

On the two iron centers of desulfoferrodoxin

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Desulfoferrodoxin from *Desulfovibrio vulgaris*, strain Hildenborough, is a homodimer of 28 kDa; it contains two Fe atoms per 14.0 kDa subunit. The N-terminal amino-acid sequence is homogeneous and corresponds to the previously described *Rbo* gene, which encodes a highly charged 14 kDa polypeptide without a leader sequence. Although one of the two iron centers, Fe_A, has previously been described as a 'strained rubredoxin-like' site, EPR of the ferric form proves very similar to that of the pentagonal bipyramidally coordinated iron in ferric complexes of DTPA, diethylenetriaminepentaacetic acid: both systems have spin $S = 5/2$ and rhombicity $E/D = 0.08$. Unlike the Fe site in rubredoxin the Fe_A site in desulfoferrodoxin has a pH dependent midpoint potential with $pK_{ox} = 9.2$ and $pK_{red} = 5.3$. Upon reduction ($E_{m,7.5} = +2$ mV) Fe_A exhibits an unusually sharp $S = 2$ resonance in parallel-mode EPR. The second iron, Fe_B, has $S = 5/2$ and $E/D = 0.33$; upon reduction ($E_{m,7.5} = +90$ mV) Fe_B turns EPR-silent.

Bioelectrochemistry; EPR; Rubredoxin; Redox; *Desulfovibrio vulgaris* (H)

1. INTRODUCTION

Rubredoxin is a small, monomeric, mononuclear iron protein. It is generally present in fermentative and in sulfate reducing anaerobic bacteria, where it probably functions in secondary electron transport. There is refined structural data on several rubredoxins to 1.2–1.5 Å resolution. The mononuclear iron is coordinated by four cysteinate ligands forming a highly distorted tetrahedron. The reduction potential at neutral pH is close to 0 mV ([1] and refs quoted therein).

Three different, and mutually quite distinct classes of iron proteins have been described in sulfate reducers as intriguing variants to the rubredoxin system: (1) rubrerythrin/nigerythrin, (2) desulforedoxin, and (3) desulfoferrodoxin. The reddish rubrerythrin and the similar, blackish protein nigerythrin appear to contain a regular Fe(Cys)₄ rubredoxin site, however, with an unusually high reduction potential; they also contain a high-potential dinuclear iron cluster presumably with His/Glu coordination [2,3]. Both desulforedoxin and desulfoferrodoxin have been proposed to contain an Fe(Cys)₄ site that is more 'strained' compared to the site in rubredoxin because two of the Cys residues are adjacent in the primary sequence [4,5]. Furthermore, desulfoferrodoxin contains a second iron site presumably with octahedral N/O coordination [5]. Acid-labile sulfide is not found in any of these proteins. No biological function has been established or even indicated.

There are several unsolved questions related to the so-called strained rubredoxin-like center in desulfore-

doxin and desulfoferrodoxin. The proposed coordination is based on a questionable analogy with rubredoxin; redox potentials at unspecified pH have been reported in preliminary reports [6,7]; the proposed subunit composition is puzzling: homodimeric for desulforedoxin [4] and monomeric for desulfoferrodoxin [5]; desulfoferrodoxin from *D. vulgaris* has been reported to be a mixture of two proteins with distinct primary sequence [5]. These questions are addressed in what follows.

2. MATERIALS AND METHODS

Desulfovibrio vulgaris, strain Hildenborough, NCIB 8303, was maintained, grown in 300 liter batch cultures, and harvested as previously described [8]. The purification scheme of desulfoferrodoxin was similar to the original one described by Moura et al. [5] with minor modifications:

After the first DEAE column the ionic strength of the sample was adjusted by dilution with 1 mM potassium phosphate buffer and the sample was loaded onto a hydroxylapatite column (Bio-Rad) (2.6 × 7 cm) equilibrated with 1 mM potassium phosphate pH 7.0. A linear phosphate gradient (1–200 mM) with a total volume of 350 ml was applied and the pooled fractions were concentrated in an Amicon cell with a YM 10 membrane. The sample was subsequently loaded onto a Superdex G-75 gel filtration column (2.6 × 60 cm) (Pharmacia) equilibrated with 50 mM potassium phosphate pH 7.0 and 150 mM NaCl. The fractions containing desulfoferrodoxin were collected using polyclonal antibodies and, after concentration and dilution, applied onto a MonoQ HR 5/5 column (Pharmacia) equilibrated with 10 mM HEPES pH 7.0. A shallow linear gradient (0–1 M NaCl) was applied and two closely spaced purple peaks were eluted from the MonoQ column and analyzed separately (see below).

2.1. Analytical determinations

Iron was determined colorimetrically according to the procedure described in [9] using 3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triaz-

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ine, disodium salt trihydrate (ferene, Aldrich) as the chelator. Protein was determined using either the microbiuret method [10] with trichloroacetic acid/deoxycholate precipitation [11] or the method described by Bradford [12]. The reagent used for the Bradford protein determination was obtained from Bio-Rad. Bovine serum albumine was used as a standard.

Electrophoresis was performed with a Midget system (Pharmacia) using 17.5% and 20% gels, or with a Phastsystem (Pharmacia) using 8–25% gradient gels. The SDS-polyacrylamide gels were according to Laemmli [13]. The gels were calibrated with Pharmacia low molecular weight markers. For Isoelectric focussing 3–9 precoated gels were used with a Phastsystem. The calibration was performed with the broad pI calibration kit (Pharmacia). The native molecular mass of the protein was determined with a superdex G-75 gel filtration column equilibrated with 50 mM potassium phosphate pH 7.0 and 150 mM NaCl using a flow rate of 1.5 ml/min. BSA (67 kDa), ovalbumine (43 kDa), myoglobin (18.8 kDa), cytochrome c_3 (14.1 kDa) and cytochrome c_{553} (9.2 kDa) were used as the markers for the calibration. The void volume was determined with Blue dextran.

2.2. Spectroscopy

UV/VIS spectra were recorded on a Aminco DW 2000 spectrophotometer using 1 cm quartz cuvettes. EPR measurements and mediated redox titrations were as previously described [8].

2.3. N-terminal sequencing

4 μ g quantities of the purified protein were freeze dried. Gas-phase sequencing of these samples was carried out at the sequencing facility of the Netherlands Foundation for Chemical Research (SON) (Dr R. Amons, University of Leiden, NL).

2.4. Antibodies

The purified desulfoferrodoxin was applied to a Laemmli gel (300 $\times$ 150 $\times$ 1.5 mm) and the protein was electroeluted after excision of the 14.0 kDa band. Antibodies were induced in mice by subcutaneous injection of a 35 μ g quantity mixed with Freund's complete adjuvant. Boosts of equal amounts of antigen mixed with Freund's incomplete adjuvant were administered twice at three-weekly intervals. The serum was used without further purification. Proteins were blotted onto a nitrocellulose support after separation on denaturing SDS gels [14]. Goat anti-mouse IgG alkaline phosphatase conjugate (Boehringer, Germany) was used for immunostaining.

2.5. Electrochemistry

The electrochemical measurements were performed as described by Hagen [15] with the following modifications: The working electrode was treated with nitric acid and washed with 0.1 M dipotassiumhydrogenphosphate and water. Instead of polishing, the electrode was held in a methane flame until it became red-hot. After cooling down, 20 μ l of a 20 mM neomycin solution was applied onto the electrode. Subsequently the electrode was dried with a tissue and mounted in the electrochemical cell. The sample was applied and the cyclic voltammetry was started using a BAS CV 27 potentiostat (BioAnalytical Systems Indiana, USA) connected to an X-Y recorder (Kipp & Zonen, NL). After one scan neomycin was added to the sample in a final concentration of 2 mM. The pH dependence was measured using sodium acetate, MES, HEPES, TAPS, CHES and CAPS all at a final concentration of 50 mM. Temperature dependence was measured as described by Link et al. [16].

3. RESULTS

3.1. Primary structure

A mixture of two different amino-acid sequences has

	1	5	10	15
protein:	Met-Pro-Yyy-Gln-Tyr-Glu-Ile-Tyr-Lys-Xxx-Ile-Yyy-Xxx-Gly-Asn-Ile			
Rbo gene:	Met-Pro-Asn-Gln-Tyr-Glu-Ile-Tyr-Lys-Cys-Ile-His-Cys-Gly-Asn-Ile			

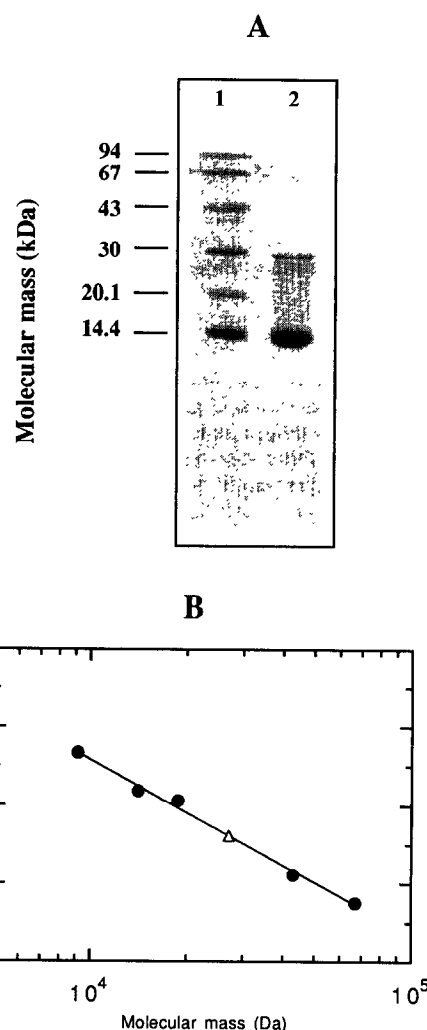


Fig. 1. (A) SDS-PAGE of purified *D. vulgaris* (Hildenborough) desulfoferrodoxin. Lane 1, molecular mass marker mixture; lane 2, 5 μ g desulfoferrodoxin. The band at 28 kDa is the dimeric form of the protein. (B) Calibration of the native molecular mass of Desulfoferrodoxin with a Superdex G-75 gel filtration column. The gel filtration was performed as described in section 2. K_{av} is the ratio of the elution volume V_e minus the void volume V_0 over the total volume V_t minus the void volume V_0 .

been reported for *D. vulgaris* desulfoferrodoxin by Moura et al. one corresponding to the sequence of the protein from *D. desulfuricans* ATCC 27774 and the second corresponding to the *Rbo* gene from *D. vulgaris* except for the first two residues [5,17]. Since in our hands the protein always eluted in two separate peaks from the final MonoQ column, samples from both peaks were freeze dried and mailed for separate analysis in the gas-phase sequenator at the University of Leiden. The two samples had identical N-terminal sequences, which fully corresponded to that of the *D. vulgaris* *Rbo* gene including the leading residues:

No indication whatsoever was found for a sequence corresponding to the *D. desulfuricans* protein. Since both samples were also found to be indistinguishable on SDS-PAGE, in isoelectric focussing, and in spectroscopy (not shown) their distinction is ignored from now on.

3.2. Subunits and antibodies

The purified protein exhibited a two-band pattern on SDS-PAGE corresponding to molecular masses of approximately 14 and 28 kDa (see Fig. 1A). The 14 kDa band was excised and used to raise polyclonal antibodies in mouse. When these antibodies were used in a Western blot of the purified protein both the 14 kDa and the 28 kDa proved to exhibit comparable reactivity suggesting that the 28 kDa band is a dimeric aggregate of the 14 kDa band (data not shown). It was subsequently found that the dimerization in the SDS gel was dependent on the lapse time after boiling of the protein in SDS, but was independent of the boiling time and of the excess amount of SDS. Application of the sample onto the gel directly after boiling and running the gel immediately afforded mainly a 14 kDa band pattern. When the sample was left for 1 h after boiling and/or

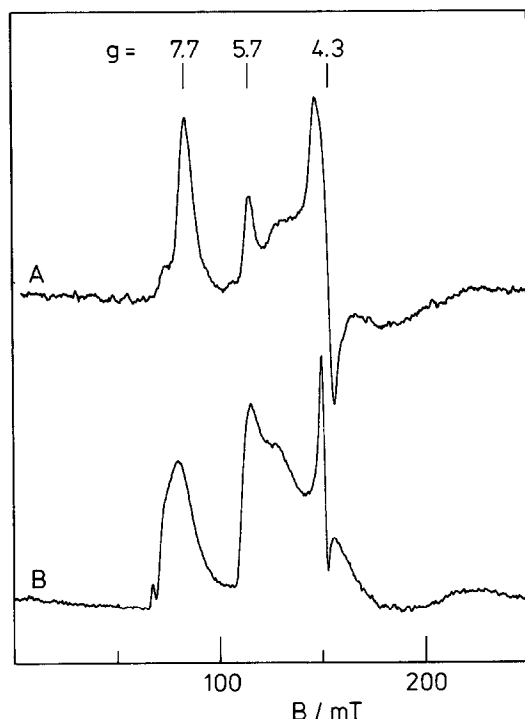


Fig. 2. Comparison of the EPR spectral shapes from desulfoferrodoxin (trace A) and ferric DTPA (trace B). The 'as isolated' desulfoferrodoxin from *D. vulgaris* (H) was 2.4 mg/ml in 10 mM Tris/HCl pH 8.0. Ferric DTPA was 1 mM FeCl_3 plus 10 mM DTPA with the pH adjusted to 5.0. EPR conditions: microwave frequency, 9.14 GHz; microwave power, 200 mW; modulation frequency, 100 KHz; modulation amplitude, 1.25 and 1.6 mT; temperature, 10 and 16 K. The microwave power level is somewhat saturating for the middle part of the FeDTPA spectrum resulting in an emphasized peak at $g = 5.7$.

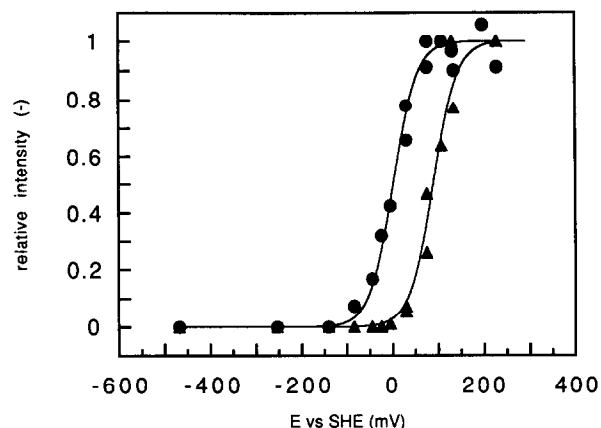


Fig. 3. Redox titration of purified desulfoferrodoxin from *D. vulgaris* (H). The protein was mixed with mediators in 100 mM HEPES pH 7.5. The reduction was performed with sodium dithionite, the oxidation with ferricyanide. Samples were withdrawn at different potentials and frozen in liquid nitrogen. The reduction of Fe_A was followed at $g = 7.7$. The reduction of Fe_B was followed at $g = 4.3$ after correction for the $g = 4.1$ feature in the spectrum. The data points were fitted with two different Nernst curves with $n = 1$ and $E_m = +2$ and $+90$ mV respectively.

when the gel was run on low voltage overnight a 14 + 28 kDa two-band pattern would evolve.

The *D. desulfuricans* desulfoferrodoxin has previously been reported to be a monomer based on analytical gel filtration [5]. In view of its unusually strong tendency to dimerize under denaturing conditions we repeated the gel-filtration experiment for the *D. vulgaris* protein. On the Superdex G-75 hiload column the native protein ran with an apparent molecular mass of 27.1 kDa (Fig. 1B). We conclude that *D. vulgaris* desulfoferrodoxin is a homodimer.

The antibodies were used to explore the expression of desulfoferrodoxin protein as a function of growth phase. Cells were harvested from a 1 liter culture of *D. vulgaris* from 16 to 64 h after inoculation. Cell-free extracts were prepared and samples (with a volume corrected for the 600 nm optical density of the culture at the time of harvesting) were used for Western blotting. No significant variation in expression was found (not shown) suggesting that expression of the protein is not dependent on the growth phase.

3.3. Optical and analytical data

The optical spectrum of the protein as isolated was virtually identical to that reported for the *D. desulfuricans* protein [5]: there are peaks at 279, 367, 502 nm and shoulders at 317 and 567 nm. The purity index of the *D. vulgaris* protein, $A_{279}/A_{502} = 6.7$, is also comparable to that reported for the *D. desulfuricans* protein, $A_{297}/A_{495} = 7.8$ [5]. When the protein concentration is determined with the Biuret method the extinction coefficient at 279 nm is $\epsilon = 57.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the 28 kDa dimer. The Bradford method (Coomassie brilliant blue colouring) of protein determination gives a 10% overestima-

tion compared to the Biuret method. The ferene coloring method quantitates to 2.2 iron atoms per 14.0 kDa subunit ($n = 2$). This number is not significantly reduced upon overnight dialysis against 25 mM HEPES pH 7.0 with 1 mM EDTA. In iso-electric focussing the protein (i.e. both forms from the MonoQ column) consistently exhibited two well resolved bands with pI = 5.3 and 5.5.

3.4. EPR of ferric sites

The low-temperature EPR spectrum of ferric DTPA (diethylenetriaminepentaacetic acid) at neutral pH is presented in trace B of Fig. 2. This spectrum is essentially independent of the pH in the range 1.9 to 10.0. At elevated pH > 11.0 the spectrum is converted into the familiar $g = 4.3$ type signal. The spectra of ferric TTHA (triethylenetetraaminehexaacetic acid) are essentially identical to those of the DTPA complexes (not shown). The Fe-DTPA spectra have been published before because of their qualitative similarity with pH-dependent spectra from the ferric ion in soybean lipoxygenase [18]. The EPR was not analyzed, however, the crystal structure of $[\text{FeHDTPA}]^{1-}$ and $[\text{FeDTPA}]^{2-}$ was determined (ibidem).

In trace A of Fig. 2 the spectrum is given of desulfoferrodoxin as it is isolated. Its similarity with the Fe-DTPA spectrum is striking, and is much stronger than that between lipoxygenase and Fe-DTPA. In fact, the only significant difference between the two spectra is one of line width. The effective g -values of both spectra can be understood in terms of the $S = 5/2$ spin Hamiltonian

$$H = D(S_z^2 - 35/12) + E(S_x^2 - S_y^2) + 2.00\beta B \cdot S$$

in the weak-field regime and with rhombicity $E/D = 0.08$ [19]. This description predicts $g \approx 7.7, 4.1, 1.8$ for the $|\pm 1/2\rangle$ doublet and $g = 5.7$ for the $|\pm 3/2\rangle$ doublet.

From a Mössbauer study Moura et al. concluded that the second iron site in *D. desulfuricans* desulfoferrodoxin was ferrous, however, in recent preliminary reports the isolation has been announced of fully oxidized protein; those preparations exhibit extra EPR features at $g = 9.74$ and 4.3 from high-spin ferric ion [6,7]. The *D. vulgaris* desulfoferrodoxin spectrum exhibits a weak feature at $g = 4.3$. When the protein is incubated with redox mediators and carefully (see below) reduced with sodium dithionite this feature decreases in intensity. We identify this signal with the second iron site, Fe_B .

3.5. EPR redox titration

The protein was incubated with a mixture of 13 redox mediators [8] and then stepwise reduced with additions of sodium dithionite or oxidized with potassium ferricyanide. EPR samples were drawn at redox equilibrium, and the oxidation state of the two iron centers was

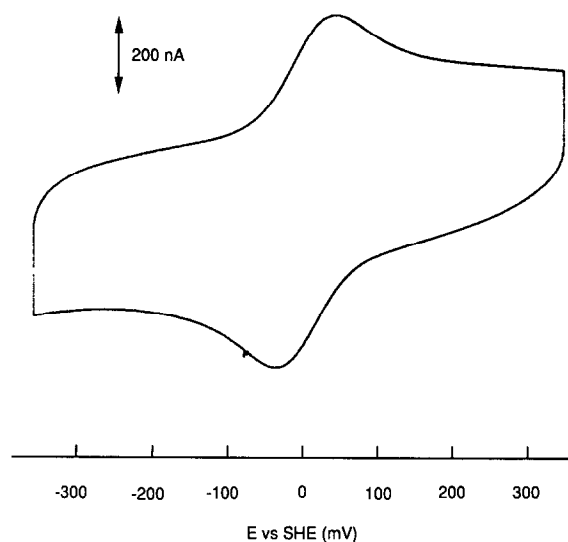


Fig. 4. Cyclic voltammogram of 3 mg/ml desulfoferrodoxin in 50 mM HEPES pH 7.0 and 2 mM neomycin. The scan rate was 10 mV/s; temperature, 23°C; working/reference/counter electrodes, glassy carbon/SCE/Pt. The potential axis is defined versus the standard hydrogen electrode.

determined from the amplitude of the $g = 7.7$ peak (Fe_A) or the $g = 4.3$ derivative feature (Fe_B). The result is presented in Fig. 3 with the potential axis relative to the standard hydrogen electrode. Both iron centers behave as independent one-electron transferring entities; the potentials are $E_{m,7.5} = +2$ mV for Fe_A and $+90$ mV for Fe_B . When the potential is raised with ferricyanide to values greater than approximately 250 mV erratic results are obtained: the signal from Fe_A decreases and the Fe_B signal is replaced by a sharp, intense signal at $g = 4.3$. We have recently reported a similar effect for the ferricyanide-oxidation of the nitrogenase MoFe-protein beyond some $+245$ mV [20].

3.6. Electrochemistry

The electrochemistry of desulfoferrodoxin results in well defined cyclic voltammograms (Fig. 4) between pH 5.0 and pH 9.5. Beyond pH 9.5 the voltammograms become less defined. The low potential iron (Fe_A) gives rise to a quasi reversible diffusion controlled electron transfer. The potential is pH dependent as shown in Fig. 5 and can be described by the equation:

$$E_m = E_{\text{low pH}} + \frac{RT}{nF} \cdot \ln \left\{ \frac{(K_{\text{red}} \cdot [\text{H}^+] + [\text{H}^+]^2)}{(K_{\text{ox}} + [\text{H}^+])} \right\}$$

A least squares fit of this equation to the data points results in a $E_{\text{low pH}}$ of 307 mV, a $\text{p}K_{\text{ox}}$ of 9.21 and a $\text{p}K_{\text{red}}$ of 5.25. The temperature coefficient is $-1.4 \text{ mV} \cdot ^\circ\text{C}^{-1}$ in the range between 10 and 40°C. The midpoint potential at 25°C and pH 7.0 is calculated to be -4 ± 5 mV versus SHE. The high potential iron (Fe_B) is not detected in the cyclic voltammograms. Since this site can be reduced in a chemical titration using dithionite and mediators we

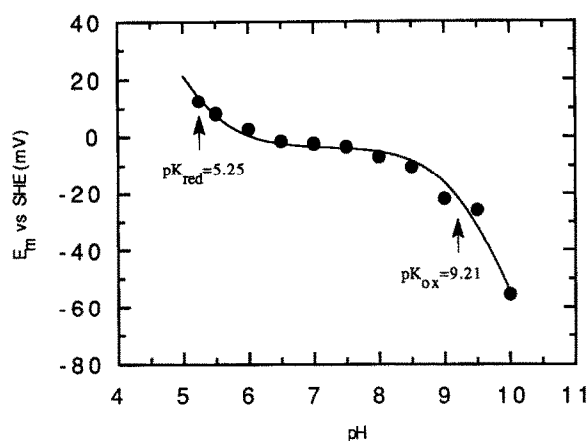


Fig. 5. pH dependence of the midpoint potential of desulfoferrodoxin. Voltammograms were taken from -600 to $+100$ versus SCE at a scan rate of 10 mV/s. The concentration of desulfoferrodoxin was 3 mg/ml in 50 mM buffer of the appropriate pH and 2 mM neomycin.

suggest that Fe_B is buried within the protein. Together with the high potential of Fe_B and the destruction of the protein upon oxidation beyond 250 mV this might indicate that Fe_B has a structural or a coordination function rather than a redox function.

3.7. Ferrous EPR

A very broad EPR feature with zero crossing at $g_{\text{eff}} \approx 10.9$, indicative of an integer spin system, was found by Moura et al. with *D. desulfuricans* desulfoferrodoxin after reduction with excess dithionite [5]. Integer spin systems are usually more readily studied in parallel-mode EPR [21]. Therefore, dithionite-reduced *D. vulgaris* desulfoferrodoxin was studied in the dual-mode cavity, which allows to do both experiments sequentially under identical conditions. The two spectra are given in Fig. 6. Not only the position of the observed line at $g_{\text{eff}} = 11.6$ but also the narrow line width and the considerable intensity difference of the signal between parallel-mode and normal-mode are unusual for ferrous $S = 2$ [21]. The latter two properties are reminiscent of the EPR from a putative $S = 2$ system in O_2 -pulsed cytochrome oxidase [22], from a putative $S = 3$ system in nitrogenase [20] and from a putative $S = 4$ system in the prismane protein [23]. This pattern is indicative of a system with a very narrow distribution in zero-field splitting parameters; it is a fingerprint for Fe_A as it sets it apart from other ferrous $S = 2$ systems.

4. DISCUSSION

4.1. Structural aspects

Desulfoferrodoxin from *D. vulgaris* has been reported to be a mixture of two proteins with different primary sequence [5]. We find only a single homogeneous sequence corresponding to the *Rbo* gene [17]. We suggest that the previously described preparation has been iso-

lated from a mixed culture of *D. vulgaris* and *D. desulfuricans*. Also, in contrast to previous work [5] we find desulfoferrodoxin to be a 2×14 kDa dimer under non-denaturing conditions. The protein carries two iron sites with putatively novel coordination. Particularly, a strained coordination by four cysteines has been proposed for the Fe_A site [5]. This idea is based on an unsubstantiated inference from the well-known rubredoxin structure. However, the midpoint potential of desulfoferrodoxin shows a complicated dependence on the pH. This is completely unlike rubredoxin that has a pH independent midpoint potential between pH 5.5 and 9.0 (our unpublished results). From a sequence comparison with desulforedoxin it follows that two of the putatively coordinating Cys are adjacent residues. From our present EPR spectral comparison with iron complexes it would appear that a higher coordination number is not excluded. In the DTPA complex the iron is seven-coordinated in a pentagonal bipyramide. Just recently, the crystal structure of soybean lipoxygenase L-1, for which this DTPA complex functioned as a model compound, was solved at a resolution of 2.5 Å. The iron was found to be coordinated by three histidines, the C-terminal isoleucine and possibly an asparagine. With asparagine as a ligand the coordination was described as approximate octahedral with one vacant position [24]. Without the coordination of asparagine the iron atom was described to be at the center of a highly distorted octahedron with two adjacent, unoccupied positions [25]. From this we conclude that, despite

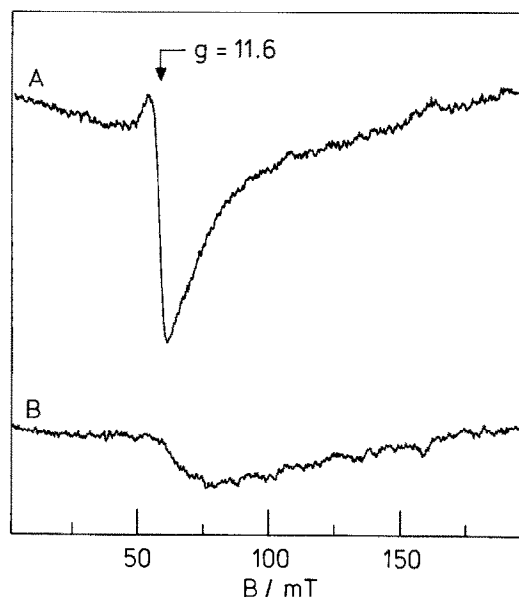


Fig. 6. Parallel-mode (trace A) and perpendicular mode (trace B) EPR spectrum of dithionite-reduced desulfoferrodoxin from *D. vulgaris* (H). The protein concentration was 2.4 mg/ml in 10 mM Tris-HCl pH 8.0. Dithionite was added to a final concentration of 2 mM. EPR conditions: microwave frequency, 9085 MHz (parallel mode) and 9138 MHz (perpendicular mode); microwave power, 200 mW; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; temperature 9 K.

the ultimate differences, the spectral properties of FeDTPA initially gave a reasonable indication of the symmetry around the iron atom in lipoxxygenase. We therefore believe that the similarity between the spectral properties of FeDTPA and desulfoferrodoxin is also indicative for a novel type of coordination of the iron atom in this protein. Comparison of the primary sequences of *D. gigas* desulfoferrodoxin, *D. desulfuricans* desulfoferrodoxin, and *D. vulgaris* desulfoferrodoxin [5] shows several conserved residues with potential metal binding capacity near the Cys cluster: Tyr-8, Cys-10, Cys-13, Cys-29, Cys-30, Glu-32, Met-34. Thus, even for a full oxygen/sulfur seven coordination the involvement of both Cys-29 and Cys-30 is not a mandatory condition.

4.2. Functional aspects

The biological function of desulfoferrodoxin is unknown. A function in secondary electron transfer is not likely in view of its strong tendency to dimerize. A function in the biosynthesis of other proteins (e.g. iron insertion) is less likely as expression of desulfoferrodoxin is not dependent on the bacterial growth phase. Brumlik and Voordouw named the gene encoding *D. vulgaris* desulfoferrodoxin the *Rbo*, or rubredoxin oxidoreductase encoding gene because it forms a transcriptional unit with the gene encoding rubredoxin [17]. This suggests that rubredoxin may be a natural redox partner of desulfoferrodoxin, however, it does not really suggest a function, as rubredoxin oxidoreductase is not a self-evident biological activity. The name *Rbo* does suggest that desulfoferrodoxin is a redox enzyme. Our determination of the iron redox potentials puts a thermodynamic limit on this suggestion. The E_m 's are at +2 and +90 mV, and oxidation beyond 250 mV is irreversible. The substrate would have to be one or two electron accepting with a reduction potential of the order of +0.1 V.

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REFERENCES

- [1] Adman, E.T., Sieker, L.C. and Jensen, L.H. (1991) *J. Mol. Biol.* 217, 337–352.
- [2] LeGall, J., Prickril, B.C., Moura, I., Xavier, A.V., Moura, J.J.G. and Huynh, B.-H. (1988) *Biochemistry* 27, 1636–1642.
- [3] Pierik, A.J., Wolbert, R.B.G., Portier, G.L., Verhagen, M.F.J.M. and Hagen, W.R. (1993) *Eur. J. Biochem.*, 212, 237–245.
- [4] Moura, I., Huynh, B.-H., Hausinger, R.P., Le Gall, J., Xavier, A.V. and Münck, E. (1980) *J. Biol. Chem.* 255, 2493–2498.
- [5] Moura, I., Tavares, P., Moura, J.J.G., Ravi, N., Huynh, B.-H., Liu, M.-Y. and LeGall, J. (1990) *J. Biol. Chem.* 265, 21596–21602.
- [6] Tavares, P., Ravi, N., Liu, M.Y., LeGall, J., Huynh, B.H., Moura, J.J.G. and Moura, I. (1991) *J. Inorg. Biochem.* 43, 264.
- [7] Tavares, P., Moura, J.J.G., Ravi, N., Huynh, B.H., LeGall, J. and Moura, I. (1993) *J. Inorg. Biochem.* 51, 478.
- [8] Pierik, A.J., Hagen, W.R., Redeker, J.S., Wolbert, R.B.G., Boersma, M., Verhagen, M.F.J.M., Grande, H.J., Veeger, C., Mutsaers, P.H.A., Sands, R.H. and Dunham, W.R. (1992) *Eur. J. Biochem.* 209, 63–72.
- [9] Pierik, A.J., Wolbert, R.B.G., Mutsaers, P.H.A., Hagen, W.R. and Veeger, C. (1992) *Eur. J. Biochem.* 206, 697–704.
- [10] Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* 5, 218–222.
- [11] Bensadoun, A. and Weinstein, D. (1976) *Anal. Biochem.* 70, 241–250.
- [12] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [13] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [14] Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4350–4354.
- [15] Hagen, W.R. (1989) *Eur. J. Biochem.* 182, 523–530.
- [16] Link, T.A., Hagen, W.R., Pierik, A.J., Assmann, C. and von Jagow, G. (1992) *Eur. J. Biochem.* 208, 685–691.
- [17] Brumlik, M.J. and Voordouw, G. (1989) *J. Bacteriol.* 171, 4996–5004.
- [18] Finnen, D.C., Pinkerton, A.A., Dunham, W.R., Sands, R.H. and Funk, M.O., Jr. (1991) *Inorg. Chem.* 30, 3960–3964.
- [19] Hagen, W.R. (1992) in: *Advances in Inorganic Chemistry*, Vol. 38: Iron-Sulfur Proteins, (Cammack, R. and Sykes, A. G., Eds.) Academic Press, San Diego, pp. 165–222.
- [20] Pierik, A.J., Wassink, H., Haaker, H. and Hagen, W.R. (1993) *Eur. J. Biochem.* 212, 51–61.
- [21] Hagen, W.R. (1982) *Biochim. Biophys. Acta* 708, 82–98.
- [22] Hagen, W.R., Dunham, W.R., Sands, R.H., Shaw, R.W. and Beinert, H. (1984) *Biochim. Biophys. Acta* 765, 399–402.
- [23] Pierik, A.J., Hagen, W.R., Dunham, W.R. and Sands, R.H. (1992) *Eur. J. Biochem.* 206, 705–719.
- [24] Minor, W., Steczko, J., Bolin, J.T., Otwinowski, Z. and Axelrod, B. (1993) *Biochemistry* 32, 6320–6323.
- [25] Boyington, J.C., Gaffney, B.J. and Amzel, L.M. (1993) *Science* 260, 1482–1486.