders-Loehr & Loehr, 1979; Elam et al., 1982; Hendrickson et al., 1982; Wilkins & Harrington, 1983; Stenkamp et al., 1984; Maroney

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et al., 1986; Wilkins & Wilkins, 1987). The usual oligomeric form of Hr is octameric and has a molecular weight of approximately 108 000. Each subunit contains an antiferromagnetically coupled high-spin iron pair, which can bind a single oxygen molecule reversibly. X-ray crystallographic data show that the two high-spin iron atoms are bridged by two carboxylates from the protein and one oxo ion from a water molecule (Stenkamp et al., 1984, 1985). Besides the two carboxylate ligands, this binuclear iron center is bound to the protein by five additional histidine ligands. One of the iron atoms is six-coordinated, and the other is five-coordinated. The five-coordinated iron is the oxygen-binding site. This binuclear center can exist in three stable oxidation states: [Fe(II), Fe(II)] in deoxy-Hr, [Fe(II), Fe(III)] in semimet-Hr, and [Fe(III), Fe(III)] in oxy-Hr or met-Hr. The deoxy-Hr, oxy-Hr, and met-Hr are EPR silent, but the different forms of the semimet-Hr are EPR active and show characteristic EPR signals of spin-coupled $S = {}^1/_2$ systems (Muhoberac et al., 1980; Lukat et al., 1984). Similar binuclear iron centers were also found in ribonucleotide reductase (Sjöberg & Gräslund, 1983), uteroferrin, and purple acid phosphatase (Antanaitis & Aisen, 1983).

In this paper, we report the isolation of a novel non-heme iron protein from the periplasmic fraction of *D. vulgaris* (Hildenborough NCIB 8303). [The presence of this protein in *D. vulgaris* was briefly mentioned by Bruschi and LeGall (1972); it was described as a rubredoxin with a high molecular weight.] The protein has been purified to homogeneity, and its amino acid composition, molecular weight, redox potential, iron content, and optical, Mössbauer, and EPR properties were determined. We found that this protein has a most unusual composition of prosthetic groups; it contains two Rd-like FeS_4 centers and a binuclear iron center similar to that of Hr. This discovery of a Hr-type center in a protein isolated from an anaerobic bacterium is very intriguing. We have tentatively named this new protein *Rubrerythrin*.

Materials and Methods

Growth of Organisms and Preparation of Crude Extracts. Cells of *D. vulgaris* (Hildenborough NCIB 8303) were grown for 38 h in lactate–sulfate media containing the following components per liter: sodium lactate (60%), 12.5 mL; Na_2SO_4, 4 g; NH_4Cl, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; yeast extract (DIFCO), 1 g; K_2HPO_4, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g; $CaCl_2$, 0.25 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and cysteine hydrochloride, 0.25 g. The pH of the media was adjusted to 7.6 before autoclaving. ^{57}Fe-enriched cells were grown for the same time in the same media with addition of 1 mg of ^{57}Fe (95% enrichment, New England Nuclear) per liter. Cells (470 g) were suspended in 470 mL of 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.6, frozen at −80 °C for several days, and thawed at room temperature for 14 h. The suspension was treated with 1 mg each of DNase I and DNase II for 30 min and centrifuged at 20000*g* for 1 h. The supernatant containing the periplasmic fraction was called crude extract.

Purification of Rubrerythrin. All purification procedures were performed at 4 °C and pH 7.6. During the initial steps, the presence of rubrerythrin was judged by change of the absorbance at 490 nm after ascorbate reduction. Purity was determined during the later steps by the ratio of A_{280}/A_{490}.

The crude extract (700 mL) was loaded onto a diethylaminoethyl (DEAE)-Bio-Gel column (4.5 × 35 cm) equilibrated with 10 mM Tris-HCl buffer and washed with 2 L of the same buffer. A fraction containing rubrerythrin and cytochromes was collected during this washing and was loaded onto a hydroxylapatite column (6 × 24 cm) equilibrated with 0.01 M Tris-HCl buffer. The column was washed successively with 200 mL each of 0.01 and 0.001 M Tris-HCl buffer and then 0.001 M potassium phosphate buffer (KPB). Then two linear KPB gradients were applied (0.001–0.2 M with a total volume of 3 L and 0.2–0.4 M with a total volume of 2 L), and a rubrerythrin- and cytochrome-containing fraction was eluted at about 0.03 M. This fraction was concentrated to a volume of 10 mL, degassed, and loaded onto a Sephadex G-75 column (5.4 × 8.5 cm) equilibrated with 0.05 M Tris-HCl buffer. At a flow rate of 25 mL/h the rubrerythrin, which formed a distinct rose-colored band followed by a cytochrome band, was eluted from the column. This rubrerythrin fraction, which had a A_{280}/A_{490} ratio of 15, was twice concentrated to 5 mL and diluted with water to 50 mL to lower the ionic strength.

The rubrerythrin fraction was then loaded onto a DE-52 column (4.5 × 29 cm), and a linear Tris-HCl gradient was applied (0.01–0.1 M with a total volume of 1.5 L). The rubrerythrin, which eluted at about 0.03 M, was then applied to a second hydroxylapatite column (4 × 23 cm) equilibrated with 0.001 M KPB. A linear gradient was applied (0.001–0.1 M with a total volume of 1.5 L), and the purified rubrerythrin collected at about 0.03 M had an A_{280}/A_{490} ratio of 7.0.

Molecular Weight Determination and Electrophoretic Analysis. The molecular weight of rubrerythrin was determined by equilibrium ultracentrifugation at 20 °C with a Beckman Model E analytical ultracentrifuge. Subunit molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE) using Bio-Rad low molecular weight standards (M_r 14 400–92 500). Isoelectric focusing was performed by using an analytical thin-layer polyacrylamide gel with an ampholyte pH gradient between pH 3 and 10 on a LKB multiphor apparatus.

Amino Acid Analysis. Protein samples were analyzed for individual amino acids on a Beckman Model CL25 amino acid analyzer. The analysis was performed after hydrolysis of the protein in 6 N HCl for 24, 48, and 72 h in vacuum-sealed Pyrex tubes; norleucine was used as the internal standard. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (Hirs, 1966). Tryptophan was determined after treatment with thioglycollic acid (Matsubara & Sasaki, 1969).

Iron Content Determination. Iron content was determined by plasma emission spectroscopy using a Jarrell-Ash Atomcomp 750 spectrometer and also by chemical determination with bathophenanthroline after trichloroacetic acid precipitation (Brumby & Massey, 1967). Samples for plasma emission analysis were run in the presence of 50 mM Tris-HCl, pH 7.6, against blanks of equal buffer concentration.

Spectroscopies. The UV–visible absorption data were recorded on an AVIV Model 14DS spectrophotometer. EPR measurements were performed on a Bruker ER-200 tt spectrometer at the University of Lisbon or on a Bruker ER 200D-SRC spectrometer at Emory University. Both EPR spectrometers are equipped with Oxford Instruments continuous flow cryostats. The weak- and strong-field Mössbauer spectrometers are of the constant acceleration types and have been described elsewhere (Yang et al., 1987). The zero velocity of the Mössbauer spectra was referred to the centroid of the room temperature spectrum of a metallic iron foil.

Redox Titration. Redox titrations were performed on proteins in 100 mM Tris-HCl, pH 8.6, in the presence of redox mediators (70 μM each). The potential was adjusted by addition of small amounts of dithionite (0.2 M) or ferricyanide solution (0.2 M). After equilibration at a fixed potential, a sample was transferred into an EPR tube under argon at-

Table I: Amino Acid Composition of *D. vulgaris* Rubrerythrin Subunit[a]

Lys	11–12	Ala	29
His	8	Cys	4
Arg	12	Val	4
Trp	0	Met	2
Asp	15	Ile	7
Thr	8	Leu	11
Ser	9	Tyr	6
Glu	31–32	Phe	14
Pro	8		
Gly	17–18	total	196–199

[a] Calculation based upon a molecular weight of 21 900.

mosphere and frozen immediately at 77 K. The reduction of the Rd center was monitored by the disappearance of the sharp and intense signal at $g = 4.3$.

Materials. DEAE-Bio-Gel A, hydroxylapatite, and low molecular weight protein standards were purchased from Bio-Rad. Sephadex G-75 and ampholytes were purchased from LKB. DE-52 was purchased from Whatman. All other chemicals were of reagent grade or of the highest available purity.

Results

Homogeneity, Molecular Weight, Iron Content, and Amino Acid Composition. The purified rubrerythrin from *D. vulgaris* was judged to be homogeneous according to the following criteria: electrophoresis on a polyacrylamide gel (pH 8.8) indicated the presence of a single red band, and only this band was visible after the protein was stained with Coomassie brilliant blue R-250. Similar results were obtained on a 10% SDS–PAGE slab gel and with thin-layer gel isoelectric focusing. The molecular weight of the purified rubrerythrin was determined to be 45 200 by equilibrium ultracentrifugation, and the subunit molecular weight was 21 900 by SDS–polyacrylamide gel electrophoresis, suggesting an α_2 composition for the native protein.

Two separate preparations of rubrerythrin were used for total iron content determination: a ^{57}Fe-enriched preparation and a naturally abundant ^{56}Fe preparation. A value of 3.9 ± 0.4 iron atoms/molecule (45.2 kDa) was found for the ^{57}Fe-enriched rubrerythrin and 4.1 ± 0.2 iron atoms/molecule for the naturally abundant ^{56}Fe protein. In the following sections, evidence is presented to show that these four iron atoms are organized into two Rd-like FeS_4 sites and a Hr-like binuclear iron center.

The amino acid composition of the purified rubrerythrin is listed in Table I. Large amounts of basic and hydrophobic residues were found for rubrerythrin, consistent with the isoelectric focusing result, which shows an isoelectric point of 7.1. The fact that four cysteine residues were found for each subunit indicates that each subunit may bind one Rd center. The large amount of histidine residues observed for rubrerythrin is consistent with the presence of a Hr-like binuclear iron center since most of the ligands of the binuclear center in Hr are histidine.

Spectral Studies. Figure 1 shows an UV–visible absorption spectrum of the as-isolated rubrerythrin from *D. vulgaris*. The spectrum exhibits maxima at 492, 365, and 280 nm with shoulders at 570 and 350 nm. Except for the higher A_{365}/A_{492} ratio, the rubrerythrin spectrum appears very similar to those of the Rds (Moura et al., 1977). The molar extinction coefficient at 492 nm was determined to be 10.4 mM^{-1} cm^{-1}, which is slightly less than twice the value found for Rds. In order to examine whether the higher A_{365}/A_{492} ratio is caused by the presence of the Hr-like center, we have performed the

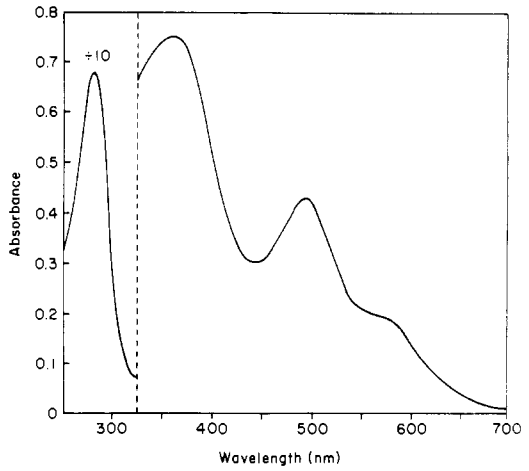

FIGURE 1: Absorption spectrum of the purified rubrerythrin from *D. vulgaris* measured in 0.1 M phosphate buffer, pH 7.6, with 1.9 mg of protein/mL.

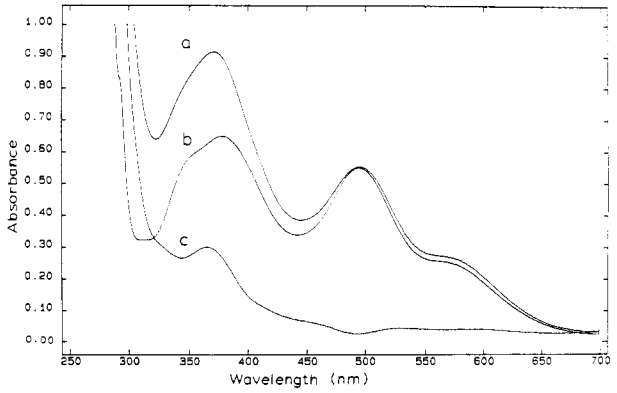

FIGURE 2: Absorption spectra of the purified rubrerythrin (a) and the Rd (b) from *D. vulgaris*. Spectrum b is normalized so that its intensity at the 490-nm peak matches that of spectrum a. Trace c is the difference spectrum between spectra a and b, showing the contribution from the Hr-like center in rubrerythrin.

following manipulation of the data. First, we assumed that the absorption contribution from the Rd-like centers in rubrerythrin can be approximated by the optical spectrum of Rd from *D. vulgaris*. We then proceeded to remove the contribution of the Rd sites from the rubrerythrin spectrum by subtracting the spectrum of *D. vulgaris* Rd from the rubrerythrin spectrum using the 492-nm peak as a reference. The resulting spectrum (shown in Figure 2) exhibits an absorption maximum at 365 nm and a shoulder at 460 nm. The estimated molar extinction coefficient at 365 nm is 5.3 mM^{-1} cm^{-1}. This spectrum bears some resemblance to that of met-Hr, which was reported to show an absorption maximum at 355 nm (ϵ_{355} = 6.4 mM^{-1} cm^{-1}) and shoulders at 480 and 580 nm (Garbett et al., 1969). Consequently, the spectrum of the native rubrerythrin is reasonably consistent with the picture that rubrerythrin contains two Rd-like and one Hr-like iron centers.

EPR and Midpoint Redox Potential. A low-temperature (8 K) EPR spectrum of the purified rubrerythrin from *D. vulgaris* is shown in Figure 3. The dominant EPR signals observed have g values of 9.4 and 4.3, which are characteristic of a high-spin ferric ion in a rhombic crystal field (E/D = 0.26), and are similar to those observed for oxidized Rds (Peterson & Coon, 1968; Peisach et al., 1971; Moura et al., 1978). The $g = 9.4$ signal arises from the ground-state Kramers doublet and the $g = 4.3$ signal from the excited middle doublet. Even though these signals are commonly observed for the Rds, it is important to point out that they are not unique for Rd. Other proteins, such as protocatechuate

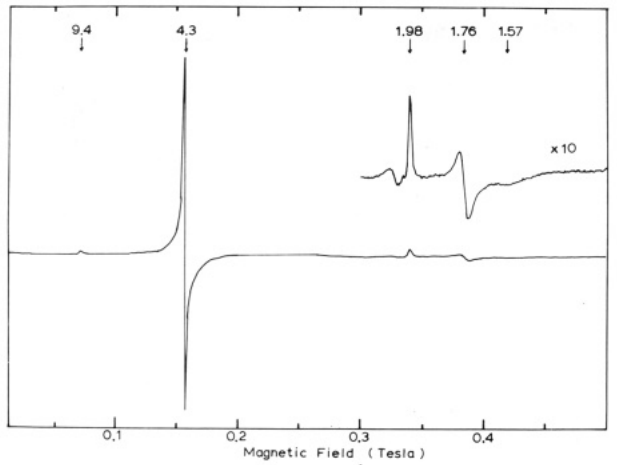

FIGURE 3: EPR spectrum of the purified rubrerythrin from *D. vulgaris* measured at 8 K in 0.01 M phosphate buffer, pH 7.6, with 0.8 mM protein concentration. Other experimental conditions are microwave frequency 9.435 GHz, microwave power 2 μW, modulation amplitude 1 mT, and receiver gain 5×10^4.

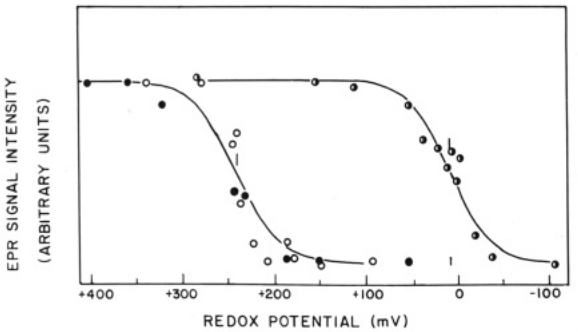

FIGURE 4: EPR redox titration of rubrerythrin and rubredoxin from *D. vulgaris*: (○, ●) rubrerythrin; (◑) rubredoxin. The solid lines are the calculated Nernst curves for 1e reduction. The midpoint redox potentials are +230 mV for rubrerythrin and 0 mV for rubredoxin.

3,4-dioxygenase (Wittaker et al., 1982), also exhibit similar EPR signals. It is by gathering information obtained from all the different techniques reported in this paper that we have concluded that rubrerythrin contains Rd-like FeS_4 centers.

In order to determine the midpoint oxidation–reduction potential for the Rd-like center, redox titration was performed on the purified rubrerythrin. The reduction of the FeS_4 center was monitored by the sharp and intense EPR signal at $g = 4.3$ observed at 77 K. For comparison, redox titrations were also performed on the Rd isolated from *D. vulgaris*. In Figure 4, the intensities of the $g = 4.3$ signals were plotted as a function of the redox potential. The data were fitted with calculated Nernst curves for 1e reduction. The midpoint potentials were estimated to be +230 ± 10 mV for the Rd-like center in rubrerythrin and 0 ± 10 mV for the Rd. The value obtained for *D. vulgaris* Rd is within the range of midpoint potentials found for Rds from other organisms, but the redox potential for the Rd-like center in rubrerythrin is exceptionally high in comparison with other electron-transfer proteins found in *D. vulgaris*, suggesting that rubrerythrin may not function in the electron-transfer pathway leading from sulfate to sulfide.

From the following Mössbauer studies, we realize that in addition to the Rd-like centers in rubrerythrin there exists an Hr-like center composed of a pair of antiferromagnetically coupled high-spin ferric ions. This Hr-like center is diamagnetic in the native state and should yield no EPR signal. Nevertheless, the presence of an Hr-like center is revealed by a very weak EPR signal at the $g < 2$ region. When the intensity of this region is amplified by a factor of 10, a well-

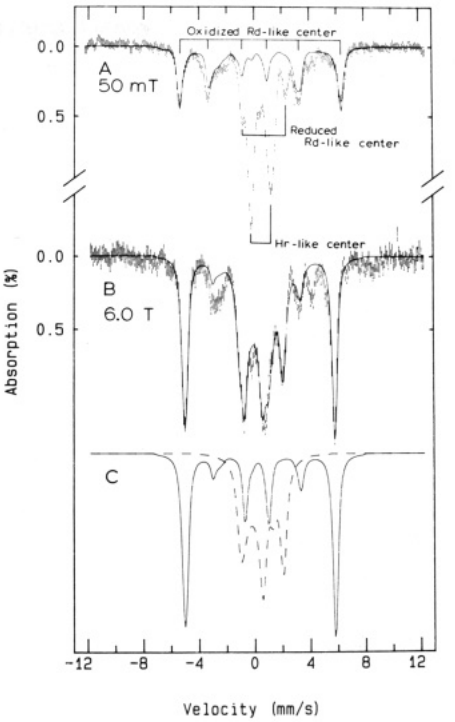

FIGURE 5: Mössbauer spectra of the purified rubrerythrin from *D. vulgaris*. The data were recorded at 4.2 K in parallel applied fields of the indicated strengths. The solid line in (A) is a theoretical simulation of the oxidized Rd-like center and is normalized to 45% of the total iron absorption. The spectra shown in (C) are theoretical simulations of the Rd-like center (—) and the diamagnetic Hr-like center (- - -) in an applied field of 6 T. The solid line in (B) is the summation of the spectra shown in (C).

defined EPR signal with g values at 1.98, 1.76, and 1.57 is observed. (See Figure 3.) These g values and the shape of this EPR signal are highly similar to those observed for the semimet-Hr (Muhoberac et al., 1980; Lukat et al., 1984) and support the existence of an Hr-like center in rubrerythrin.

Mössbauer Results. Figure 5A shows a Mössbauer spectrum of the as-purified rubrerythrin from *D. vulgaris* recorded at 4.2 K with an external magnetic field of 50 mT applied parallel to the γ-beam. Two major spectral components are observed. The six-line magnetic spectral component extending from −5.5 to +6.4 mm/s is very similar to that observed for *C. pasteurianum* Rd (Schulz & Debrunner, 1976) and is attributed to the Rd-like FeS_4 center. The central doublet has Mössbauer parameters ($\Delta E_Q = 1.47 \pm 0.05$ mm/s and $\delta = 0.52 \pm 0.03$ mm/s) that are typical for high-spin ferric ions with nitrogenous and/or oxygenous ligands. These high-spin ferric ions are labeled Hr-like for reasons that will become obvious. A minor component (approximately 5% of the total absorption) is also observed as a quadrupole doublet with parameters identical with those of the reduced Rd-like center (see below). Since the Rd-like center has a redox potential of +230 mV, it is not surprising to find that a minor portion of the Rd-like centers is reduced in the as-purified protein. A more detailed analysis of spectrum 5A presented below shows that the percent absorption originating from the Rd-like iron (oxidized plus reduced) and from the Hr-like iron are approximately equal, indicating that rubrerythrin contains equal amounts of Rd-like and Hr-like iron atoms (i.e., two for each).

An unusual feature observed for the Hr-like iron is that its low-temperature spectrum is a quadrupole doublet but has parameters of high-spin ferric ions. In general, a high-spin ferric ion yields a magnetically split spectrum at low temperatures (e.g., 4.2 K). The observation of a quadrupole

Table II: Spin Hamiltonian Parameters for the Rd-like Center in *D. vulgaris* Rubrerythrin and for the Native Rd from *C. pasteurianum*

	rubrerythrin[a]	Rd[b]
D (cm^{-1})	1.5 ± 0.5	1.9 ± 0.3
E/D	0.26 ± 0.03	0.23 ± 0.02
g	2.0	2.0
$A_{xx}/g_n\beta_n$[c] (T)	−16.5 ± 1.0	−16.5 ± 1.1
$A_{yy}/g_n\beta_n$ (T)	−15.6 ± 0.5	−15.9 ± 0.3
$A_{zz}/g_n\beta_n$ (T)	−17.0 ± 0.5	−16.9 ± 0.3
ΔE_Q (mm/s)	−0.55 ± 0.10	−0.50 ± 0.05
δ (mm/s)	0.27 ± 0.04	0.32 ± 0.02
η[d]	0.7 ± 0.4	0.2 ± 0.1

[a] The uncertainties were estimated by visually comparing the theoretical simulations with the experimental data. [b] From Schulz and Debrunner (1976). [c] The symbol β_n represents the nuclear magneton, and g_n has the values 0.1806 and −0.1033 for the ground and excited states of the ^{57}Fe nucleus, respectively. [d] $\eta = (V_{xx} - V_{yy})/V_{zz}$, where V_{ii} are the principal components of the electric field gradient tensor.

doublet for the Hr-like iron indicates that either it has a fast electronic relaxation in comparison with the nuclear precession or it is in a diamagnetic ($S = 0$) environment. In order to distinguish these two situations, we recorded rubrerythrin spectra at strong applied fields up to 8 T. Figure 5B shows the 4.2 K Mössbauer spectrum of rubrerythrin recorded in a field of 6 T applied parallel to the γ-beam. The central doublet observed in Figure 5A is magnetically split by an effective field of exactly 6 T as shown in Figure 5B, indicating that the high-spin ferric ions are in a diamagnetic environment. Consequently, the two Hr-like iron atoms in rubrerythrin must be antiferromagnetically coupled and thus result in the observed diamagnetism. Similar Mössbauer characteristics have been observed for the binuclear iron cluster in the met-Hr (Okamura et al., 1969: diamagnetic, $\Delta E_Q = 1.57$ mm/s and $\delta = 0.46$ mm/s at 77 K), and this has resulted in our choice of the term "Hr-like" iron.

In order to further characterize the iron centers in rubrerythrin, we applied the following spin Hamiltonian to analyze the data in detail.

$$\hat{H} = D[S_z^2 - S(S+1)/3 + (E/D)(S_x^2 - S_y^2)] + \beta\vec{S}\cdot\tilde{g}\cdot\vec{H} + \vec{S}\cdot\tilde{A}\cdot\vec{I} + (eQV_{zz}/12)[3I_z^2 - I(I+1) + \eta(I_x^2 - I_y^2)] - g_n\beta_n\vec{H}\cdot\vec{I} \quad (1)$$

For the Rd-like iron, the spin S equals $^5/_2$, and the value of E/D is determined to be 0.26 from the EPR data. The magnetic hyperfine coupling tensor $\tilde{A}$ for a high-spin ferric ion is generally quite isotropic and can be determined with reasonable accuracy from the splitting of the weak-field spectrum. The zero-field splitting D can then be obtained from the splitting of the strong-field spectra. Other parameters were determined through a series of theoretical simulations and visual comparisons of the simulations with experiments. The parameters thus obtained for the Rd-like iron in rubrerythrin are listed in Table II and the simulated spectra for the Rd-like center are plotted in Figure 5 (the solid lines in Figure 5A,C). The theoretical spectra are normalized to be 45% of the total absorption. For comparison, the parameters obtained for the FeS_4 center in *C. pasteurianum* Rd are also listed in Table II. These two sets of parameters are almost identical, suggesting strongly that these two FeS_4 centers must have very similar structural conformations.

In order to show that the Hr-like iron atoms are in a diamagnetic environment, we use the values of ΔE_Q and δ obtained from the weak-field spectrum and simulated the corresponding strong-field spectra assuming diamagnetism for the iron sites. The dashed line in Figure 5C is the result of such a simulation. The theoretical spectrum is normalized to

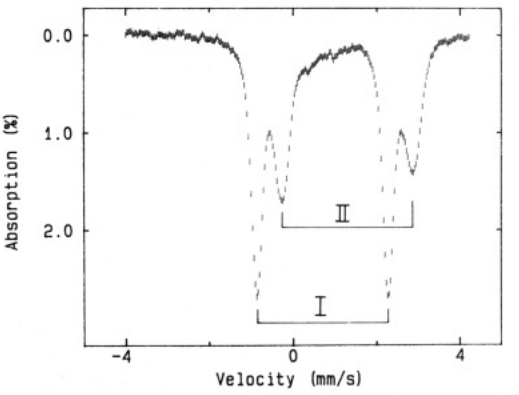

FIGURE 6: Mössbauer spectrum of the reduced rubrerythrin from *D. vulgaris* recorded at 4.2 K in the absence of a magnetic field. Doublet I is originating from the reduced Rd-like center and doublet II from the reduced Hr-like center. A very weak doublet (less than 5% of the total absorption) of undetermined origin is also observed at the center of the spectrum.

be 50% of the total absorption. The solid line in Figure 5B plotted over the data is the sum of the theoretical spectra of the Rd-like and the Hr-like centers. The good agreement between theory and experiment indicates that the Hr-like center in native rubrerythrin is indeed diamagnetic, and the percent absorptions originating from the Rd-like and Hr-like iron atoms are approximately equal.

Since the iron centers in rubrerythrin have relatively high redox potentials, they can be reduced by ascorbic acid. Figure 6 shows a spectrum of an ascorbic acid reduced rubrerythrin sample. The data were recorded at 4.2 K in the absence of a magnetic field. Two quadrupole doublets are observed and are labeled doublet I and doublet II. A least-squares fit was performed; the parameters obtained for doublet I are $\Delta E_Q = 3.15 \pm 0.04$ mm/s, $\delta = 0.70 \pm 0.03$ mm/s, and full width at half-maximum $\Gamma = 0.30 \pm 0.02$ mm/s and for double II $\Delta E_Q = 3.14 \pm 0.04$ mm/s, $\delta = 1.30 \pm 0.03$ mm/s, and $\Gamma = 0.45 \pm 0.04$ mm/s. Both sets of parameters are typical for high-spin ferrous ions ($S = 2$). The smaller isomer shift, 0.7 mm/s, for doublet I is characteristic for a high-spin ferrous ion with tetrahedral coordination of sulfur ligands. In fact, the parameters for doublet I are almost identical with those obtained for the reduced Rd from *C. pasteurianum* (Schulz & Debrunner, 1976). The larger isomer shift, 1.30 mm/s, for doublet II is similar to that of deoxy-Hr (Okamura et al., 1969; Clark & Webb, 1981) and is indicative of an octahedral coordination with oxygen/nitrogen ligands. The ratio of the percent absorptions of doublet I to doublet II is determined to be 1.07 from the least-squares-fit analysis. Consequently, the Mössbauer data of the reduced rubrerythrin provide further evidence that rubrerythrin contains equal amounts of two types of iron atoms: the Rd-like and the Hr-like irons.

In the presence of an external applied field (≥2.0 T), both doublets I and II exhibit paramagnetic properties. Paramagnetism is expected for the Rd-like mononuclear high-spin ferrous ion and has been observed for deoxy-Hr (Clark & Webb, 1981). In deoxy-Hr, the exchange coupling constant, $-J$, of the binuclear [Fe(II), Fe(II)] center was estimated to be reduced to the order of 10–20 cm^{-1} (Reem & Solomon, 1984; Maroney et al., 1986). Presently, the analysis of the strong-field spectra of the reduced rubrerythrin is not yet completed and will be the subject of a future report.

Discussion

In the previous sections, we have presented the purification scheme and some physicochemical properties of a novel nonheme iron protein isolated from the periplasmic fraction of

D. vulgaris. This protein is composed of two identical subunits with subunit molecular weight of 21 900. Each molecule was found to contain four iron atoms that are organized into two types of prosthetic groups: two Rd-like FeS_4 centers and an Hr-like antiferromagnetically coupled binuclear iron center. Due to this unusual combination of prosthetic groups, this new protein is termed rubrerythrin.

For the Rd-like iron, the observed optical, EPR, and Mössbauer data are very similar to those found for Rds (Schulz & Debrunner, 1976; Cammack et al., 1977; Moura et al., 1977 & 1980; LeGall et al., 1984). In particular, the fine and hyperfine parameters obtained for the Rd-like iron in rubrerythrin are almost identical with those reported for the Rd from *C. pasteurianum* (Schulz & Debrunner, 1976). Consequently, the spectroscopic data suggest that the Rd-like center in rubrerythrin must be structurally similar to, if not identical with, the FeS_4 center in Rd. The amino acid composition, which shows four cysteine residues per subunit, further indicates that each subunit binds one Rd-like center. The redox titration studies, however, show that the Rd-like center in rubrerythrin has a midpoint redox potential that is approximately 200 mV more positive than those found for Rds. We believe that this high redox potential does not result from variation of the structure of the FeS_4 site, as this would be reflected in the EPR and Mössbauer spectral properties. An iron–sulfur protein, desulforedoxin from *D. gigas*, was suggested to contain distorted FeS_4 centers and exhibits very different EPR and Mössbauer spectra (Moura et al., 1980). Redox potentials of other classes of proteins, such as the *c*-type cytochromes (Mathews, 1985), flavoproteins (Mueller, 1983), and iron–sulfur proteins (Carter, 1977), are known to vary over a wide range. A combination of factors, including the nature of the redox center, is likely to play important, albeit variable, roles in modulating the value of the midpoint redox potential.

In iron-containing proteins, there exist basically two types of binuclear iron centers: the Fe_2S_2 cluster found in iron–sulfur proteins (Sands & Dunham, 1975) and the Hr-type binuclear center found in hemerythrin (Wilkins & Harrington, 1983), ribonucleotide reductase (Sjöberg & Gräslund, 1983), uteroferrin, and purple acid phosphatase (Antanaitis & Aisen, 1983). In the oxidized forms, both types of centers are similar in the sense that they are composed of two antiferromagnetically coupled high-spin ferric ions, resulting in a diamagnetic system. They differ, however, in many respects. The Fe_2S_2 cluster can exist only in two stable oxidation states, the oxidized [Fe(III), Fe(III)] and the reduced [Fe(II), Fe(III)] states, while the Hr-type center can exist in three states, the oxy or met [Fe(III), Fe(III)], the semimet [Fe(II), Fe(III)], and the reduced [Fe(II), Fe(II)] states. Also, the iron atoms of the Fe_2S_2 cluster are tetrahedrally coordinated to four cysteinyl sulfur ligands, but the ligands of the Hr-like center are oxygen and nitrogen.

In native rubrerythrin, the Mössbauer data indicate that two of the iron atoms are strongly antiferromagnetically coupled to form a diamagnetic binuclear iron center. Both iron atoms are high-spin ferric and have nitrogen and/or oxygen as their ligands. In the reduced rubrerythrin, the iron atoms in this binuclear center are high-spin ferrous. Consequently, this binuclear center belongs to the type found in Hr. Other evidence supporting the Hr-like nature of this center is the observation of a characteristic EPR signal at $g = 1.98$, 1.76, and 1.57 in the native rubrerythrin. Similar EPR signals have been reported for the many different forms of the Hr-like centers in their semimet oxidation states (Muhoberac et al., 1980; Antanaitis & Aisen, 1982; Kurtz et al., 1983). The reduced Fe_2S_2 center is in an oxidation state equivalent to that of semimet-Hr and also exhibits characteristic EPR signal (Sands & Dunham, 1975). However, the EPR signal of the reduced Fe_2S_2 center is very distinct from that of the semimet-Hr. Unfortunately, the semimet-Hr-like signal observed in the native rubrerythrin represents only a very small fraction of the protein (less than 5%, estimated from the Mössbauer data). Attempts to convert a majority of the Hr-like center into the semimet form have not been successful so far.

Preliminary X-ray crystallographic data of rubrerythrin show a twofold symmetry for the molecule (Sieker et al., unpublished result). Since only one binuclear center is found for each molecule, it must be bridged between the two subunits in order to retain the twofold symmetry. In this respect, rubrerythrin is analogous to the *Escherichia coli* ribonucleotide reductase, which also has an Hr-like binuclear center bridged between the two B2 subunits (Lammers & Follman, 1983).

As we have pointed out under Results, the redox potentials of the iron centers in rubrerythrin are relatively high in comparison with other electron carriers in *D. vulgaris*, and, therefore, rubrerythrin may not participate in the electron-transfer pathway of sulfate reduction. Recently, it has been shown that certain bacteria, including *D. vulgaris*, can obtain energy for growth by disproportionation of sulfite into sulfate and sulfide (Bak & Cypionka, 1987; Bak & Pfennig, 1987). Interestingly, *D. vulgaris* was found to contain many unique proteins, such as rubrerythrin and cytochrome c_{553}. In our preliminary search for a function for rubrerythrin, we discovered that sulfite is capable of reducing rubrerythrin. The reduction process, however, took hours to complete. Although this observation may suggest that our suspicion of rubrerythrin being a sulfite oxidase may not be correct, it may also indicate that the presence of an enzymatic electron acceptor is required for the oxidation process. We have also attempted to complex rubrerythrin with small ligands, such as sulfide and azide, but with no success. Obviously, the physiological function of this new-found protein requires and deserves further scrutiny.

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