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IRON-SULPHUR CLUSTERS IN FUMARATE REDUCTASE FROM *VIBRIO SUCCINOGENES*

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(1) The fumarate reductase complex from *Vibrio succinogenes* contains one FAD molecule, one $[4Fe\text{-}4S]^{3+(3+,2+)}$ and one $[2Fe\text{-}2S]^{2+(2+,1+)}$ cluster per enzyme molecule. Both clusters can be partly reduced by succinate. In the presence of excess $Na_2S_2O_4$ and fumarate, the [2Fe-2S] cluster is completely oxidized, whereas the other cluster is largely reduced. (2) The [2Fe-2S] cluster is localized in the M_r 31 000 subunit. The EPR spectrum of the reduced cluster in the isolated subunit differs slightly in line width, but not in *g*-value, from the spectrum of the reduced, intact enzyme complex. This demonstrates that the immediate environment of the cluster is little perturbed by dissociating this subunit from the FAD-containing M_r 79 000 subunit. The temperature dependence of the power-saturation behaviour has, however, greatly decreased in the isolated subunit, the saturation at 11 K of the paramagnetic cluster being much less than in the enzyme complex. Moreover, the temperature dependence of the power-saturation behaviour of this cluster in the enzyme is greater with succinate as reducing agent, than with dithionite. (3) The [4Fe-4S] cluster is located on the M_r 79 000 subunit. This cluster is unstable in air when the subunit has been dissociated from the enzyme complex.

Introduction

The fumarate reductase complex from *Vibrio succinogenes* [1,2] consists of three different peptides (M_r 79 000, 31 000 and 25 000) present in a molar ratio of 1 : 1 : 2. The M_r 79 000 peptide contains 1 mol FAD/mol and the substrate site [2], and the M_r 25 000 subunits represent cytochrome *b*. Both the M_r 79 000 and the 31 000 subunits were found to contain non-heme iron and sulphide. We are not aware of any reports in the literature on the nature of the prosthetic groups that contain the iron and sulphur atoms.

In the present study, the enzyme was examined by EPR spectroscopy in order to elucidate the type and amount of iron-sulphur clusters present. It will be shown that fumarate reductase contains one [2Fe-2S]$^{2+(2+,1+)}$ and [4Fe-4S]$^{3+(3+,2+)}$ cluster per FAD molecule and resembles in many aspects the enzyme, succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC1.3.99.1) from bovine-heart mitochondria. Furthermore, the function of the clusters in fumarate reduction and succinate oxidation was investigated. Using the isolated subunits, it was possible to determine the location of the clusters within the enzyme.

Materials and Methods

The fumarate reductase complex containing cytochrome *b* was prepared as described earlier [1]. The M_r 79 000 subunit, which contains the FAD, and the preparation containing the M_r 31 000 peptide as well as cytochrome *b* were obtained by splitting the complex with guanidinium chloride.

The complex, (5 mg protein/ml) in 1.5 M guanidinium chloride/20 mM Tris-HCl (pH 7.7) at 0°C, was immediately layered on to a 11–25% (w/v) linear sucrose gradient in 0.05% Triton X-100/20 mM Tris-HCl/0.2 M NaCl/1 mM malonate/2 mM dithiothreitol/1 mM $Na_2S_2O_4$ (pH 7.7) at 3°C. The solution was centrifuged for 6 h at 430 000 × g and then fractionated. The fractions were analysed for the individual peptides by gel electrophoresis in the presence of SDS. The fractions containing the M_r 79 000 peptide were pooled and concentrated by precipitation with poly(ethyleneglycol) (M_r 6000) and those containing a mixture of the M_r 31 000 peptide and cytochrome b were subjected to pressure dialysis under N_2 before EPR analysis. The latter preparation contained 2–3 mol Fe/mol M_r 31 000 peptide and less than 5% of the M_r 79 000 peptide. The M_r 79 000 peptide preparation contained 3–5 mol Fe/mol and was contaminated by the M_r 31 000 peptide (0.05–0.1 mol/mol FAD).

EPR spectra were recorded on a Varian E-9 EPR spectrometer and stored on a magnetic disc with a HP 2100 computer. Other experimental details were described earlier [3,4]. Quantification of the EPR signals was carried out either directly [5] or indirectly by comparison with computer-generated spectra [4], in both cases against a reference containing 10 mM $CuSO_4$/2 M $NaClO_4$/10 mM HCl [6].

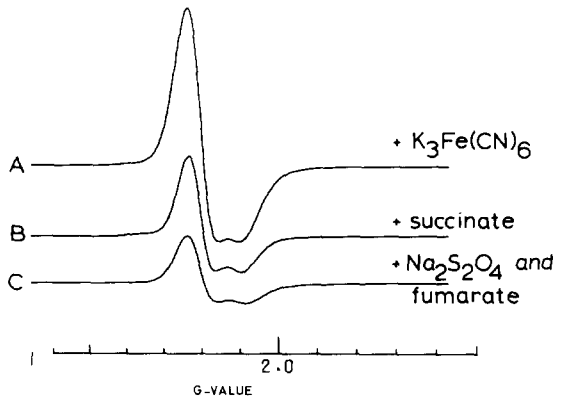

Fig. 1. EPR spectra of fumarate reductase complex at 11.5 K. (A) Fumarate reductase complex (22 μM FAD, , 4 mg protein/ml) was mixed with 350 μM $K_3Fe(CN)_6$ for 30 s at 0°C and then frozen in liquid N_2. (B) The enzyme was mixed with 35 mM succinate at 20°C and the solution was then frozen after 2 min. An EPR spectrum was recorded (data not shown) after which the tube was thawed and incubated at 30°C for 15 min. The EPR spectrum after this treatment is shown. (C) The enzyme was reduced with excess of solid $Na_2S_2O_4$ for 1 min at 0°C and then frozen. No signal was detected under the present EPR conditions. The tube was then thawed and the contents were incubated with 35 mM fumarate for 15 s at 20°C. EPR conditions: microwave frequency, 9339.3 MHz; temperature, 11.5 K; microwave power, 2.2 mW; modulation amplitude, 0.5 mT, scanning rate, 10 mT/min. The g-value scale is in units of 0.01. Spectra are corrected for differences in gain, tube calibration factors and experimental microwave frequencies.

Results

EPR spectra of the fumarate reductase complex

The EPR spectrum of the cytochrome b-containing fumarate reductase complex is presented in Fig. 1, trace A. Since the preparation did not contain reagents (such as dithiothreitol), that may partly reduce the enzyme, addition of $K_3Fe(CN)_6$ did not affect the spectrum. At this amplification, no further signals could be detected in the field region 0–400 mT.

When the amplification was increased 1000-fold, two more absorption lines could be detected in the sample in the absence of $K_3Fe(CN)_6$: (a) a signal at g = 4.3, usually observed in protein samples, due to a high-spin d^5-ion in a rhombic ligand field and presumably caused by contaminating Fe^{3+}; (b) a faint absorption peak around g = 3.6, probably a g_z peak of cytochrome b. It is known that b-cytochromes can have g_z values in the region g = 3.4–3.8 [7–9]. Addition of $K_3Fe(CN)_6$ did not affect these absorptions, but addition of $Na_2S_2O_4$ abolished the peak at g = 3.6.

The signal in Fig. 1 trace A disappeared when the temperature was increased to 30 K or when $Na_2S_2O_4$ was added. Together with the knowledge that the enzyme contains iron and sulphide [1], it can be concluded that the signal originates from a [4Fe-4S]$^{3+(3+,2+)}$ cluster, although at present a [3Fe-3S] cluster, which seems to have similar EPR properties [10–12], cannot be excluded. The detailed line shape of the signal in Fig. 1, trace A, in particular that between g = 2.01 and 2.018, varied slightly with the preparation. Simulation of the signal as a S = 1/2 system, using a Gaussian shape function, could reproduce the signal quite well, except for the flanks which

are broader in the experimental trace (parameters for closest fit: $g_{x,y,z}$ = 2.0227, 2.0214, 2.0084 and widths (x, y, z) = 1.3, 1.2 and 1.3 mT).

When excess of succinate was added, the signal amplitude decreased by 50%, irrespective of the incubation conditions used (Fig. 1, trace B). Addition of excess $Na_2S_2O_4$ completely removed the signal, but subsequent addition of fumarate caused the reappearence of 28% of the signal (Fig. 1, trace C). Under these conditions a steady state of electron transport from dithionite to fumarate is established. Addition of more $Na_2S_2O_4$ to this mixture had no further effect.

A second effect of succinate addition was the appearance of two new signals that could only be observed well at higher temperatures. The signal of the [4Fe-4S] cluster then disappeared due to lifetime broadening (Fig. 2, trace A). The signal intensities were independent of the incubation conditions with succinate. The radical signal at g = 2.0, that is greatly saturated under the present EPR conditions, is probably due to half-reduced flavin. It is not present when $Na_2S_2O_4$ is used as reductant (Fig. 2, trace B, solid line) or under fully oxidized conditions.

The other lines, around g = 2.035, 1.945 and 1.903, are 2.5-times more intense with $Na_2S_2O_4$ (Fig. 2, trace B) and remain clearly detectable up to 80 K. The g-values as well as the temperature dependence indicate that the signal is due to a [2Fe-2S]$^{2+(2+,1+)}$ cluster. The signal completely disappeared on subsequent addition of fumarate. Addition of more $Na_2S_2O_4$ had no further effect. No other signals could be detected in the magnetic field region 0–400 mT with either succinate or dithionite present, except for a weak g = 4.3 signal only showing up at a 1000-fold amplification.

The signal of the [2Fe-2S] cluster could easily be simulated as one S = 1/2 signal (Fig. 2, trace B, dotted line, and trace C). The position of the g_x line around g = 1.9 and to a lesser extent that of the g_z line varied with the preparation. Two extreme examples encountered during a study of nine different preparations are presented in Fig. 3. There was no apparent difference in the temperature dependence of the power-saturation behaviour.

Stoichiometry of the Fe-S clusters and FAD

The signal of the [4Fe-4S] cluster (Fig. 1, trace A) is of sufficient quality to permit direct double integration for obtaining the spin concentration. This is

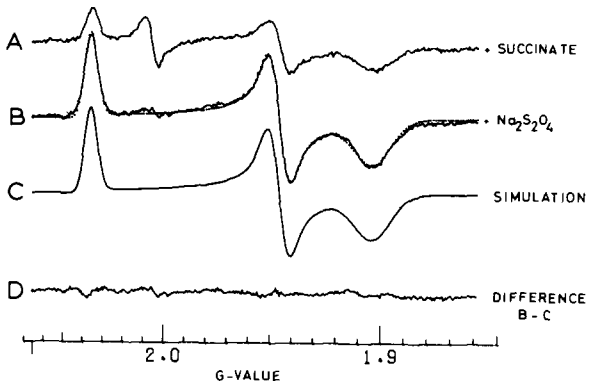

Fig. 2. EPR spectra of fumarate reductase complex at 42 K. (A) Same tube as used for Fig. 1, trace B. (B, ——) Enzyme reduced with excess of solid dithionite for 1 min at 0°C. (B, · · · · · ·) and (C) simulation as an S = 1/2 system using a gaussian line shape function and the following parameters: $g_{x,y,z}$ = 1.9032, 1.9455, 2.0347 and width (x, y, z) = 2.9, 1.46 and 1.07 mT. (D) Difference spectrum (B, ——) minus (C). EPR conditions: microwave frequency, 9339.3 MHz; temperature, 42 K; microwave power, 2.2 mW, modulation amplitude, 0.5 mT, scanning rate, 10 mT min.

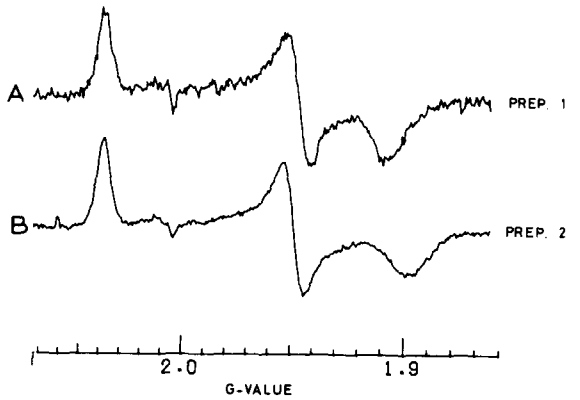

Fig. 3. Fluctuations in EPR line shape of dithionite-reduced preparations of fumarate reductase complex. The preparations were mixed with excess of solid $Na_2S_2O_4$ for 1 min at 0°C. (A) Preparation containing 14 μM FAD and 2.6 mg protein/ml. (B) Preparation containing 15.9 μM FAD and also 2.6 mg protein/ml. EPR conditions: microwave frequency, 9339.5 MHz, temperature, 47 K; microwave power, 2.2 mW; modulation amplitude, 0.5 mT; scanning rate, 10 mT/min.

not true for the anisotropic signal of the [2Fe-2S] cluster (Fig. 3). In this case, each individual spectrum was simulated as in Fig. 2, and the simulation was then used for double integration. A copper standard was used to compute the cluster concentrations. These were compared with the FAD, iron and sulphide contents. The results from nine different preparations are given in Table I. Two types of preparations of the fumarate reductase complex were examined. Preparations 1–6 were analysed as obtained from step 3 of the isolation procedure [1], while preparations 7–9 received additional treatment. This treatment was designed to remove iron and sulphide which is not accounted for by the EPR signals, and to remove dithionite and dithiothreitol which interfered with the measurement of sulphide. The contents of the binuclear cluster varied between 0.55 and 1.29 mol/mol FAD. This variation was paralleled by the contents of the tetranuclear cluster.

The average ratio of the contents of the two clusters was 0.99, while the average contents of the binuclear and the tetranuclear clusters were 0.88 and 0.89 mol/mol FAD, respectively. The variations were not paralleled by the contents of total iron and on average, the iron contents were twice those detected by EPR. The contents of total sulphide were close to those calculated from the EPR signals in preparations 8–9. On average, 90% of the content detected chemically was accounted for by the EPR signals.

Localization of the prosthetic groups

The M_r 79 000 peptide, as well as a preparation of the M_r 31 000 peptide that also contained cytochrome *b*, had been obtained in a sufficient quantity to be studied by EPR. In Fig. 4, the two preparations are compared with the enzyme complex, in a search for the [4Fe-4S] cluster. In the oxidized state, only the M_r 79 000 peptide showed a clear signal compar-

TABLE I

STOICHIOMETRY OF THE PROSTHETIC GROUPS IN FUMARATE REDUCTASE COMPLEX

FAD was determined as previously [1]. Total iron and sulphide were determined chemically as non-heme iron and as acid-labile sulphur as previously [1]. The amounts of EPR-iron and EPR-S^{2-} were obtained by multiplication of the present clusters with their iron and sulphide contents. All the preparations were obtained from step 3 of the purification procedure [1]. Preparations 7–9 have received additional treatment at 0°C under anaerobic condition. The activity of succinate oxidation by methylene blue was not altered by these treatments. Preparation 7 was dialyzed for 15 h against 20 mM Tris-HCl buffer (pH 7.7). Preparations 8a and 9a were filtered through a Sephadex G-25 column which was equilibrated with a buffer (pH 7.7) containing 0.05% Triton X-100/20 mM Tris-HCl/1 mM malonate. Preparation 8b was dialyzed for 30 h against a buffer (pH 7.7) containing 0.05% Triton X-100/10 mM Tris-HCl/1 mM malonate/2 mM EDTA, and subsequently subjected to gel filtration as described above. Preparation 9b was treated in the same way. However, 1 mM EDTA was used in the dialysis. S.D., standard deviation.

Preparation	[2Fe-2S] / FAD	[4Fe-4S] / FAD	[2Fe-2S] / [4Fe-4S]	total iron / EPR-iron	total S^{2-} / EPR S^{2-}
1	0.88	1.03	0.86	2.52	–
2	0.67	0.79	0.85	2.31	–
3	1.04	0.84	1.24	1.94	–
4	0.89	0.84	1.07	2.53	–
5	0.55	0.58	0.96	1.75	–
6	0.63	0.60	1.05	3.11	–
7	1.29	1.25	1.03	0.96	–
8a	1.02	1.02	1.01	1.60	1.16
8b	1.04	0.94	1.11	1.72	1.23
9a	0.63	0.83	0.77	2.27	1.18
9b	1.01	1.02	0.99	1.33	0.86
Mean of prep. 1–9	0.88	0.89	0.99	2.00	–
S.D. of prep. 1–9	0.23	0.20	0.13	0.62	–
Mean of prep. 8–9	0.93	0.95	0.97	1.73	1.11
S.D. of prep. 8–9	0.20	0.09	0.14	0.40	0.17

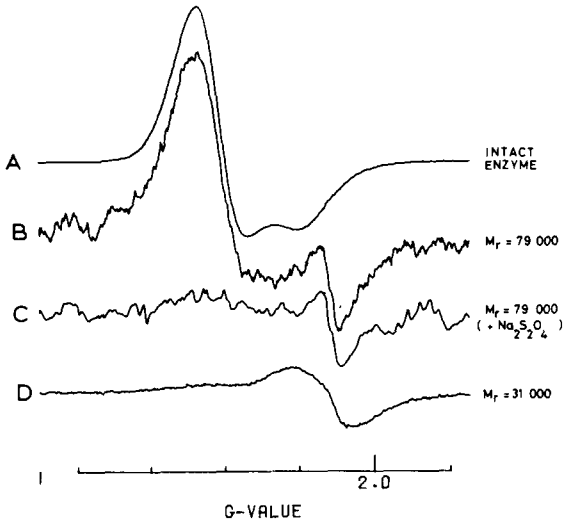

Fig. 4. EPR spectra of the subunits of fumarate reductase in the presence of excess $K_3Fe(CN)_6$. The subunits are compared with the enzyme complex. (A) Spectrum of the fumarate reductase complex (14.6 μM FAD and 2.7 mg protein/ml) mixed with 1 mM $K_3Fe(CN)_6$ for 30 s at 0°C. (B) A preparation of the M_r 79 000 subunit of fumarate reductase (0.35 mg/ml), mixed with 3 mM $K_3Fe(CN)_6$ for 30 s at 0°C. (C) Same preparation as (B) but now reduced with $Na_2S_2O_4$. (D) A preparation of the M_r 31 000 subunit of fumarate reductase also containing cytochrome *b* (5 mg protein/ml), mixed with 5 mM $K_3Fe(CN)_6$ for 30 s at 0°C. EPR-conditions: microwave frequency, 9339.1 MHz; temperature, 7 K; microwave power, 0.26 mW, modulation amplitude, 0.4 mT; scanning rate, 5 mT/min. The relative gains for the traces A, B, C and D were 1, 170, 170 and 6.2, respectively. The *g*-value scale is in units of 0.01.

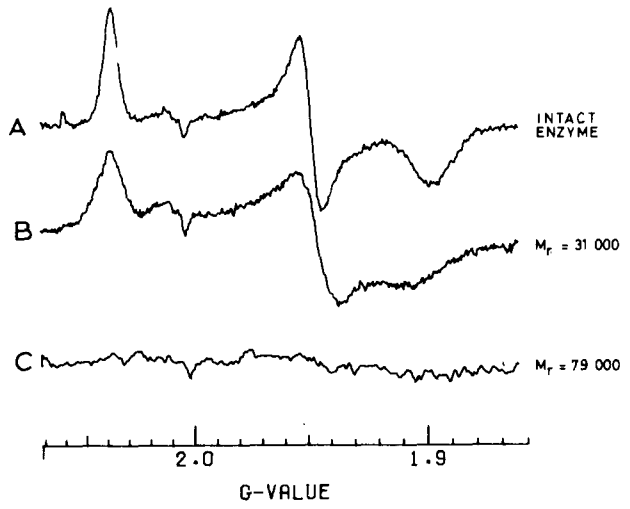

Fig. 5. EPR spectra of the subunits of fumarate reductase in the presence of excess of dithionite. The subunits are compared with the enzyme complex. All preparations were reduced with excess of $Na_2S_2O_4$ for 1 min at 0°C. (A) Spectrum of the fumarate reductase complex (15.9 μM FAD, 2.6 mg protein/ml). (B) Sample of the same preparation as used for Fig. 4, trace D. (C) Spectrum of the same tube as used for Fig. 4, trace C. EPR conditions: microwave frequency, 9334.3 MHz; temperature, 47 K; microwave power, 2.2 mW; modulation amplitude, 0.5 mT; scanning rate, 10 mT/min. The relative gains for the traces A, B and C were 1, 0.98 and 4.9, respectively.

able to that of the enzyme complex. The signal disappeared on addition of $Na_2S_2O_4$ (Fig. 4, trace C) and this treatment left the radical signal at $g = 2$ unchanged. The signal intensity amounted to only 0.023 mol [4Fe-4S] cluster per mol M_r 79 000 peptide. The preparation contained about four iron atoms per peptide molecule.

The dithionite-reduced preparations, in which the signal of the [2Fe-2S] cluster is expected to appear, showed that only the M_r 31 000 peptide containing preparation showed a signal comparable to that of the enzyme complex (Fig. 5 trace B). Although the g_x value had shifted in comparison to the reference enzyme (Fig. 5, trace A), it is within the region of g_x-values found for the various enzyme preparations shown in Fig. 3. Typically, the g_x-value was the same as that of the signal of the enzyme in Fig. 3, trace A.

It can be seen that the line widths of the signal of the M_r 31 000 peptide-containing preparation are greater than those of the signal of the enzyme complex (parameters for best-fitting simulation $g_{x,y,z}$ = 1.9059, 1.9448, 2.0373 and widths (x, y, z) = 5.0, 2.42 and 1.94 mT). This is probably not due to lifetime broadening, since spectra taken at lower temperatures were exactly the same. Also quantification of the signal at 47 and 11 K, using non-saturating microwave powers, gave identical results. The spin concentration represented in this signal amounts to 0.25 mol [2Fe-2S] cluster/mol 31 000 peptide. The preparation contained about 2 mol non-heme iron/mol M_r 31 000 peptide.

Temperature dependence of the power saturation behaviour of the [2Fe-2S] cluster

During the course of this study it was noted that on changing the temperature of a fumarate reductase

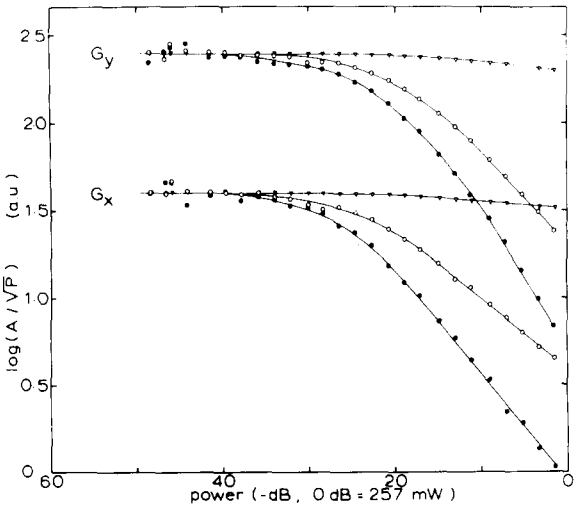

Fig. 6. Power saturation behaviour of the signal of the [2Fe-2S] cluster of fumarate reductase at 11 K. Upper traces: Amplitude of the g_y line; fumarate reductase complex reduced with excess of succinate (●——●); fumarate reductase complex reduced with excess of dithionite (○——○); a preparation of the M_r 31 000 subunit of fumarate reductase reduced with excess of dithionite (▽——▽). Lower traces: Amplitudes of the g_x line. The symbols used are as in the upper traces. Abbreviations: A, signal amplitude; P, incident microwave power in mW, a.u. arbitrary units. For a good comparison the non-saturating, horizontal part of the curves with the same g-values have been shifted such that they coincide.

sample from 50 K to lower values, the signal induced by succinate could be saturated more easily than that induced by dithionite. In contrast, the signal induced by dithionite in the M_r 31 000 subunit-containing preparation could be saturated much less readily than the signal of the dithionite-reduced enzyme. This effect was already observed at 25 K, but was more clearly seen at 11 K (Fig. 6). At 50 K, there was almost no saturation in any of these samples in the power region studied.

Discussion

In a previous report [1] we have mentioned that, from a structural point of view, the fumarate reductase of *V. succinogenes* resembles the succinate dehydrogenase of mitochondria [13] and bacteria [14, 15]. In this discussion, we shall make comparisons with known properties of succinate dehydrogenase.

The properties of the EPR signal of the oxidized fumarate reductase (Fig. 1) are indicative for a [4Fe-4S]$^{3+(3+,2+)}$ cluster and can be directly compared to the analogous signal in mitochondrial complex II (EC 1.3.99.1) [16,17], although the latter signal shows greater line widths [4]. Also the features and temperature dependence of the EPR signal of the reduced enzyme compare well with spectra of [2Fe-2S] clusters, and again resemble the spectrum of reduced succinate dehydrogenase [17–20], although the latter is less anisotropic.

There is about one [2Fe-2S] cluster as well as one [4Fe-4S] cluster present per FAD molecule. The average content of 6.3 mol sulphide/mol FAD corresponds to the content of Fe-S clusters. As the EPR detectable Fe-S clusters can account for 90% of this sulphide, it is very unlikely that there is another functional Fe-S cluster present in the fumarate reductase complex. The proportion of non-heme iron is distinctly higher (10.2 ± 2.5 atoms/FAD molecule) and can not be reduced by gel filtration and complexing agents. The origin and function of the iron which is not bound in the Fe-S clusters is not known. It is likely to be an impurity of the preparation. The finding of one [4Fe-4S] cluster and one [2Fe-2S] cluster/FAD molecule (Table I) in the fumarate reductase complex strengthens the suggestion of resemblance with the membrane-bound succinate dehydrogenase, which also shows EPR signals of one of each cluster/FAD [4,21].

The redox potentials of the clusters in fumarate reductase are lower than those in succinate dehydrogenase, since excess of succinate can only reduce 50% of the 4Fe cluster (Fig. 1) and 40% of the 2Fe cluster (Fig. 2). This also means that the [2Fe-2S] cluster is about 10 mV more negative than the [4Fe-4S] cluster. In succinate dehydrogenase, both clusters are fully reduced with succinate [4,21].

When fumarate was added to the enzyme, previously reduced with excess of dithionite, the [2Fe-2S] cluster was completely reoxidized, whereas the [4Fe-4S] cluster remained reduced by 72%. This shows that the [2Fe-2S] cluster is more rapidly oxidized with fumarate than it is reduced. Since the reduced FAD is the most probable direct donor of reducing equivalents for fumarate, this implies that under these conditions the [2Fe-2S] cluster can more rapidly react with the flavin than with its electron

donor, which might be the [4Fe-4S] cluster or dithionite or both. Reduction of the [4Fe-4S] cluster apparently is faster than the reoxidation of this cluster. As the site of interaction of dithionite with the enzyme is not known, no further conclusions about the possible pathway of electrons can be made at this stage.

As shown in Fig. 5, the M_r 31 000 subunit preparation, which also contains cytochrome *b*, shows the [2Fe-2S] signal. It was previously shown that no sulphide is associated with the M_r 25 000 subunit of cytochrome *b* [1], so that the cluster must be attached to the M_r 31 000 subunit. Only one-fourth of the chemically determined iron is represented in the EPR spectrum of Fig. 5.

The EPR line shape as well as the *g*-values of the cluster in the isolated M_r 31 000 subunit very closely match those of the cluster in the enzyme complex. This means that the symmetry of the direct environment of the cluster has not changed. From recent experiments with succinate dehydrogenase, where the covalently-bound FAD could be partly separated from the EPR detectable, intact [2Fe-2S] cluster, it was concluded that the [2Fe-2S] cluster is attached to the smaller, M_r 28 000 subunit of this enzyme [4]. This is supported by our direct observations with fumarate reductase.

The M_r 79 000 peptide contains the [4Fe-4S] cluster (Fig. 4). Also, in this case, the shape and *g*-values of the signal of the cluster in the isolated peptide are like those of the cluster in the enzyme complex. The signal represents only 2.3% of the amount of iron in the preparation. This indicates that the [4Fe-4S] cluster in fumarate reductase has stability properties similar to the equivalent cluster in succinate dehydrogenase, i.e., the cluster is stable in air in the fumarate reductase complex, but sensitive towards oxygen or $K_3Fe(CN)_6$ in more purified preparations and in the isolated subunit, resulting in a greatly diminished intensity of the characteristic EPR signal.

The temperature dependence of the power-saturation behaviour of the $[2Fe\text{-}2S]^{1+}$ cluster is greater with succinate than with dithionite as reducing agent (Fig. 6). This has also been observed with succinate dehydrogenase [22,4]. It is remarkable that the power-saturation behaviour of the cluster in the isolated M_r 31 000 subunit of fumarate reductase is nearly independent of the temperature in the region 10–50 K. This is probably not due to a decrease of the relaxation time itself. If this had decreased considerably, life-time broadening would be expected to occur at higher temperatures. This was not observed, since the line widths are the same at 11, 25 and 50 K. Also the normalized signal intensity is the same at both 50 and 11 K. Thus, the transfer of excess spin-energy from the cluster to the surrounding ice crystals remains efficient in the free subunit while decreasing the temperature, whereas this process slows down in the enzyme complex. This indicates the existence of a pronounced influence of the association with the M_r 79 000 peptide on this process in the enzyme complex. If this association is in some way perturbed by dithionite reduction, this could easily manifest itself as a change in the temperature dependence of the power-saturation behaviour of the [2Fe-2S] cluster and we interpret the differences in Fig. 6 in this sense.

In comparing all the properties of fumarate reductase of *Vibrio succinogenes* with those of the mitochondrial succinate dehydrogenase, the similarity between the two enzymes is striking. Although they clearly differ in catalytic characteristics, we feel that further comparison of these enzymes in the future could lead to better understanding of their mechanisms of action.

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