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ON THE REDOX PROPERTIES OF THREE BISULFITE REDUCTASES FROM THE SULFATE-REDUCING BACTERIA

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SUMMARY: The redox properties of purified bisulfite reductases from *Desulfovibrio gigas, D. desulfuricans* (Norway) and *Desulfotomaculum ruminis,* containing non-heme iron and siroheme have been studied by EPR spectroscopy. Each enzyme shows ferric siroheme EPR signals which are not completely reduced by dithionite after 20 min, but are readily reduced within 1 min by dithionite plus methyl viologen. With the latter reducing system, each reductase also reveals a variable Beinert "g=1.94" type iron-sulfur signal. Reaction of each reductase with reduced methyl viologen results in reduction of only the siroheme. These results suggest different redox potentials for the iron-sulfur and siroheme moieties, and indicate that their functional properties are similar for each reductase.

INTRODUCTION:

The sulfate-reducing bacteria contain three sulfite-reducing enzymes desulfoviridin (1,2), desulforubidin (3) and P-582 (4) which serve identical functions in respiratory sulfate reduction but can be readily differentiated on the basis of their spectral and redox properties. These enzyme reduce bisulfite (HSO_3^{-1}) (5) and have been termed bisulfite reductases. Each reductase contains 14 non-heme irons and sulfides, two sirohemes (determined as siroporphyrin in the case of desulfoviridin) (6) and forms thiosulfate, sulfide and trithionate as products depending on the concentrations of substrate and electron donor (7-9).

Desulfoviridin is not reduced immediately by dithionite or borohydride (1). The isolated enzyme exhibits a highly variable and distorted ferric hemes EPR absorption (10,11). The variability of the EPR absorbance seems to be due to differing amounts of two forms of desulfoviridin (1,12) in different preparations (6). Desulforubidin and P-582 exhibit spectral shifts in the presence of dithionite but it is not clear whether the enzymes are fully or partially reduced (4,6). Both reductases show different but characteristic high-spin rhombically distorted ferric heme EPR resonances (11). None of the reductases show optical changes which can be related to the reduction of non-heme iron.

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In this paper, we report the changes in the EPR spectra of purified desulfoviridin, desulforubidin and P-582 which occur in the presence of dithionite, reduced methyl viologen and dithionite plus methyl viologen.

METHODS:

Desulfoviridin, desulforubidin and P-582 were purified by essentially identical procedures (6). The two electrophoretically distinguishable forms of desulfoviridin, Desulfoviridin-S (slow) and Desulfoviridin-F (fast) were separated by chromatography on DEAE-Biogel A (6). Hydrogenase from *Desulfovibrio vulgaris* was prepared according to Van der Westen et al (13).

EPR spectroscopy at liquid-helium temperatures was performed as previously described (14). EPR conditions are found in the figure legends. Quantitation by double integration of the heme and iron-sulfur signals for each reductase was performed using Cupric-EDTA and myoglobin fluoride as standards and correcting for g-value dependence on transition probability by the procedure of Aasa and Vanngard (15).

RESULTS AND DISCUSSION:

Desulforubidin from *D. desulfuricans*

Oxidized State: Desulforubidin contains a siroheme chromophore, forms an alkaline pyridine hemochromagen and reacts slightly with sodium dithionite. The improved purification procedure results in an enzyme preparation exhibiting a simpler ferric EPR spectrum than previously reported (11). The EPR spectrum of desulforubidin (Fig. 1,A) consists of a predominant rhombic high-spin ferric heme resonance with g_z at 6.331 and g_y at 5.289 and a minor rhombic high-spin ferric heme species at g_z=6.918 and at about g=5.721. This EPR spectrum is the least complex of the three bisulfite reductases considered in this study. A recovery of two hemes was found which is in good agreement with analytical values (6).

The g=2 region EPR signal (Fig. 1,E) arises from a high-potential type iron-sulfur center with g_y at 2.024. The signal is asymmetric suggesting at least two different iron-sulfur centers.

Reduced State: One of the problems with the bisulfite reductases has been the relative unreactivity of the reductases to reducing agents. Optically the enzymes react with sodium dithionite or borohydride only after 10 to 20 min. This non-reactivity obviously raised questions regarding the structural and mechanistic properties of the reductases. Fig. 1,B shows the EPR resonances on the addition of dithionite to desulforubidin after a 30 min reaction. Significant changes in the EPR spectra are observed with the most pronounced effect being the 82% decrease in signal intensity of the mi-

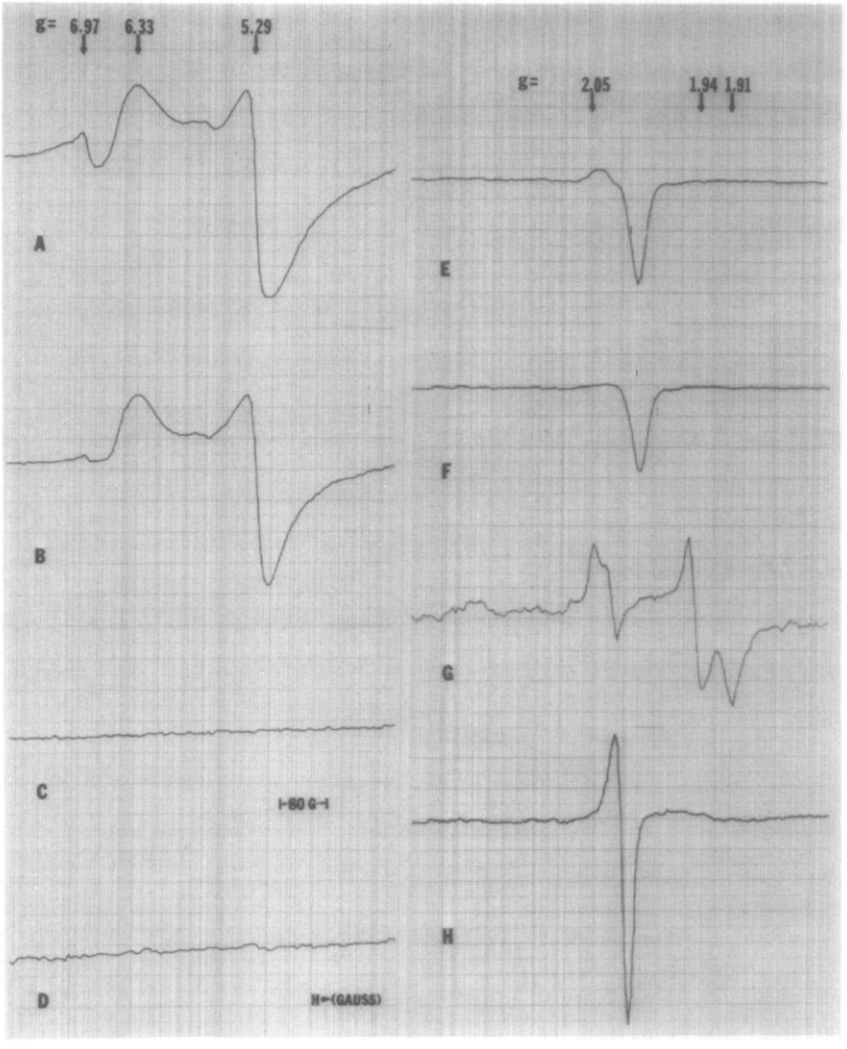

Figure 1. EPR spectra of Desulforubidin from *D. desulfuricans* (Norway). EPR conditions: microwave frequency, 9.188 GHz (frequency-matched EPR tubes); microwave power, 10 mW for the siroheme signals and 0.3 mW for the iron-sulfur signals; modulation amplitude, 5.2 gauss; time constant, 0.1 sec; scanning rate, 400 gauss per min; temperature, 11°K and Gain = 100 unless otherwise specified.

A, E Isolated enzyme (11.8 mg/ml) dissolved in 50 mM potassium phosphate buffer (pH 7.6).

B, F Reduced under anaerobic conditions with slight excess of sodium dithionite for 30 min.

C, G Reduced under anaerobic conditions with sodium dithionite plus 1 µM methyl viologen for 1 min.

D, H Reduced under anaerobic conditions with hydrogen, 1 µM methyl viologen and 0.1 mg of purified hydrogenase from *D. vulgaris* for 20 min.

nor species at g_z=6.918 and only an 8% decrease in signal intensity for the major species (g_z=6.331). A slight decrease in signal intensity was also found for the g=2.024 signal (Fig. 1,F).

When methyl viologen is reacted with dithionite and desulforubidin, all high-spin ferric heme resonances disappear within 1 min (Fig. 1,C) due to reduction to the diamagnetic ferrous state. In the g=2 region (Fig. 1,G) a small low-potential Beinert "g=1.94" type iron-sulfur signal appears at g_z=2.034, g_y=1.927 and g_x=1.892. The double-integrated value of this latter signal corresponded to a 3% recovery of EPR-detectable iron as compared to the chemically determined non-heme iron content.

The reaction of desulforubidin with methyl viologen reduced by hydrogenase and hydrogen resulted in complete loss of the initial high-spin ferric heme resonances (Fig. 1,D) and the appearance of the methyl viologen radical but no g=1.94 type iron-sulfur signal. The EPR response with the two distinct reducing agents indicated a possible difference in oxidation-reduction potential for the heme and iron-sulfur systems.

P-582, the Bisulfite Reductase from *Desulfotomaculum ruminis*

Oxidized State: In the presence of dithionite, P-582 undergoes slow optical changes (within 5 min) resulting in the shift of the maximum peak from 582 to 610 nm (4). Murphy and Siegel (10) reported that a siroheme chromophore can be isolated on extraction with acetone-HCl. The isolated enzyme exhibits a complex set rhombically distorted high-spin ferric heme resonances (Fig. 2,A) with two significant EPR-detectable species. The major species has g-values at g_z=7.087, g_y=5.997 and g_x=4.779 while the minor species has g-values at g_y=6.332 and g_x=5.606. Quantitation by double integration of the heme signals for ferric P-582 indicates a recovery of 2.2 hemes which is higher than that found by light-absorption measurements (1.2 hemes) (6.)

The EPR spectrum in the g=2 region (Fig. 2,E) is very similar to that found for oxidized desulforubidin and desulfoviridin and consists of an asymmetric (at least two species) high-potential iron-sulfur signal at g=2.023.

Reduced State: Reaction with dithionite for 30 min of P-582 results in substantial changes in both the g=6 and g=2 EPR spectral regions. In both regions the heme (Fig. 2,B) and iron-sulfur centers (Fig. 2,F) are 85% reduced. A trace "g-1.94" type EPR signal is observed. The degree of reduction of P-582 with dithionite was the most

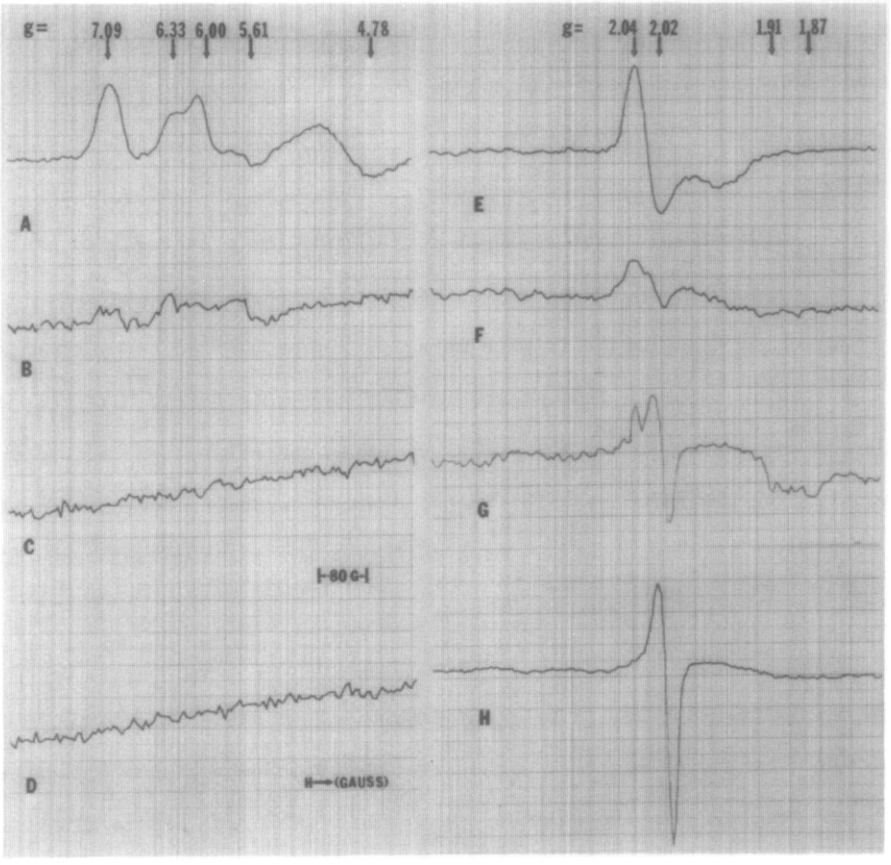

Figure 2. EPR spectra of P-582 from *Desulfotomaculum ruminis*. EPR conditions as in Figure 1.

A, E Isolated enzyme (8.8 mg/ml) dissolved in same buffer as Desulforubidin.
B, F As Figure 1, B, F but Gain = 200.
C, G As Figure 1, C, G but Gain = 200.
D, H As Figure 1, D, H but D at Gain = 200.

pronounced for the bisulfite reductases examined, and may be correlated with the light absorption spectral changes noted when P-582 was reduced with only dithionite.

Reaction of P-582 with dithionite and methyl viologen for 1 min resulted in loss of the EPR signals in the g=6 region (Fig. 2,C) as is observed for desulforubidin and desulfoviridin. Under these conditions a weak "g=1.94" type signal appears (Fig. 2,G) with g_z at 2.045, g_y=1.928 and g_z at 1.877. Double integration of this signal results in the lowest EPR recovery (1-2%) of all the bisulfite reductases examined when compared to the chemically determined non-heme iron content.

The reaction of P-582 with methyl viologen reduced by hydrogenase and hydrogen resulted in the complete loss of EPR signals in the g=6 region (Fig. 2,D) while in the g=2 region (Fig. 2,H) only the methyl viologen radical species was observed.

Desulfoviridin from *D. gigas*

Oxidized State: The two forms of desulfoviridin are designated as S and F (for slow and fast moving). They have nearly identical physical and kinetic properties (12); however, the EPR spectra of the ferric states for the two desulfoviridins are quite different. Ferric desulfoviridin S (Fig. 3, I-A) shows a multiple set of rhombically distorted resonances consisting of two major and one minor species. The most intense species (arbritarily termed heme I) has a g_z value at 6.390 and a g_y value at 5.619 while the other major heme species (termed heme II) has a g_z value of 7.048. The minor species has a g_z value of 5.856 and a g_x value of 5.210. The ferric EPR spectrum of desulfoviridin F (Fig. 3, I-E) is still more complex and consists of multiple rhombic and axial high-spin ferric heme resonances with at least two more additional heme species than observed with desulfoviridin S. The g-values for the various hemes are slightly different than those found for desulfoviridin S except for the minor species with g_x at 5.219 which is the same for both desulfoviridins. The g-values of the heme systems in desulfoviridin F are: heme I, g_z=6.217, g_y=5.693; heme II, g_z=6.948; heme III (axial) g_y=5.910. Two minor species are found with g_z= 6.704 for one species and g_z=7.467 for the second species. A reasonable agreement was found for the EPR double-integrated heme value and that determined by light-absorption. For example, desulfoviridin S by EPR double-integration shows 2.1 hemes and by light-absorption spectroscopy 2.0 hemes.

The other significant EPR signal in oxidized desulfoviridin S (Fig. 3, II-A) and desulfoviridin F (Fig. 3, II-D) is that found in the g=2 region. As in the case of desulforubidin and P-582 this signal is due to superimposition of two different high-potential iron-sulfur centers with g_y at 2.024.

Reduced State: The reaction of dithionite with desulfoviridin S (Fig. 1-B) for 30 min caused the heme signal intensity (II) at g_z=7.048 to double and new signals appeared at g_z=6.070 and at g_z=4.743 (broad). The increase in the intensity of the g=6 signal on reduction is strongly reminiscent of beef heart cytochrome c oxidase which on partial reduction shows a previously absent g=6 type ferric heme EPR signal (16). The intensity of heme signal (I) declined about 80%.

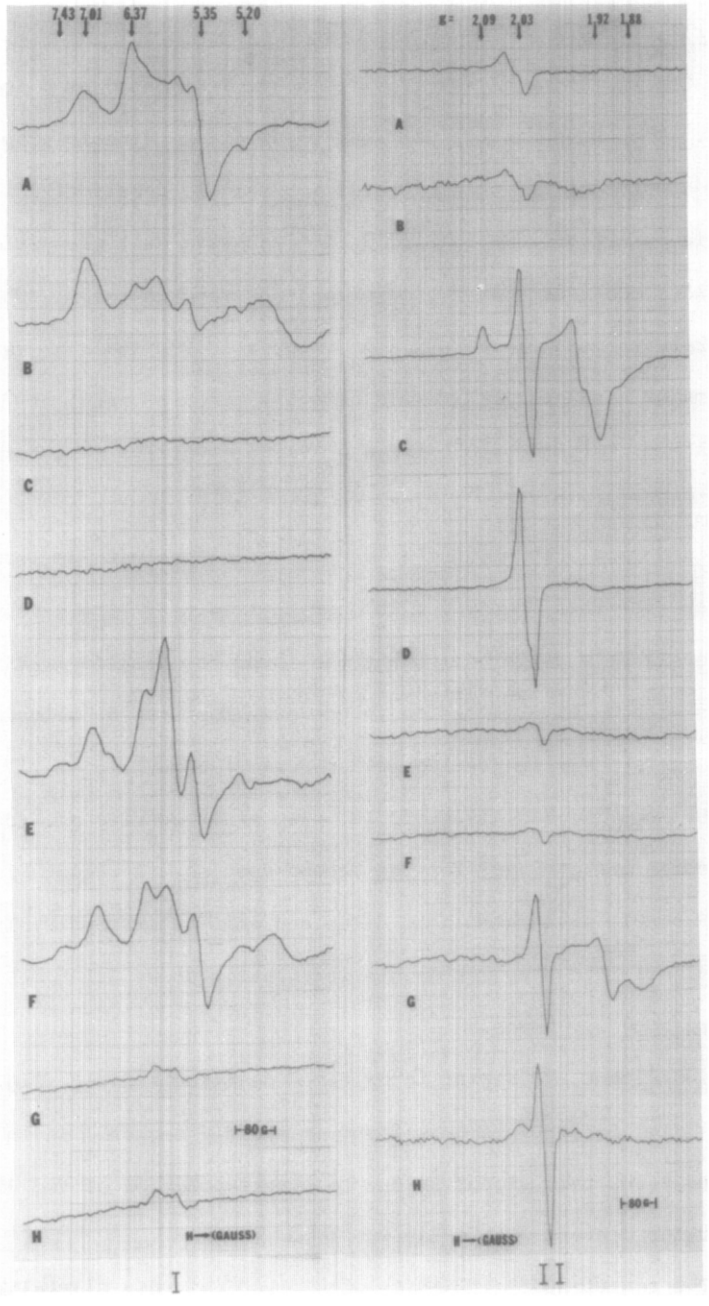

Figure 3. EPR spectra of Desulfoviridins S and F from *D. gigas*. EPR conditions as in Figure 1.

I-A, II-A Isolated Desulfoviridin S (11.3 mg/ml) dissolved in same buffer as Desulforubidin.
I-B, II-B As Figure 1, B, F.
I-C, II-C As Figure 1, C, G but Gain = 50.
I-D, II-D As Figure 1, D, H but Gain = 50.
I-E, II-E Isolated Desulfoviridin F (8.4 mg/ml) in same buffer as Desulforubidin.
I-F, II-F As Figure 1, B, F.
I-G, II-G As Figure 1, C, G.
I-H, II-H As Figure 1, D, H.

A similar 30 min reduction of desulfoviridin F with dithionite caused slightly different EPR changes. The signal intensities of heme II (g_z=6.948) and the minor species with g_z=7.467 were unchanged. The signal of heme III (g_y=5.910) decreased 71% while those assigned to heme I (g_z=6.217, g_y=5.693) were unchanged. A new broad signal similar to that found with desulfoviridin S appeared at g_x=4.741. These EPR changes are quite complex yet surely the significant aspect of the observed EPR changes is that lengthy reduction with dithionite alone did not completely reduce either type of desulfoviridin.

In the g=2 region (Fig. 3, II-B, II-F) a 30 min reduction with dithionite of either desulfoviridin did not result in any significant changes. However the reaction of methyl viologen with dithionite-reduced desulfoviridin S or F (within 1 min) resulted in dramatic EPR changes. In the g=6 region all EPR resonances essentially disappeared (Fig. 3, I-C, I-G) while in the g=2 region an intense "g=1.94" type iron-sulfur signal appeared (Fig. 3, II-C, II-G). The intensity of the g=1.94 type signal was more intense for desulfoviridin S than for F. The g-values for the rhombic-type low-potential iron-sulfur signal for desulfoviridin S were g_z, 2.092; g_y, 1.997 and g_x, 1.924 while those for desulfoviridin F differed and were g_y, 1.921; g_x, 1.880 (the g_z component was not resolved). Quantitation of the iron-sulfur signal for desulfoviridin S represented 16.8% of the non-heme iron content while a similar calculation recovery for desulfoviridin F accounted for only 6.0%.

Reduction of either desulfoviridin S or F with methyl viologen reduced by hydrogenase and hydrogen for 20 min resulted in essentially the same EPR spectral changes as found for desulforubidin and P-582, i.e., loss of the EPR signals in the g=6 region (Fig. 3, I-D, I-H) and in the g=2 region (Fig. 3, II-D, II-H) appearance of the methyl viologen radical species but no iron-sulfur signal of the g=1.94 type.

In conclusion, the data presented for the three bisulfite reductases indicate that the oxidation reduction potentials of the heme and iron-sulfur systems are significantly different and that the functional properties of the two different iron centers must be similar for each reductase.

Acknowledgements

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