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A SURVEY OF EPR-DETECTABLE COMPONENTS IN SULFUR-REDUCING BACTERIA

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A survey was made of components of sulfur-reducing bacteria that can be detected by EPR spectroscopy. To this purpose, whole cells were examined and a membrane and cytoplasm fraction obtained by centrifugation at 120000 × *g* after cell lysis by suitable procedures. Attempts were made to obtain spectra on these materials with their electron acceptors in the oxidized and reduced states, respectively. *Desulfuromonas* strains 5071, 2873, Gö 10, Gö 20, NZ 27, Spirillum 5175, and *Desulfovibrio baculatus* strain 9974 were studied. Fe-S proteins were recognized from their characteristic EPR behavior; and low-spin cytochromes, largely of the *c* type, were clearly observed. Signals obviously originating from unknown types of components were also found. Partial segregation of some of the observed species into the cytoplasm or membrane fraction was achieved. Tentative assignments of the various resonances seen in the EPR spectra, based on behavior with fractionation, on oxidation-reduction or on change of microwave power are presented.

Introduction

The cytoplasmic fraction of the sulfur-reducing bacterium *Desulfuromonas* strain 5071 [1] contains large amounts of *c*-type cytochromes and iron-sulfur proteins. Ferredoxin, rubredoxin and cytochrome c_7 have been purified and described [2,3] previously. Separation of the soluble proteins by column chromatography indicates that there are additional electron-carrier proteins, which have not yet been purified or identified [4]. One may reasonably expect that at least some of these proteins are necessary for electron transport to elemental sulfur. Biebl and Pfennig [5] showed that some strains of the sulfate-reducing bacterium *Desulfovibrio* are also able to conserve energy for growth by reducing elemental sulfur. A number of their metalloproteins have been characterized by EPR spectroscopy [6], and it was demonstrated in vitro that cytochrome c_3 reduces elemental sulfur [7]. The microaerophilic bacterium Spirillum 5175, which is known to gain energy from nitrate reduction, can also easily grow with elemental sulfur as an electron acceptor [8].

In view of this capability for reducing sulfur apparently common to different bacterial groups, we have undertaken in the present study to screen seven representative microorganisms for EPR-detectable electron acceptors, which presumably will belong to the classes of heme and iron-sulfur proteins. The results of this survey are presented below with the expectation that it may serve as a preliminary guide for the selection of one or more organisms, with which purification of components, functional tests and eventually reconstitution of metabolic pathways may be attempted. Our experience as to ease of growth, yield, ease of fractionation and either simplicity or multiplicity of components detected will all have to be considered for an appropriate choice.

Material and Methods

Source of organisms

The following four strains were isolated in pure culture from enrichment cultures, inoculated with anoxic sediment samples: (1) Strain Gö 10 from a brackish water ditch near the Jadebusen (North Sea); (2) strain Gö 20 from a seawater lagoon near Montpellier (Southern France); (3) strain NZ 27 from Ngawha-Springs, Moerewa (New Zealand); and (4) strain 2873 from a sludge digester of the Göttingen sewage treatment plant (F.R.G.). *D. acetoxidans*, strain 5071, was isolated and described by Pfennig and Biebl [1]. Spirillum 5175 (*Campylobacter*) was described by Wolfe and Pfennig [8]. *Desulfovibrio baculatus*, strain 9974 [5], was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen.

Chemicals

Most chemicals were purchased from E. Merck, Darmstadt, F.R.G.; lysozyme and DNAase from Sigma, Munich, F.R.G.; sodium succinate from Fluka, Buchs, Switzerland.

Growth of Organisms

Freshwater or salt medium was prepared as described by Pfennig and Biebl [1] except for the following modifications: NH_4Cl was increased to 0.5 g/l. From a trace element solution, according to Laanbroek and Pfennig [9], which contained no copper and manganese, 2 ml/l were added. Instead of biotin, 5 ml/l of a vitamin solution [10] were added. Cells were cultivated in 10-1 flasks and growth was followed by measuring the absorbance at 578 nm. For the different strains the following substrates and electron donors (sodium salts) were given to the growing cultures (mmol/l): Strains Gö 10, Gö 20, 2873 and Spirillum 5175 – acetate 15, fumarate 40, *D. acetoxidans*, strain 5071 – pyruvate 10, DL-malate 40. *D. baculatus* strain 9974 – fumarate 60. Strain NZ 27, pyruvate 20, elemental sulfur 150. In the presence of elemental sulfur as electron acceptor, growth-limiting amounts of H_2S are formed. Sulfide was extruded continuously by nitrogen after growth started. The pH was adjusted to 7.3 by 2 M H_2SO_4.

Preparation of samples for EPR spectroscopy

About 8 g of cells per 10 l culture medium were harvested by centrifugation ($6000 \times g$, 10 min) and washed once with 40 mM Tris-HCl buffer, ph 7.4. A portion of the cell paste (referred to as whole cells) was transferred to sample tubes. To the 1 : 2 diluted pellet of cells lysozyme and DNAase were added; in case of Spirillum 5175, Desulfovibrio and strain NZ 27 a French pressure cell was used at 1500 kPa/cm^2 and DNAase was then added to obtain a disrupted cell suspension (referred to as homogenate). From this fraction, cell debris was removed ($6000 \times g$, 10 min). A particulate fraction (referred to as membranes) and supernatant (referred to as cytoplasmic fraction) were obtained by centrifugation at $120\,000 \times g$ for 2 h. The 'oxidized' samples from each of the four preparative steps were thoroughly stirred in the presence of air with a nichrome wire which had a loop at its end. Addition of a few crystals of sodium dithionite yielded a 'reduced' protein fraction. The oxidized samples were immediately frozen in liquid nitrogen; a few minutes were allowed for dissolution of dithionite and reduction for reduced samples; they were usually frozen when changes of visible color (cytochromes) could no longer be detected.

EPR spectroscopy

EPR measurements were made with a Bruker Spectrometer BER 420 equipped with a variable modulation frequency unit, a microwave frequency counter and a B-H 12 NMR-based field marker (Bruker, Karlsruhe, F.R.G.). The samples were frozen in quartz tubes (4.5 or 5.2 ± 0.2 mm outer diameter) in liquid nitrogen. Generally spectra were recorded at 5–12 K using the Helitran LTD-110 C System (Air Products, Allentown, U.S.A.). The g values were calculated by measuring the microwave frequency and the magnetic field (calibration with diphenylpicrylhydrazyl and Mn(II) in MgO).

Prominent features of spectra such as extrema or shoulders are also reported on a g-value scale. The accuracy and precision of these values depend, if specific features of the instruments are not considered, on the signal-to-noise ratio and resolution of peaks achieved in the spectra. For sharp and well separated peaks the error will be no more than ± 0.002, when line positions are measured

from the field markers on the spectra, whereas for broad features, such as the high-field peak of low-spin heme, the error could be as large as ±0.01. Similarly, if two peaks of similar height overlap, our measurement may not represent the exact values for each of the individual components. This situation is, of course, almost inevitable with the spectra of whole cells. Almost all spectra recorded at low power show a signal typical of free radicals which is expected to be centered at *g* 2.003–2.005. We have always verified that our calculations using the field markers produced by the instrument will generate a *g* value in this range for the free radical signals in the spectra. The procedures followed for making assignments of features in the spectra are outlined in Results and Discussion with reference to Figs. 1–3. At this stage, before at least some pure components are available, we have not attempted any simulations of spectra; however, the values listed for the cytochromes have been verified as reasonable combinations according to theoretical calculations by Löw [11]. Throughout this paper we will use the recommended nomenclature [12,13] for designation of Fe-S proteins and their oxidation levels.

Results and Discussion

Assignment of spectral lines to individual components

Tentative assignments of features to individual components were mainly based on three types of observations: (1) the response of the lines to changes in microwave power, (2) their segregation with fractionation of broken cells, and (3) the differences between spectra of oxidized and reduced samples. Concerning this last point, as expected, we were not always able to reduce or reoxidize (when previously reduced) particulate fractions completely. However, even partial reduction or reoxidation often showed clearly which features of the spectrum are likely to arise from the same components or which features cannot be attributed to a single component. Examples of these three principal approaches for the preliminary resolution of spectra are illustrated in the figures presented. The figures are organized (contrary to the process of fractionation) so that we proceed from the simpler to more complicated spectra, i.e., from the cytoplasmic fraction to membranes and then to whole cells. Fig. 1 shows an example encountered with *Desulfuromonas* strain Gö 10 where it is quite clear that an Fe-S component with peaks at *g* 1.89, 1.92 and presumably 2.052 predominates in the cytoplasm, whereas one (or more) component(s) with peaks at *g* 1.887, 1.944 and 2.046 remain(s) in the membrane fraction, and whole cells show these and additional peaks. Fig. 2 presents an example, taken from the membrane fraction of strain 2873, of the

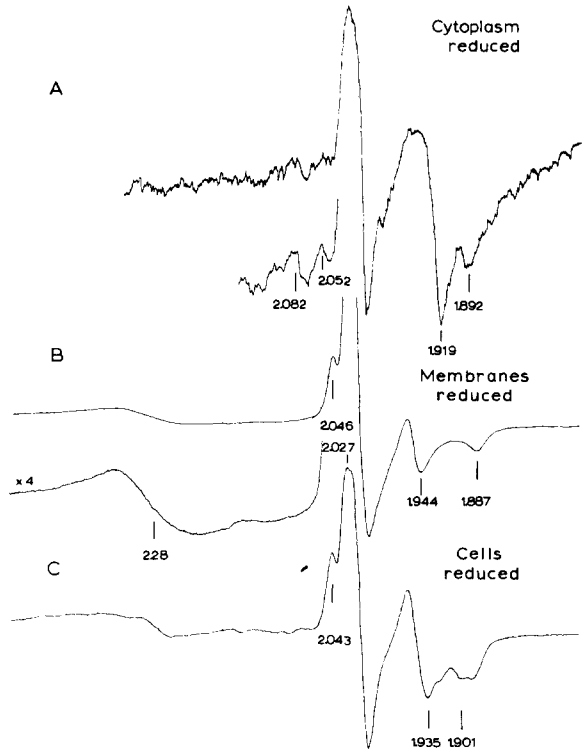

Fig. 1. Comparison of EPR spectra between approx. 250 and 400 mT from whole cells (C), cytoplasmic (A) and membrane (B) fractions of strain Gö 10 after addition of dithionite, showing a distinct separation of Fe-S components by the fractionation. The conditions of EPR spectroscopy were: microwave power, 150 mW for A and 2 mW for B and C; modulation amplitude, 1 mT; time constant, 0.2 s; scanning rate, 0.4 mT/s; and temperature, 8.0, 10.3 and 10.9 K, respectively, for A, B and C. Note that a spectrum of A at 2 mW showed the same *g* values but with poor signal-to-noise ratio. Note that the *g* values in the figures and the corresponding text refer to measurements made during that particular recording, whereas those in the table are derived from several spectra. There are, therefore, a few minor differences in the third decimal place.

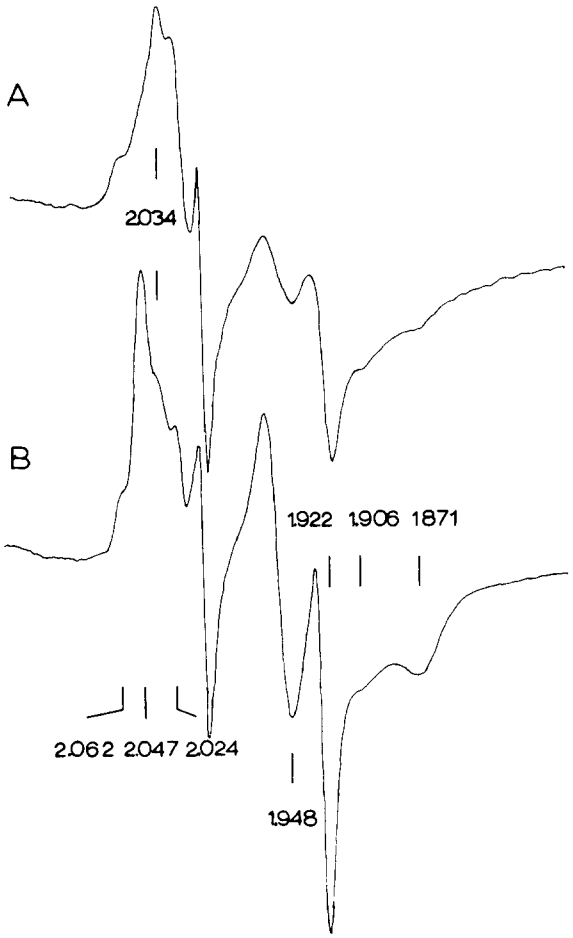

Fig. 2. EPR spectra in the g approx. 2 region of the membrane fraction from *Desulfuromonas* strain 2873 after addition of dithionite, demonstrating the resolution achievable by microwave power variation, particularly in the low-field portion of the spectra. The conditions of EPR spectroscopy were essentially as in Fig. 1 except that the temperature was 10.8 K for A and B and the microwave power 150 mW for A and 2 mW for B. The amplification for A was 3.1-times that used for B.

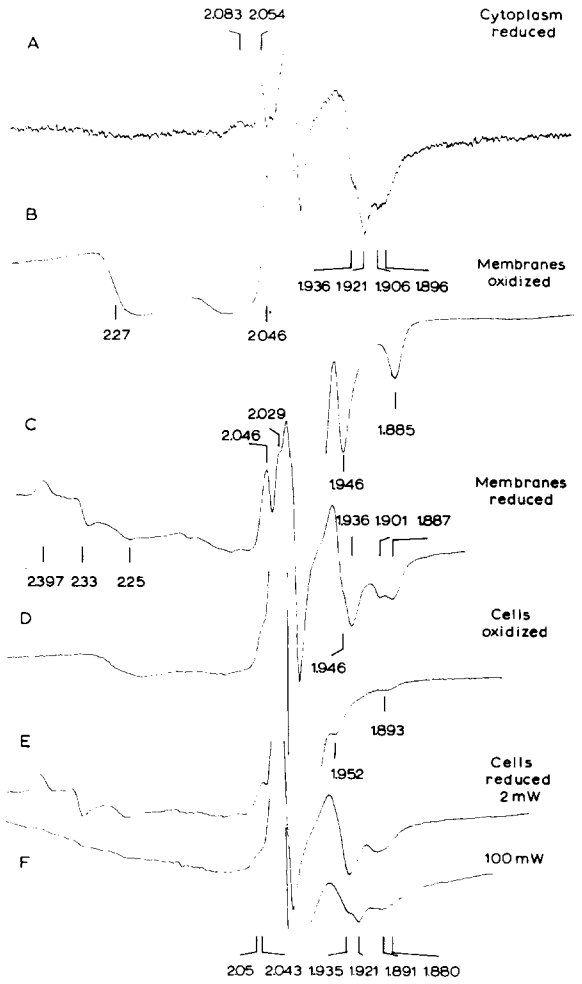

Fig. 3. Comparison of EPR spectra from whole cells and fractions as in Fig. 1 from *D. acetoxidans* strain 5071. The spectra of the membrane fraction (B, C) and whole cells (D–F) are shown as obtained (oxidized) and after addition of dithionite (reduced). The oxidized cytoplasmic fraction showed only a Hipip-type signal at g 2.01 (see text). Spectra of whole cells at high and low microwave power are also shown (E, F). The conditions of EPR spectroscopy were essentially as for Fig. 1 except that the microwave power was 2 mW for A–E and 100 mW for F and the temperature was 6.5, 8.6, 10.8, 7.0, 7.1 and 7.1 K, respectively, for A–F. The amplification of E was 3.1-times that of F.

dramatic effect that microwave power can have in providing clues as to the relation of various features in the spectra to each other. This is particularly evident at low field. At 2 mW the peaks at g 1.871, 1.948 and 2.047 are prominent, whereas at higher power the peaks at g 1.922 and 2.034 are dominant. Other peaks, e.g., at g 1.871, 1.906 and 2.062, do not show such a pronounced response to a variation in power. Fig. 3 then illustrates an example from *D. acetoxidans* strain 5071 where all three approaches, fractionation, change in oxidation state and effect of microwave power, have been brought to bear. Fig. 3A shows the relatively simple spectrum of reduced cytoplasm with principal peaks at g 1.921 and 2.054. Note also the free radical signal at g 2.005. While the reduced membrane fraction (C) shows a variety of peaks, a

comparison with the oxidized membrane fraction (B) immediately indicates that the peaks at g 1.885, 1.946 and 2.046 probably represent one component. As explained above, an oxidized particulate fraction rarely has all its electron acceptors in the oxidized state, specifically at the protein concentrations used in our experiments. The peaks indicating the presence of a reduced Fe-S cluster in the oxidized membrane fraction obviously belong to a component of relatively high oxidation-reduction potential. Similar conclusions can be drawn by comparing oxidized (D) and reduced (E) whole cells. In Fig. 3F then we show again the effect of increased microwave power in the resolution of the high-field components of the spectrum. It may also be mentioned here that the spectra of the reduced membrane fraction (C) and reduced cells (E) show one of the unknowns at low field (g 2.397 and 2.33) which deserves particular interest. It can be concluded from the spectra shown in Fig. 3 that this component readily responds to dithionite, has an EPR signal in the reduced state, follows the particulate fraction and that the signal is easily saturated with microwave power (E, F).

Tentative assignments derived from these spectra are found in Table I. In grouping together g values observed with whole cells as presumably belonging to individual components, we were principally guided by observations on these spectra of cells rather than by any knowledge from the separated fractions as to what components we should presumably find in cells. It becomes clear from an inspection of Table I that the combinations of values listed for cytoplasm and membranes, respectively, have rarely been obvious from the observations on whole cells. Whether these apparent changes in EPR parameters when going from whole cells to cytoplasm or membranes are due to overlap of lines leading to an apparent shift of a number of peaks and shoulders, to a separation of components originally present, or merely represent an effect of solubilization or other treatment applied to the cells cannot be decided without further work. Similarly, the origin of the rather intense signals for Fe-S proteins at the (3 +) oxidation level centered around g 2.01 (Hipip type, see below) remains questionable. While they might be significant native components of the organisms studied, the signals observed may have arisen artificially from such components through oxidation by air.

EPR signals observed or identified

In Table I we have only assembled our observations on cytochromes, reduced Fe-S proteins, i.e., those of the (1 +) oxidation level (cf. Refs. 12 and 13) and for signals whose origin remains unexplained. Altogether the following known types of EPR signals were observed.

(1) At g 2.01 signals with the characteristics (strong temperature dependence, high spin relaxation rate, near isotropy) of oxidized Hipip-type Fe-S clusters $[4Fe\text{-}4S]^{3+}$ or 3Fe clusters (cf. Refs. 14 and 15) were observed in both cytoplasm and membrane fractions. These signals might originate from native Hipip-type Fe-S proteins, akin to *Chromatium vinosum* Hipip, or succinate dehydrogenase; they could, however, also have arisen artificially as mentioned above, e.g., from [4Fe-4S] clusters of hydrogenases or ferredoxins. They may well originate from more than a single protein in any one of the species investigated; this seems rather likely because of their presence in membrane and soluble fractions. We considered the possibility that the signals might be due, at least partly, to a succinate or fumarate reductase (cf. Ref. 16). However, addition of 50 mM succinate, albeit aerobically, neither diminished the signals at g 2.01 in the cytoplasm fraction significantly nor did it elicit any new Fe-type signals except with *Desulfuromonas* Gö 20 where signals with g_x 1.882, g_y 1.917 and g_z 2.09 appeared in the cytoplasm with a zero line crossing at g 1.924 and in the membrane fraction of NZ 27 and *Dv. baculatus* for both with g_x 1.888; g_y 1.943; g_z 2.044 and zero line crossings at g 1.955. These are not the exact g values observed for animal succinate dehydrogenase [16–18] or *vibrio succinogenes* fumarate reductase [19]. While these observations argue for the presence of a succinate dehydrogenase-type enzyme in these organisms, it should be pointed out here that NZ 27 is not capable of utilizing fumarate as electron acceptor. It remains to be shown by further work whether with Gö 20 succinate dehydrogenase or fumarate reductase might not have been retained by the membrane fraction and happened to be partly solubilized or carried over in particulate form into the cytoplasmic frac-

TABLE I

TENTATIVE ASSIGNMENTS OF RESONANCES TO INDIVIDUAL COMPONENTS IN VARIOUS STRAINS OF SULFUR-REDUCING BACTERIA

The numbers given refer to prominent features (viz., positive or negative extrema or shoulders) observed in the first-derivative EPR spectra of the materials listed. In the case of cytochromes, the zero line crossing points of the derivative spectrum will be given as the second number of three. For other components zero line crossings, where obvious from the spectra, are indicated by the superscript z. The numbers listed will come close to $g_{x,y,z}$ for cytochromes, while for reduced Fe-S proteins g_x and g_z will be closely approximated. For Fe-S proteins we prefer to list only peaks or shoulders rather than zero line crossings even in the g_y region because of the extensive overlap of most spectra. When assignments of resonances to a single component are fairly unambiguous, the respective group of numbers is given without parentheses or brackets. When a group of numbers is in parentheses, their relationship, not the reality of the corresponding features, is in doubt. If a single number of a group of two or three is in parentheses, there is doubt whether this particular feature is related to the other one or two. Similarly, when brackets are used, a guess is made as to the relationship of the enclosed numbers. When, however, a single number is enclosed in brackets, there is doubt whether the corresponding feature is at all significant. For cytochromes, where major species are more readily differentiated from minor ones because of their similar line widths, the numbers referring to the major species are underlined. When the relationship of certain components has become particularly obvious through the use of different microwave power, the features subject to ready saturation are marked with the superscript s, those increased with increasing power with the superscript p. The superscript su indicates that the features in this row appear in response to addition of succinate. Note that the unknown component in *Dv. bacculatus* with g 6.16, 6.316 and 5.225 is also seen in the cytoplasmic fraction.

Organism	Cytoplasm						Membranes					
	Cytochromes			Fe-S Clusters			Cytochromes			Fe-S Clusters		
Desulfuromonas	1.505	2.27	2.971	–	1.923	2.052	1.505	2.26	2.94	1.887	1.946	2.046
acetoxidans	–	2.34	–	(1.903	–	2.029)	–	2.34	–	1.901	1.936	(2.029)
Strain 5071				–	1.934	–						
				–	–	2.043						
				[1.89	–	2.082]						
	1.55	2.29	2.93	1.890	1.930	2.068	1.55	2.29	2.93	(1.866^s)	1.940^s	2.049^s
Strain	–	2.26	–	1.876^p	(1.912	2.09^p)	–	–	[3.12]	–	1.922^p	2.034^p
2873				1.902	1.920	–	–	–	[2.79]	(1.871	1.906	2.062)
Spirillum 5175	[1.51]	2.16	2.33	1.896	1.940	2.044	1.51	2.16	2.33	1.907^p	1.930^p	–
				–	1.916^z	–	–	–	2.98	1.897^s	1.936^s	2.033
				1.871^p	–	2.112^p	–	–	2.91	1.868^p	–	–
				1.879	1.984	–	–	–	[3.4]	–	1.986	–
				(1.955	–	2.06)				–	1.925^p	–
				1.93	–	–						
Desulfuromonas												
Strain	[1.5]	2.24	[2.95]	1.89^p	1.920^p	2.052^p	1.5	2.28	2.946	1.887	1.944	2.046
Gö 10				–	(1.936^s	2.031^s)	–	–	3.24			
Strain	1.53	2.26	2.975	1.882	1.917	2.091^{su}	1.53	2.28	2.978	1.863	1.949	2.046
Gö 20				–	1.936	–	–	–	3.24	1.875^p	–	2.06^p
				1.877^p	1.912^p	2.099^p				1.891^p	–	–
				–	–	2.048				(1.922^p	1.945^p	2.026^p)
												2.0^p
Strain								2.32	2.948	1.908^p	(1.980^p)	2.035
NZ 27								2.35	–	1.931^s	(1.948)	(2.018)

								(2.29)	–	[1.848^{p}	–	2.081^{p}]
										1.888	1.943	2.044^{su}
										–	–	2.032
										1.853^{p}	–	2.060^{p}
Desulfovibrio	–	2.3	3.026	1.901^{p}	–	2.086^{p}	–	(2.30)	3.047	1.884^{p}	–	–
baculatus				1.903^{s}	1.941^{s}	2.085^{s}	–	(2.26)	3.002	1.909^{s}	1.941^{s}	2.084^{s}
Strain 9974				1.893^{p}	–	2.059^{p}	–	(2.41)	3.326	1.893^{p}	(1.931^{p})	2.059^{p}
				–	1.931^{p}	–				1.865^{p}	–	–
				1.908^{p}	–	–				1.888	1.943	2.044^{su}
				1.865^{p}	–	–				–	–	2.019^{p}
				–	–	2.032						

	Membranes			Whole Cells								
Organism	Unknown			Cytochromes			Fe-S Clusters			Unknown		
Desulfuromonas	(2.249)	2.33^{z}	2.397	1.505	2.26	2.94	1.89	1.945	2.045	(2.249)	2.33^{z}	2.397
acetoxidans							1.901	1.935	(2.029)			
Strain 5071							–	1.921	2.05			
Desulfuromonas												
Strain				1.55	2.29	2.93	–	1.923	2.034			
2873				–	–	2.70	(1.924)	1.949^{s}	2.046^{s}			
							–	1.938	–			
							1.872^{p}	1.902	(2.06)			
							–	–	[2.08]			
							1.862	–	–			
Spirillum 5175	–	2.150^{z}	2.197	1.51	2.16	2.33	(1.910^{p}	1.934^{p}	2.042^{p})	–	2.150^{z}	2.197
				–	–	2.93	(1.908^{s}	1.937^{s}	2.036^{s})			
				–	–	[2.6]	[1.917^{p}	1.925^{p}	2.039]			
							1.870^{p}	–	–			
Desulfuromonas							1.895^{p}	–	–			
							1.983	–	–			
							–	–	2.06			
Strain				1.54	2.28	2.94	1.89^{s}	1.940^{s}	2.045^{s}			
Gö 10				–	–	3.01	1.903^{s}	1.937	–			
					[2.33]	–	1.923^{p}	1.945^{p}	–			
					[2.38]	–						
						2.81						
Strain				1.53	2.26	2.914	1.885	1.92	2.084			
Gö 20							1.921^{p}	1.936^{p}	2.056^{p}			
							1.894	–	–			
							–	1.949	2.045			
							–	–	2.026			
							–	(1.922^{s}	2.044^{s})			
							1.83	–	–			

TABLE I (continued)

Organism	Membranes			Whole Cells								
	Unknown			Cytochromes			Fe-S Clusters			Unknown		
Strain NZ 27				–	2.32	2.944	1.876[P]	(1.913)	2.086[P]	–	–	6.52
							1.884[S]	(1.940)	2.080[S]			
							(1.885[P])	(1.935)	2.053[P]			
							1.886	1.941	2.042[SU]			
							1.932[S]	–	2.017[S]			
							1.921[S]	(1.945)	2.026[S]			
Desulfovibrio baculatus	5.225[Z]	6.16	6.316	–	–	3.047	1.899[S]	–	2.084[S]	5.225[Z]	6.16	6.316
				–	–	3.002	1.857[P]	–	–			
				–	–	3.326	(1.89[P]	1.922[P]	2.054[P])			

tion. Addition of NADH also elicited the same signals in the cytoplasm of strain Gö 20, whereas it was without effect with the other organisms. It is known from work on mammalian systems that NADH will reduce succinate dehydrogenase in particulate fractions of the electron-transport system. With most organisms addition of succinate or NADH to the membrane fraction was not very useful, since these preparations were largely in the reduced state as prepared.

(2) At g 2.0–2.1 and 1.8–1.9 with baseline crossing at g approx. 1.93, signals typical of reduced Fe-S clusters in the $[2Fe\text{-}2S]^{1+}$ or $[4Fe\text{-}4S]^{1+}$ state were seen in almost all fractions. Many of these signals were present in oxidized samples, indicating autoreduction by endogenous substrates.

(3) At g 6 axial or slightly rhombic high-spin ferric heme signals which could originate from native high-spin cytochromes or other heme proteins or also from products derived from heme proteins by degradation in cells or in other fractions by denaturation.

(4) At g 3.5–2.3 and approx. 1.5 with baseline crossing at g approx. 2.2, signals typical of low-spin heme compounds such as most cytochromes. The principal cytochrome in all organisms studied (except maybe for Spirillum 5175) seems to be that with g_x 1.50–1.54, g_y 2.26–2.29 and g_z 2.91–2.98. These g values are fairly similar to those of mammalian cytochrome c [20]. A sample of purified cytochrome c_7 from *d. acetoxidans* showed $g_{x,y,z}$ 1.506, 2.254, 2.971. The values we observed with some of the organisms, though depending on the state, viz., whether in the cytoplasm or in the membrane, were somewhat scattered around the values given above; however, all of these cytochrome species may be considered to be minor variants of cytochrome c_7 except those minority species with g_z above 3.1 or below 2.8. The findings of an entirely different cytochrome ($g_{x,y,z}$ 1.51 (?), 2.16, 2.33) in Spirillum 5175 only, which followed the membrane fraction, is a significant observation. Spectrophotometry at room temperature showed that both *b*- and *c*-type cytochromes are present in whole cells. In membranes only a *b*-type cytochrome was detected.

(5) At g 4.3 rhombic high-spin ferric signals possibly in part from rubredoxin(s) in cells and from iron-EDTA complexes after cell lysis in presence of EDTA. In conjunction with these signals the expected signals for the corresponding ground state were seen at g approx. 9.

(6) Signals of Mn^{2+} of varied intensity in all preparations. Mn^{2+} was enriched considerably in the membrane fraction. These signals, which had the same appearance wherever they were seen, probably are of no significance but represent Mn^{2+} present in the nutrient medium although no Mn^{2+} had been added. However, it became obvious from the recorded spectra that the features contributed by Mn^{2+} complicate the evaluation even at the relatively low intensity at which they were present.

EPR signals of unknown origin

While it is not entirely certain that the signals in the g 2 region are all due to Fe-S proteins, or those at g 2.005 to free radicals (presumably semiquinones), this is, in our opinion, a reasonable assumption. At least, we think, it may be futile at this point to search in this spectral region for unknown signal types until it is entirely certain that the observed signals cannot be explained in terms of the mentioned types. On the other hand, we cannot explain the signals seen in reduced cells and membranes of *D. acetoxidans* strain 5071 and Spirillum 5175 at g 2.33, 2.397 and 2.150, 2.197, respectively. Obviously, according to their shape, these resonances do not constitute a complete signal and a third line would have to be found at higher fields. There is an indication of such a line in *d. acetoxidans*, namely, at g 2.249. However, the missing line may also be buried under the stronger lines around g approx. 2. One observation concerning these unexplained signals is striking: they respond promptly and completely to oxidation and reduction in contrast to other electron acceptors in the preparations in which they are found, whose oxidation state is not readily changed by aeration or addition of dithionite. Since the unexpected signals are only seen in reduced preparations, they cannot be due to low-spin heme compounds. While they are of similar appearance in the two organisms where they are found, their g values differ sufficiently so as to make one wonder whether they originate from the same or related types of material. Signals in that region of the spectrum have recently been reported from Ni(III)

in several proteins [21–24] and from sulfite reductase on reduction [25]. The *g* values differ, however, and the Ni signal disappears on reduction, whereas the signals observed by us are typical for the reduced state.

A second unexplained type of signal of significant intensity was observed in *Dv. baculatus* whole cells and both cytoplasmic and membrane fractions with two closely lying peaks at *g* 6.16 and 6.316 and a derivative-type zero line crossing at *g* 5.22. While this is the general region of the spectrum where high-spin heme compounds absorb (see item 3 above), the observed line positions are unusual for high-spin heme and would require the respective heme compounds (there would have to be two similar compounds, each represented by one of the lines at *g* 6, presumably with a common zero line crossing at *g* 5.22) to be in an unusual state. If the lines at *g* 5.22, 6.16 and 6.316 were due to high-spin heme compounds, a third line would be expected in the *g* approx. 2 region. Despite the relatively high intensity of resonances at *g* approx. 6, the line(s) at *g* approx. 2 would be difficult to detect except in a purified preparation.

Outlook

As expected from their metabolic activities, from preliminary work on these organisms and from the sheer appearance of their extracts, the sulfur-reducing bacteria are a rich source of Fe-S proteins and cytochromes. Further analysis of individual components and insights into their function will require efforts of additional fractionation and purification using suitable representatives of this class of organisms. Recently discovered sulfur-reducing archaebacteria have not been studied so far with respect to electron-transport components [26,27].

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