The Dissimilatory Sulfite Reductase from *Desulfosarcina variabilis* Is a Desulforubidin Containing Uncoupled Metalated Sirohemes and $S = {}^9/_2$ Iron–Sulfur Clusters†

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ABSTRACT: The active site of *Escherichia coli* NADPH–sulfite reductase has previously been modeled as a siroheme with its iron bridged to a nearby iron–sulfur cubane, resulting in antiferromagnetic exchange coupling between all iron atoms. The model has been suggested to hold also for other sulfite reductases and nitrite reductases. We have recently challenged the generality of the model with the finding that the EPR of Fe/S in dissimilatory sulfite reductase (desulfoviridin) from *Desulfovibrio vulgaris* indicates that an $S = {}^9/_2$ system is not subject to coupling. Siroheme in desulfoviridin is to a large extent demetalated, and therefore coupling is physically impossible. We have now studied examples from a second class of dissimilatory sulfite reductases, desulforubidins, which have their siroporphyrins fully metalated. Desulforubidin from *Desulfosarcina variabilis* is a 208-kDa $\alpha_2\beta_2\gamma_2$ hexamer. The α- and β-subunits are immunologically active with antibodies raised against the corresponding subunits from *D. vulgaris* desulfoviridin, whereas the γ-subunit is not. The desulforubidin contains two fully metalated sirohemes and a total of ≈15 Fe and ≈19 S^{2-}. Quantification of high-spin plus low-spin heme EPR signals accounts for all sirohydrochlorin. The frequency-independent (9–35 GHz) effective perpendicular g-values of the high-spin $S = {}^5/_2$ siroheme (6.33, 5.19) point to quantum mixing with an excited (≈770 cm^{-1}) $S = {}^3/_2$ multiplet. Similar anomalous g-values are observed with sulfite reductases from *Desulfovibrio baarsii* and *Desulfotomaculum acetoxidans*. The *D. variabilis* enzyme exhibits very approximately stoichiometric $S = {}^9/_2$ EPR ($g = 16$). None of the EPR signals give indication for dipolar and/or exchange coupling between siroheme and iron–sulfur clusters. $S = {}^9/_2$ EPR is not detected in concentrated samples of assimilatory sulfite reductases from *E. coli* and from *D. vulgaris*. Thus, the functional difference between dissimilatory and assimilatory sulfite reductases appears to have a structural parallel in the presence or absence, respectively, of an $S = {}^9/_2$ EPR iron–sulfur cluster.

The reduction of the bisulfite ion is catalyzed in biology by sulfite reductases for two purposes. Sulfur must be assimilated for the biosynthesis of organosulfur compounds, *e.g.*, cysteine. On the other hand, the electron acceptor sulfite may be dissimilated in respiration. The overall reaction involves the transfer of six reducing equivalents:

$$6H^+ + 6e^- + HSO_3^- \rightarrow 3H_2O + HS^- \qquad (1)$$

The relevant pKs are 6.91 (HSO_3^-/SO_3^{2-}) and 7.04 (HS^-/H_2S) at 18 °C. All sulfite reductases contain siroheme and iron–sulfur. *In vitro*, the assimilatory sulfite reductases supposedly produce sulfide in a single six-electron step, *i.e.*, with no intermediate sulfur compounds released (Asada, 1967; Yoshimoto et al., 1967; Lee et al., 1973). The *in vivo* released product is not indentified. By far the most thoroughly studied system is the heterododecameric $\alpha_8\beta_4$ NADPH–sulfite reductase from *Escherichia coli* (Siegel et al., 1973). Monomeric 66-kDa β-subunit isolated from this complex contains siroheme and iron-sulfur and has reduced methyl viologen–sulfite reductase activity (Siegel & Davis, 1974). The *E. coli* enzyme shows (primary) structural and also enzymological homology with spinach leaf assimilatory nitrite reductase (Krueger & Siegel, 1982; Back et al., 1988; Ostrowski et al., 1989).

Other putative assimilatory sulfite reductases consist of only a single subunit. Some are low-molecular-mass, apparently monomeric enzymes with relatively high specific activity. They differ from all other sulfite reductases in that their siroheme is low-spin in the purified protein. The sequence of the assimilatory *D. vulgaris* enzyme has two Cys-containing motifs with some homology to the sequences of the heme–protein subunits from *E. coli* and *Salmonella typhimurium* sulfite reductase and spinach leaf nitrite reductase (Back et al., 1988; Ostrowski et al., 1989; Tan et al., 1991).

There is a different group of sulfite reductases with a *dissimilatory* function. These proteins are heteropolymers with relatively high iron contents. On the basis of visible absorption spectroscopy (especially the peak position in the red for oxidized enzyme), four classes have been reported: desulfoviridin (628 nm), desulforubidin (545 nm), P-582 (582 nm), and desulfofuscidin (576 nm). After over two decades of dispute, it is still an unsettled question whether these enzymes release intermediates, *e.g.*, trithionate or thiosulfate, when assayed *in vitro* (Kobayashi et al., 1972, 1974; Akagi et al., 1974; Schedel & Trüper, 1979; Hatchikian & Zeikus, 1983) or even *in vivo* (Fitz & Cypionka, 1990). However, whatever

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the precise mechanism will turn out to be, it is obvious that the reaction catalyzed by any sulfite reductase enzyme involves the transfer of one, two, or three *pairs* of reducing equivalents. By consequence, the redox active site has the capacity to take up *two* (or a multiple of two) electrons. The ferric/ferrous transition of a single siroheme does, therefore, not suffice.

For the oxidized *E. coli* enzyme, a putative multielectron-accepting site has been proposed by the groups of Siegel, Münck, and co-workers in the form of a coupled cubane–siroheme unit: a $[4Fe–4S]^{2+}$ cluster with one Fe bridged through an unidentified ligand, X, to the Fe(III) of the siroheme and with an additional "bridge" in the form of a van der Waals contact point between one of the acid-labile sulfurs and the porphyrin periphery (Siegel et al., 1973; Cristner et al., 1981). The coupled cubane–siroheme model is an inference from a remarkable pair of spectroscopic observations. The EPR spectrum of the oxidized *E. coli* protein shows only a high-spin ferric heme signal ($S = {}^5/_2$), consistent with the $[4Fe–4S]^{2+}$ cluster being diamagnetic ($S = 0$). Amazingly, the Mössbauer spectrum shows that *all* iron is paramagnetic (Christner et al., 1981).

The cubane–siroheme model attempts to reconcile these two apparently mutually inconsistent observations in the proposition of a novel type of exchange coupling, *i.e.*, that between an $S = {}^5/_2$ paramagnet and an $S = 0$ diamagnet (Christner et al., 1981; Münck, 1982). In spite of the unprecedented nature of this interaction, the model has, in the ensuing years, been the framework for the interpretation of all spectroscopy on sulfite reductase enzymes (Murphy & Siegel, 1973; Huynh et al., 1984, 1985; Moura et al., 1988). Unfortunately, although the *E. coli* β-subunit has been crystallized (McRee et al., 1986) and the primary structure is known (Ostrowski et al., 1989), a complete interpretation of the electron density map (3-Å resolution) in terms of a 3D structure encompassing the heme and the iron–sulfur has not yet been put forth.

We seek to answer two central questions regarding sulfite reductases: (1) is the Siegel–Münck model of a coupled cubane–siroheme correct and (2) is the model generally valid for all sulfite reductases? Our approach is to systematically (re)investigate sulfite reductases from different classes by EPR spectroscopy and to scan for inconsistencies between the EPR data and the cubane–siroheme model. We have previously studied the dissimilatory sulfite reductase from *D. vulgaris*, a desulfoviridin, and we have found that the iron–sulfur is *not* diamagnatic but has the unusually high system spin $S = {}^9/_2$ (Pierik & Hagen, 1991). No interaction between the iron-sulfur ($S = {}^9/_2$) and the siroheme ($S = {}^5/_2$) was detected. Thus, the coupled cubane–siroheme model does not appear to hold for this enzyme. However, matters are complicated by the fact that desulfoviridin-type reductases have their siroheme to a considerable extent (>70%) demetalated (Murphy & Siegel, 1973; Moura et al., 1987, 1988; Pierik & Hagen, 1991). Therefore, the theoretical possibility of coupling is physically limited to a minor part of the molecules.

We have now studied examples of dissimilatory sulfite reductases that have essentially fully metalated siroporphyrin. We describe one highly purified enzyme from *D. variabilis* and two partially purified enzymes from *D. baarsii* and *D. acetoxidans*. These three species all belong to the subgroup of sulfate reducers that oxidize acetate via the acetyl-CoA route; they all contain carbon monoxide dehydrogenase activity (Jansen et al., 1984, 1985; Schauder et al., 1986; Spormann & Thauer, 1988, 1989). Sulfite reductase has not hitherto been described for this subgroup. Below we describe the dissimilatory sulfite reductase to be of the desulforubidin type. The EPR data are in disagreement with the coupled cubane–siroheme model. We have also reinvestigated two assimilatory-type sulfite reductases, the complex *E. coli* enzyme and the low-molecular-mass, monomeric *D. vulgaris* enzyme.

MATERIALS AND METHODS

Strains, Growth, and Harvesting. D. variabilis, DSM 2060 (Widdel, 1980), was grown at 30 °C on 10 mM pyruvate. *Desulfotomaculum acetoxidans*, DSM 771 (Widdel & Pfennig, 1977), and *Desulfovibrio baarsii*, DSM 2075 (Widdel, 1980), were both grown at 37 °C on 10 mM butyrate plus 5 mM acetate. The growth medium contained Na_2SO_4, 21 mM; KH_2PO_4, 1.5 mM; NH_4Cl, 5.6 mM; $CaCl_2$, 1.4 mM; NaCl, 17 mM; $MgCl_2·6H_2O$, 2 mM; KCl, 6.7 mM; $NaHCO_3$, 30 mM; and Na_2S, 1 mM. For the brackish water organism *D. variabilis*, NaCl was 220 mM and $MgCl_2·6H_2O$ was 10 mM. In all cases, resazurin, 2.2 μM, was present as a redox indicator. Trace elements were H_3BO_3, 0.1 μM; $AlCl_3·6H_2O$, 40 nM; $MnCl_2·4H_2O$, 0.5 μM; $FeCl_2·4H_2O$, 10 μM; $CoCl_2·6H_2O$, 0.8 μM; $NiCl_2·6H_2O$, 0.1 μM; $CuCl_2$, 20 nM; $ZnCl_2$, 0.5 μM; $Na_2SeO_3·5H_2O$, 11 nM; Na_2MoO_4, 150 nM; $Na_2WO_4·2H_2O$, 15 nM. EDTA was also added to 1.3 μM. Vitamins used were biotin, 2 μM; folic acid, 2 μM; nicotinamide, 5 μM; thiamin HCl, 5 μM; riboflavin, 5 μM; pyridoxine HCl, 10 μM; cyanocobalamine, 5 μM; *p*-aminobenzoic acid, 5 μM; lipoic acid, 5 μM; and pantothenic acid, 5 μM.

Cells were grown in 20-L batch cultures (10% inoculum v/v) to an absorbance of approximately 0.25 at 660 nm. Growth period and wet-cell yield respectively were 10 days and 17 g for *D. variabilis* and 14 days and 8 g for both *D. acetoxidans* and *D. baarsii*. Cells were harvested by continuous centrifugation. The pellet was immediately frozen in liquid N_2 and then stored at –20 °C until use.

Purification of D. variabilis Dissimilatory Sulfite Reductase. Frozen cells of *D. variabilis* were suspended in three volumes of standard buffer (20 mM Tris-HCl, pH 8.0) plus 5 mM $MgCl_2$ and a spatula of DNase. The cells were broken in a French pressure cell by passing at 135 MPa. Cell-free extract was obtained as the supernate after a 1-h spin at 100 000*g*. The extract was dialyzed overnight against standard buffer and loaded onto a 60-mL DEAE-Sepharose Fast Flow anion-exchange column. After a 100-mL wash with standard buffer, a 1-L gradient was applied of 0–1 M NaCl in standard buffer. Fractions with desulforubidin were pooled on the basis of complete UV–visible spectra (peaks at 398 and 545 nm; see the Results section). After concentration (16×) over an Amicon YM-100 filter and overnight dialysis against 5 mM potassium phosphate, pH 7.5, the sample was applied onto a 40-mL hydroxylapatite affinity column and washed with 80 mL of potassium phosphate buffer. A 0.5-L gradient was applied of 5–200 mM potassium phosphate, pH 7.5. The pooled fractions were 10× concentrated over Amicon YM-100 and applied onto a 200-mL Sephacryl S-400 molecular sieve and eluted with standard buffer plus 500 mM KCl. The desulforubidin peak was 10× concentrated and dialyzed overnight against standard buffer. The last purification step was by FPLC (Pharmacia) at ambient temperature on a 1-mL Mono-Q anion-exchange column. The desulforubidin was eluted with a 0–1 M KCl stepped gradient as "fast" and a "slow" forms, and these fractions were concentrated (15×) separately over Amicon YM-100. Since the two fractions proved to be indistinguishable by SDS-PAGE, UV–visible spectroscopy, and siroheme EPR spectroscopy, they were pooled.

Purification of D. vulgaris Assimilatory Sulfite Reductase. D. vulgaris assimilatory sulfite reductase was purified ac-

cording to the method of Huynh et al. (1984), with a modification in the order of purification steps: the crude extract was first loaded on a DEAE anion-exchange column, and then on a G-75 molecular sieve, and finally on a hydroxylapatite affinity column.

Purification of E. coli NADPH–Sulfite Reductase. E. coli, strain C 600, was grown batchwise at 37 °C in the medium described by Siegel and Kamin (1971), with the modification glucose, 10 g/L, and the additions of L-leucine, 100 mg/L, and L-threonine, 50 mg/L. Also, *O*-acetyl-L-serine [synthesized according to the method of Sheenan et al. (1956)], a stimulant of sulfite reductase biosynthesis (Siegel & Kamin, 1971; Jones-Mortimer et al., 1968), was added to 2 mM, except in the final 200-L batch (bioreactor, Bioengineering). The purification from 700 g of cells was essentially according to the classical method of Siegel at al. (1973) for wild-type *E. coli* B (DEAE-cellulose was replaced by Q-Sepharose F.F.).

Activity Determinations, Analytical Chemistry, and Immunology. For specific activity determinations, the protein was measured by Coomassie staining (Bradford, 1976). The sulfite-dependent H_2-uptake activity was measured according to the method of Lee et al. (1973), except that *E. coli* hydrogenase was replaced by 10 μg of *D. vulgaris* (Hildenborough) periplasmatic hydrogenase and the assay was done at 30 °C, because the organism was grown at that temperature. For metal, heme, and spin stoichiometry determinations, the protein was determined using the microbiuret method (Bensadoun & Weinstein, 1976). Iron was determined colorimetrically as the ferene complex (Hennessy et al., 1984). The acid-labile sulfide content of the protein was determined according to the method of methylene blue formation [Fogo & Popowski, 1949, modified by Hennessy et al. (1984)]. Siroheme was extracted with an HCl/acetone mixture and subsequently determined as the pyridine hemochrome as described in Siegel et al. (1978). SDS polyacrylamide electrophoresis was performed with a midget system (Pharmacia) holding 8- × 5- × 0.75-cm gels, according to the method of Laemmli (1970). The composition (mass/volume) of the stacking gel was 4% acrylamide and 0.1% bisacrylamide; the running gel was 17.5% acrylamide and 0.07% bisacrylamide. Optical gel scanning was performed using the Gel Analysis Program of a Cybertech CS1 CAM 1.0. Western blotting, Immobilon blotting, and polyclonal antibodies against the α-, β-, and γ-subunits of *D. vulgaris* desulfoviridin were as previously described by Pierik et al. (1992a).

Spectroscopy. UV–visible data were obtained with a DW-2000 spectrophotometer. X-band EPR spectroscopy was done with a Bruker EPR 200 D spectrometer with peripheral equipment and data handling as described in Pierik and Hagen (1991). Q-band EPR spectroscopy was done in the laboratory of Dr. S. P. J. Albracht (The University of Amsterdam) on a Varian E-9 spectrometer with a 35-GHz cylindrical cavity. The modulation frequency was always 100 kHz.

RESULTS

D. variabilis Sulfite Reductase Is a Metalated Desulforubidin. The color of purified *D. variabilis* sulfite reductase is red to red-brown, which indicates that the protein is of the desulforubidin type. The UV–visible spectrum is presented in Figure 1. Indeed, it exhibits the peak at 545 nm that is the desulforubidin fingerprint. Other peaks are observed at 280, 398, and 582 nm (Table I). It has been shown that the siroheme in desulfoviridin from *D. gigas* and from *D. vulgaris* is to a considerable extent demetalated (Murphy & Siegel, 1973; Moura et al., 1987, 1988; Pierik & Hagen, 1991). To determine the metalation of the porphyrin in the *D. variabilis* enzyme, the heme was extracted with HCl/aceton and subsequently complexed with pyridine (Siegel et al., 1978). The resulting pyridine hemochrome spectrum is given in Figure 2. The spectrum is that of metalated siroheme (Siegel et al., 1978; Murphy et al., 1974); its intensity corresponds to approximately 0.7 heme per αβγ unit of 104 kDa.

Iron and acid-labile sulfur were determined colorimetrically (Table I). The numbers are typically in the range reported for other dissimilatory sulfite reductases.

The sulfite-dependent H_2-uptake activity was 41 munit per mg of protein, which is somewhat lower than values reported for dissimilatory sulfite reductases, although still in the same range (Table I).

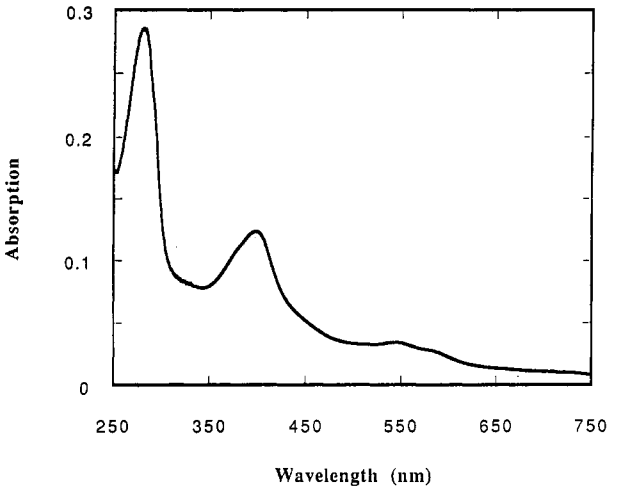

FIGURE 1: UV–visible absorption spectrum of *D. variabilis* dissimilatory sulfite reductase. The protein was 0.127 mg/mL in 20 mM Tris buffer, pH 8.

Table I: Optical and Analytical Data of *D. variabilis* Desulforubidin[a]

absorption coefficients (mM^{-1} cm^{-1})	at 280 nm	468
	at 398 nm	184
	at 545 nm	52
purity indices	398 nm/545 nm	3.65
	398 nm/280 nm	0.35
stoichiometries	siroheme	1.4
	iron	15 ± 2
	sulfide	19 ± 3
specific activity (munits/mg of protein)		41 ± 6

[a] All values refer to the $\alpha_2\beta_2\gamma_2$ holoprotein.

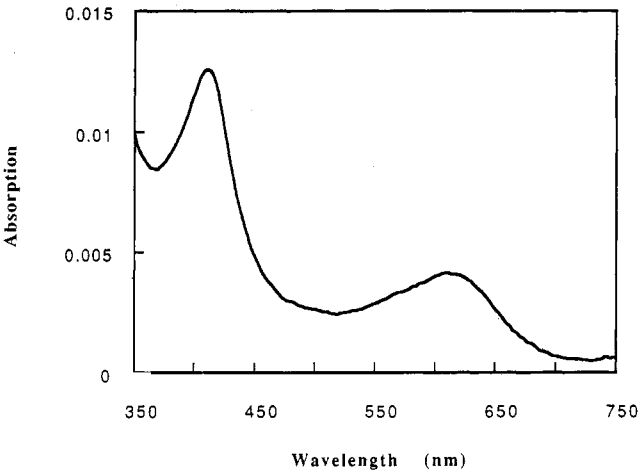

FIGURE 2: Absorption spectrum of pyridine hemochrome after heme extraction from *D. variabilis* dissimilatory sulfite reductase.

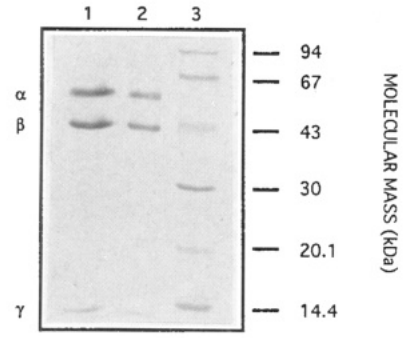

FIGURE 3: Hexameric $\alpha_2\beta_2\gamma_2$ subunit composition of *D. variabilis* desulforubidin as indicated by SDS-PAGE, followed by Immobilon blotting on a PVDF filter and subsequent staining with Coomassie Brilliant Blue. Lanes 1 and 2 contained 3 and 1.5 μg of desulforubidin, respectively; lane 3, molecular mass marker mixture.

Subunit Composition and Immunological Cross Reactivity. All dissimilatory sulfite reductases have long been taken to contain two types of subunits in the canonical $\alpha_2\beta_2$ heterotetrameric composition (Lee et al., 1973; Kobayashi et al., 1972; Schedel & Trüper, 1979; Hatchikian & Zeikus, 1983). We have recently reported multiple evidences for the occurrence of a third, small γ-subunit in $\alpha_2\beta_2\gamma_2$ hexameric desulfoviridin from *D. vulgaris* (Hildenborough) (Pierik et al., 1992a). Immunological evidence indicated that this subunit structure also applies to desulfoviridins from other species; and it was suggested that the subunit structure of other dissimilatory should be (re)examined.

We have subjected *D. variabilis* desulforubidin to analysis in SDS-PAGE. The final step in the purification over FPLC Mono-Q anion exchanger was repeated to obtain a highly purified enzyme. SDS-PAGE and subsequent Immobilon blotting, followed by Coomassie staining, revealed three bands with mobilities corresponding to molecular masses of 50, 42.5, and ≈12 kDa. Integration of densitometric scans of both the Coomassie-stained SDS-PAGE gel and the Coomassie-stained Immobilon blot resulted in a stoichiometry of 1:1.28:1.09(α:β:γ) after correction for the molecular masses (Figure 3). With a subunit composition of $\alpha_2\beta_2\gamma_2$, the molecular mass of desulforubidin from *D. variabilis* would be 208 kDa. This fits well with the molecular mass of the holoenzyme of ≈200 kDa, as determined by gel filtration.

For each of the three subunits, the immunological relation between desulforubidin from *D. variabilis* and desulfoviridin from *D. vulgaris* was explored in the cross reactivity of desulforubidin with polyclonal antibodies raised against the desulfoviridin subunits (Figure 4). Each lane of the SDS polyacrylamide gel was loaded with 650 ng of desulforubidin, corresponding to 325 ng of the α-subunit, 275 ng of the β-subunit, and 78 ng of the γ-subunit. After being blotted onto nitrocellulose membranes, the membranes were incubated with mouse antiserum raised against the individual subunits from purified desulfoviridin. The anti-α antiserum was diluted 500-fold, anti-β 2000-fold, and anti-γ 1000-fold. Goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad, Richmond, VA) was used for immunostaining. On immunoblots the antisera exhibited specific responses with the α- and the β-subunits. The response of the β-subunit was more pronounced than the responses of the α-subunit. No cross reactivity could be detected for the γ-subunit. Cross reactivity with the β-subunit was observed for both the anti-α and the anti-γ antisera (Figure 4).

EPR Spectroscopy Identifies Quantum Mixed Spin Siroheme. In Figure 5, we compare the *g*-perpendicular regions of the siroheme $S = {}^5/_2$ EPR from *D. variabilis* desulforubidin and the partially purified dissimilatory sulfite reductase from

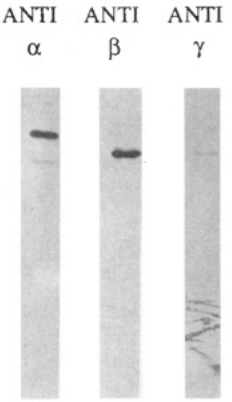

FIGURE 4: Antigenic cross reactivity of dissimilatory sulfite reductases from *D. variabilis* and *D. vulgaris*. Immunostained nitrocellulose blot; each lane was loaded with ≈650 ng of desulforubidin from *D. variabilis*, corresponding to 310 ng of α-, 260 ng of β-, and 75 ng of γ-subunit. Anti-α was 500× diluted; anti-β, 2000×; anti-γ, 1000×.

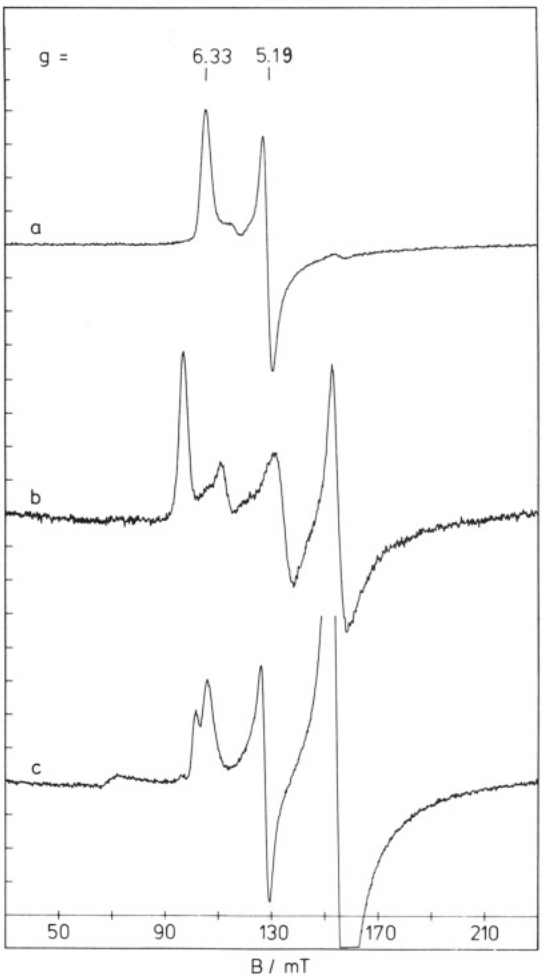

FIGURE 5: High-spin siroheme EPR spectra of desulforubidin from (a) *D. variabilis*, (b) *D. acetoxidans*, and (c) *D. baarsii*. All three spectra are indicative of quantum spin mixing. See text for details. EPR conditions: microwave frequency, 9301 MHz; microwave power, 0.2 (trace a) or 0.5 (trace b) mW; modulation amplitude, 1 mT; T, 4.2 K.

D. acetoxidans and *D. baarsii*. The spectra of the latter two also contain a rubredoxin-like signal ($S = {}^5/_2$, $E/D \approx 1/3$, $g = 4.3$ and ≈9.6); however, the high-spin siroheme signal is easily identified. All three proteins exhibit relatively simple spectra, dominated by a single major component of small rhombicity $E/D = 0.02$–0.04 [$E/D \approx \Delta g_\perp/48$ (Peisach et al., 1971)]. A similar spectrum has been observed by Moura et al. (1988) for *Desulfovibrio baculatus* desulforubidin. This simple spectrum can be contasted with the complex pattern

Table II: EPR g-Values of Siroheme and Fe/S from Desulforubidin[a]

	g_x	g_y	g_z
$S = {}^5/_2$ Siroheme			
D. variabilis	6.33(2)	5.19(2)	1.98
D. variabilis (Q-band)	6.33(8)	5.19(5)	nd[b]
D. acetoxidans	6.89	4.95	nd
D. baarsii	6.30	5.20	nd
$S = {}^1/_2$ Siroheme (*D. variabilis*)			
signal 1	1.782	2.374	2.474
signal 2	1.75	2.340	2.530
signal 3	nd	2.26	2.63
$S = {}^9/_2$ Fe/S (*D. variabilis*)			
$\|\pm{}^1/_2\rangle$ doublet	nd	16.1	nd
	(0.53)	(16.1)	(1.10)
$\|\pm{}^3/_2\rangle$ doublet	nd	≈8.6	nd
	(3.87)	(8.43)	(6.12)
$\|\pm{}^5/_2\rangle$ doublet	≈8.6	nd	nd
	(8.95)	(1.88)	(2.13)

[a] All values refer to X-band unless otherwise indicated. Values in parentheses are calculated for $S = {}^9/_2$, E/D = 0.13, g = 1.90. [b] Not determined.

of several rhombicities observed in desulfoviridins (Moura et al., 1988; Pierik & Hagen, 1991; Hall & Prince, 1981; Liu et al., 1979).

The spectra in Figure 5 and that of *D. baculatus* desulforubidin all have another striking property in common: the average of the two perpendicular g-values falls significantly short of the value of 6.0 predicted for a pure sextet state of Fe^{3+}. Moura et al. (1988) found (6.43 + 5.34)/2 = 5.89 for g_{av}; they do not comment on this unusual value. For the spectra in Figure 5, we find g_{av} = 5.76, 5.92, 5.75, respectively (*cf.* Table II).

In order to check whether these shifts from the theoretical g-value reflect intermolecular magnetic interaction, we have recorded the spectrum of *D. variabilis* high-spin heme in the presence of increasing amounts of urea (0.4, 1.2, and 3.6 M). Minor changes in the line width were observed; however, no significant change in the effective g-values was found (not shown).

Dipolar interactions between paramagnets, whether intra- or intermolecular, can usually be identified by taking EPR data at two different microwave frequencies. At the higher frequency, the relative strength of dipolar interactions *versus* the Zeeman interaction is changed in favor of the latter one. We have, therefore, recorded the spectrum of *D. variabilis* desulforubidin at Q-band frequency (Figure 6). The effective g-values observed in Q-band are identical within 0.05% to those found in X-band (*cf.* Table II). Therefore, the anomalous g-values are not caused by magnetic coupling. Deviations from g_{av} = 6 have been observed previously in cytochromes c' and in peroxidases, and they have been interpreted in terms of quantum-mechanical mixing of an $S = {}^5/_2$ and an $S = {}^3/_2$ state [reviewed by Maltempo and Moss (1976)]. The present case of desulforubidin would be the first example of a quantum mixed spin state in a protein from an anaerobic bacterium. Based on the model calculations of Maltempo and Moss (1976; Figure 5), we find the excited S = $^3/_2$ state at 770 cm^{-1} above the $S = {}^5/_2$ ground state.

Quantification of Siroheme EPR. The complete EPR spectrum of the high-spin siroheme in *D. variabilis* desulforubidin is given in Figure 7, trace a. The accurate quantification of these types of spectra is usually not trivial for two reasons. Depopulation of the $|\pm{}^1/_2\rangle$ ground doublet of the $S = {}^5/_2$ multiplet occurs with increasing temperature. Secondly, slight disturbances of the base line between the middle and the highest g-value result in relatively large errors in the second

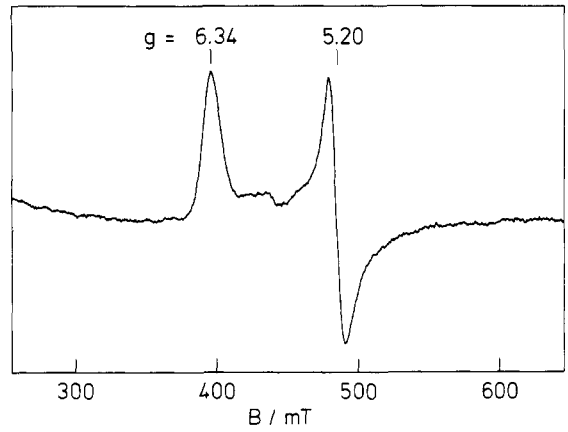

FIGURE 6: Q-band EPR spectrum of the *D. variabilis* desulforubidin high-spin siroheme. The spectrum establishes the effective g-values to be frequency independent. The sample is the same as for Figure 5, trace a. EPR conditions: microwave frequency, 35 221 MHz; microwave power, 50 mW; modulation amplitude, 1.25 mT; T, 15 K. The spectrum is an average of four scans.

integral. The depopulation problem is easily circumvented by taking data at a temperature of 4.2 K, where the ground doublet is nearly 100% populated. A possible way to avoid unreliable integrals over wide magnetic field scans is to numerically simulate the spectrum.

The angular-dependent inhomogeneous EPR line width in high-spin hemoproteins is a complicated entity encompassing contributions from several different broadening mechanisms (Hagen, 1981). However, at X-band frequencies, a major contribution is from unresolved superhyperfine interactions (Hagen, 1981; Scholes et al., 1972). This observation goes some way toward explaining why simulations of these types of effective $S = {}^1/_2$ spectra employing a Gaussian line shape with little angular dependency give satisfactory results [*cf.* Aasa and Vänngård (1975)]. We have used this approach to quantify the desulforubidin high-spin heme spectrum.

The simulation in Figure 7, trace b is the end result of minimization on a rhombic effective **g**-tensor and a near-isotropic Gaussian. Figure 7 also contains the experimental and simulated spectra of the external standard $[Cu(H_2O)]^{2+}$ in aqueous perchlorate. This latter simulation includes variation of the inhomogeneous line width as a function of m_I (*i.e.*, the $I = {}^3/_2$ ^{63,65}Cu nuclear orientation) according to the expansion

$$W(\theta,\phi) = W_0(\theta,\phi) + b(\theta,\phi)m_I + \text{higher order terms} \quad (2)$$

This pattern appears to be general for copper and low-spin cobalt complexes (Hagen, 1981; Froncisz & Hyde, 1980) and has been argued to be directly related to the phenomenon of g-strain (Hagen, 1981).

The advantage of simulating both the spectrum of the unknown and that of the standard is that the transition probability for all powder orientations is included. Therefore, quantification involves only the direct comparison of second integrals of the simulations with amplitudes normalized to those of the experimental spectra. The end result of this procedure was 0.65 high-spin siroheme per half-molecule of desulforubidin, with the concentration of the latter determined with the microbiuret method using a molecular mass of 208 kDa.

In the 4.2 K spectrum of high-spin siroheme in Figure 7, trace a, some low-spin heme is just detectable around $B \approx 280$ mT. Under the conditions employed, the low-spin heme signal is strongly saturated. Figure 8 gives the nonsaturated low-spin heme spectrum at 25 K. As we have observed before with *D. vulgaris* desulfoviridin, there are several (at least three;

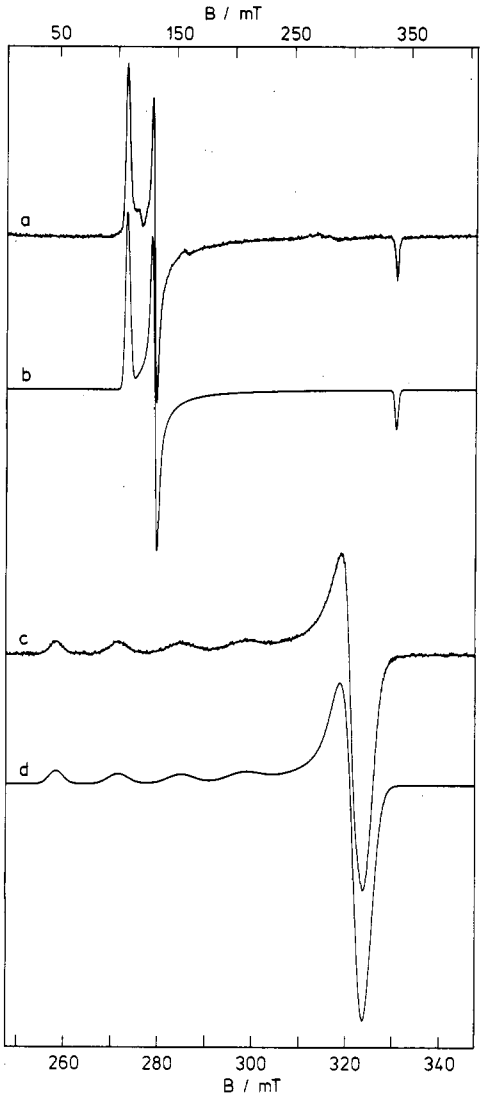

FIGURE 7: Computer simulation of the complete high-spin siroheme EPR spectrum from *D. variabilis* desulforubidin and from the external copper standard. Trace a is from desulforubidin, trace b is the simulation, trace c is the copper standard, and trace d is the simulation. EPR parameters for traces a and c: microwave frequency, 9.30 GHz; microwave power, 0.2 and 0.008 mW; modulation amplitude, 1.6 mT; T, 4.2 K. Simulation parameters for trace b: 1000 × 20 orientations; *g*-values, 6.30, 5.22, 1.98; line widths, 3.36, 2.8, 2.7 mT. Simulation parameters for trace d: 40 orientations; *g*-values, 2.071, 2.385; line widths, 3.8, 2.7 mT; asymmetry parameters (*b* in eq 2), –0.075, 0.350; copper ($I = {}^3/_2$) hyperfine splittings, 0.63, 13.2 mT.

cf. Table II) slightly different forms of low-spin heme. We have quantitated the sum of all forms by doubly integrating the spectrum of Figure 8 and correcting for the signal around $g \approx 2$ (the g_z of high-spin heme plus a minor contaminant, possibly a trace of copper). We thus find 0.20 low-spin siroheme per half desulforubidin. Added up to the high-spin heme, this gives a total of 0.85 siroheme per half desulforubidin, in reasonable agreement with the chemical heme determination. All data are summarized in Table II.

Iron–Sulfur $S = {}^9/_2$ EPR. Low-field EPR as a function of temperature is presented in Figure 9. Several weak resonances are observed with effective *g*-values up to 16. A Kramers system with effective an *g*-value > 14 implies $S \geq {}^9/_2$ (Hagen, 1992). In the weak-field limit (Pierik & Hagen, 1991; Hagen, 1992), the line at $g = 16$ and the broad feature at $g \approx 8$–9 can be fitted to the standard spin Hamiltonian

$$H_S = D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) + g\beta\mathbf{B}\cdot\mathbf{S} \quad (3)$$

for $S = {}^9/_2$, $|E/D| = 0.13$, and $g_{real} = 1.9$ (*cf.* Table II). When

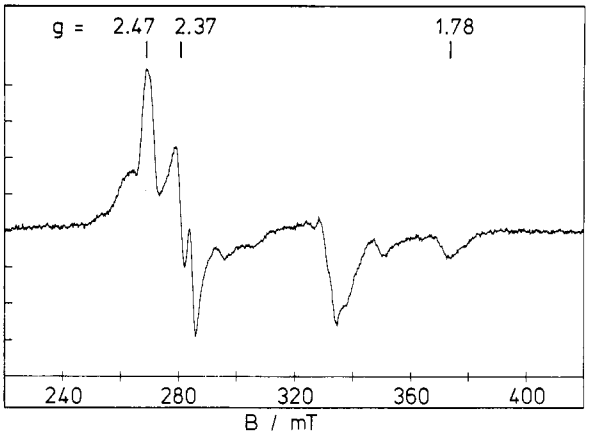

FIGURE 8: EPR of multiple low-spin siroheme components from *D. variabilis* desulforubidin. The three *g*-values of the major component are indicated. EPR conditions: microwave frequency, 9301 MHz; microwave power, 8 mW; modulation amplitude, 1.6 mT; T, 25 K.

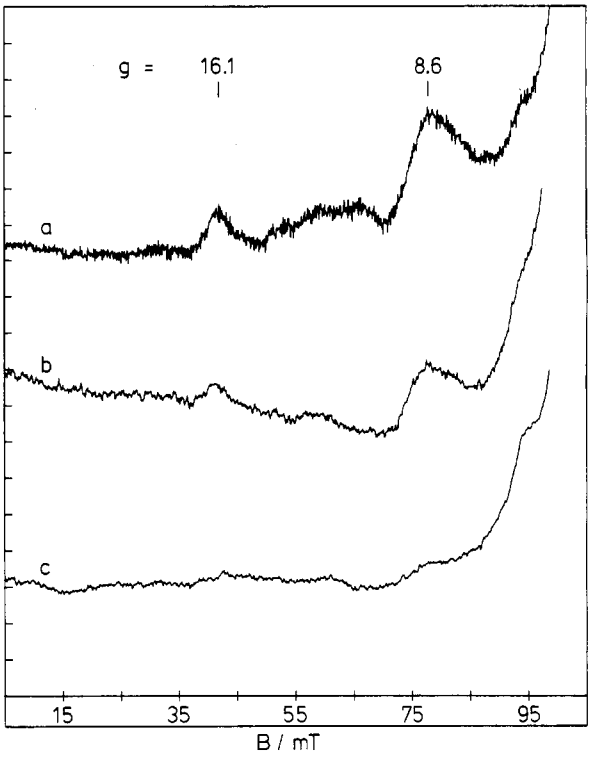

FIGURE 9: Temperature dependence of $S = {}^9/_2$ EPR from Fe/S in *D. variabilis* desulforubidin. Trace a (4.2 K), trace b (9 K), and trace c (16 K) were all recorded with the same amplification. Other settings: microwave frequency, 9.30 GHz; microwave power, 200 mW; modulation amplitude, 2 mT.

g_{real} is kept at 2.00, a fit is obtained only when allowing for a distribution in |E/D| from 0.08 to 0.12. Additional weak absorptions in the range g_{eff} = 10–13 also point to the occurrence of several rhombicities.

The lines are somewhat broader than those previously found for an $S = {}^9/_2$ system in *D. vulgaris* desulfoviridin (Pierik & Hagen, 1991); however, the temperature dependence is similar. After correction for depopulation, the intensities of the lines *increase* from $T = 4.2$ to 9 K, indicating a negative sign for the zero-field interaction. Lifetime broadening also sets in around 9 K. This combination of a fast spin–lattice relaxation and an inverted spin multiplet results in rather unfavorable conditions for the detection of this type of EPR, and only a very rough estimate of the spin count can be made.

From the intensity of the $g = 16$ peak at 4.2 and 9 K, we estimate $D \approx -0.3$ cm^{-1} when assuming $S = {}^9/_2$. This means

that the $|\pm^1/_2\rangle$ doublet is $2D \approx 6$ cm^{-1} above the $|\pm^9/_2\rangle$ ground state and has a population within the $S = {}^9/_2$ multiplet of some 7% at $T = 4.2$ K. We estimate the ratio of high-spin siroheme over $S = {}^9/_2$ iron–sulfur cluster using doubly integrated effective $S = {}^1/_2$ simulations (just as in Figure 7). The simulation for the $S = {}^9/_2$ system was for the $|\pm^1/_2\rangle$ doublet with the effective g-tensor given in Table II. As only one g-value ($g = 16$) is actually observed and no complete data sets for other systems available and no theory for $S = {}^9/_2$ powder line shapes are yet developed, our present quantification should be considered a rough order-of-magnitude estimate. After correction for the population at 4.2 K, we find about 0.4–2.5 $S = {}^9/_2$ per high-spin siroheme.

Upon reduction with excess dithionite, the protein becomes EPR silent.

Assimilatory Sulfite Reductases. In our previous work on *D. vulgaris* desulfoviridin, we have also reported on attempts to detect $S \geq {}^7/_2$ EPR from the Fe/S in $\alpha_4\beta_8$ *E. coli* NADPH–sulfite oxidoreductase and from the Fe/S in monomeric *D. vulgaris* assimilatory sulfite reductase (Pierik & Hagen, 1991). We have now made significantly more concentrated preparations of these proteins, namely, *E. coli* enzyme, 285 μM in total siroheme, and assimilatory *D. vulgaris* enzyme, 570 μM in low-spin siroheme, as determined by EPR integrations. With extensively signal-averaged EPR on these samples at 4.2 K, we have still not found any signal in the low-field range, where we do find $S = {}^9/_2$ signals for desulfoviridin (Pierik & Hagen, 1991) and for desulforubidin (Figure 9).

DISCUSSION

Disparity of Assimilatory versus Dissimilatory Sulfite Reductases. Sulfite reductase contains siroheme and iron–sulfur. The 3D structure of the enzyme, and therefore of the active site, is not known. The catalysis involves (multiple) electron pair transfer; therefore, a single heme is not sufficient. In the Siegel–Münck proposal, the active site is modeled as a 5Fe complex formed by a siroheme bridged to an Fe/S cubane (Siegel et al., 1973; Chistner et al., 1981). The model follows from an unorthodox interpretation (*cf.* exchange coupling between an $S = {}^5/_2$ and an $S = 0$ system) of extensive spectroscopic data on the heteropolymeric ($\alpha_8\beta_4$) assimilatory *E. coli* enzyme. By comparative spectroscopy, the model has been argued to be valid also for enzymes from other sources and with a very different subunit composition, namely, homopolymeric (α_2) spinach leaf nitrite reductase (Wilkerson et al., 1983), monomeric assimilatory sulfite reductase (low-spin) from sulfate reducers (Huynh et al., 1984, 1985), and heteropolymeric ($\alpha_2\beta_2\gamma_x$) dissimilatory sulfite reductases from sulfate reducers (Huynh et al., 1985; Moura et al., 1987, 1988).

We have previously questioned the validity of the Siegel–Münck model in general, and we have specifically provided EPR spectroscopic evidence (the detection of $S = {}^9/_2$ EPR from Fe/S) against its validity for desulfoviridin, the dissimilatory sulfite reductase from *D. vulgaris* (Pierik & Hagen, 1991). Our argumentation was complicated by the fact that the siroheme in desulfoviridin is to a considerable extent demetalated. Therefore, we have searched for $S = {}^9/_2$ EPR from Fe/S in other types of sulfite reductases. We have now found this clue also in fully metalated desulforubidin, the dissimilatory sulfite reductase from *D. variabilis*. On the other hand, we have been unable to detect any EPR whatsoever from Fe/S in oxidized assimilatory enzymes, *viz.* the monomeric *D. vulgaris* protein and the heteropolymeric *E. coli* protein. The finding of uncoupled $S = {}^9/_2$ Fe/S in dissimilatory sulfite reductases provides evidence against the validity of the Siegel–Münck model for these group of enzymes. For the class of assimilatory sulfite reductases, the coupled siroheme–[4Fe–4S] model is not contradicted by our EPR results since no high-spin Fe/S is detected.

On the basis of these observations, we propose that the functional difference between assimilatory and dissimilatory sulfite reductases is paralleled by structural and magnetic differences of the iron–sulfur structures. The dissimilatory enzymes have uncoupled Fe/S and siroheme prosthetic groups. In the oxidized assimilatory enzymes, the Fe/S cluster is different, as it is not detected by EPR. Its nature remains to be established.

On the basis of a Mössbauer spectroscopic study, Moura et al. have previously proposed that both desulfoviridin and desulforubidin contain a ferric siroheme exchange-coupled to a $[4Fe\text{-}4S]^{2+}$ cluster (Moura et al., 1988). An implicit ad hoc assumption in that analysis (and all previous ones) was that all iron–sulfur clusters in sulfite reductases are of the cubane type. The Mössbauer data were, by consequence, interpreted in terms of mixtures of diamagnetic and paramagnatic cubanes with the exclusion of any other interpretation. Remarkably, the authors were forced to invoke the presence of a stoichiometric extra mononuclear iron site in both proteins, although their EPR data do not show additional resonances attributable to a ferric site. It would seem to be worthwhile to readdress this question without *a priori* limitations on the modeling of the clusters, the more so since our recent Mössbauer study on a putative 6Fe cluster containing protein pointed to the hitherto not considered possibility that within a single paramagnetic cluster some of the iron ions behave as very nearly diamagnetic (Pierik et al., 1992b). We have initiated work in this direction.

Unity of Dissimilatory Sulfite Reductases. We have now determined that the dissimilatory sulfite reductase from *D. variabilis* is a metalated desulforubidin with the hexameric subunit composition $\alpha_2\beta_2\gamma_2$. The α- and β-subunits exhibit cross reactivity with antiserum raised against the corresponding subunits from *D. vulgaris* desulfoviridin. The γ-subunit, although detectable on SDS-PAGE, shows no specific response to the corresponding antibody from *D. vulgaris*. In our previous work, we have shown cross reactivity with the α-, β-, and γ-subunits of desulfoviridin from three other *Desulfovibrio* species (Pierik et al., 1992a). Especially the γ-subunit proved to be strongly cross reactive. Of all four strains tested [*D. vulgaris* (Hildenborough), *D. vulgaris oxamicus* (Monticello), *D. gigas*, and *D. desulfuricans* ATCC 27774], the anti-γ antiserum exhibited very specific responses on immunoblots. It should be noted that all four *Desulfovibrio* strains contain desulfoviridin, whereas *Desulfosarcina variabilis* contains the desulforubidin-type dissimilatory sulfite reductase.

We have found $S = {}^9/_2$ EPR in *D. variabilis* desulforubidin and in *D. vulgaris* desulfoviridin. Comparable signals are present in published spectra from *D. gigas* desulfoviridin and *D. baculatus* desulforubidin (Moura et al., 1988).

On the basis of these observations, we propose the following working hypothesis: all dissimilatory sulfite reductases have the hexameric $\alpha_2\beta_2\gamma_2$ subunit composition; they also all have Fe/S with $S \geq {}^7/_2$ and Fe ≈ 6, *i.e.*, a structure and paramagnetism analogous to that of the prismane protein (Hagen & Pierik, 1989; Hagen et al., 1991b; Pierik et al., 1992) or to that of the nitrogenase P-cluster (Hagen et al., 1987; Hagen et al., 1991a).

Presently, this hypothesis has not been tested for two other "types" (*i.e.*, based on optical spectra) of dissimilatory sulfite reductases. For P582 from *Desulfotomaculum* spp, no EPR and no subunit composition has been reported (Trudinger, 1970). For desulfofuscidin from *Thermodesulfobacterium commune*, no EPR data are available and the earlier reported

subunit composition, $\alpha_2\beta_2$, has not yet been checked for the presence of γ-subunit (Hatchikian & Zeikus, 1983).

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