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A COMPARISON OF THE RESPIRATORY CHAIN IN PARTICLES FROM *PARACOCCUS DENITRIFICANS* AND BOVINE HEART MITOCHONDRIA BY EPR SPECTROSCOPY

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Summary

A study is presented on the EPR characteristics of the paramagnetic groups in the respiratory chain present in membrane particles of *Paracoccus denitrificans*, the respiratory system of which is very similar to that in submitochondrial particles from beef heart. All paramagnetic prosthetic groups of the mitochondrial system are also found in the bacterial plasma membrane. Their properties suggest that the respiratory groups are embedded in very similar protein environments in the two systems.

Introduction

Spectroscopic data on the prosthetic groups in each of the three phosphorylating enzyme complexes in the respiratory chain of mitochondria do not fit with the commonly made assumption that the enzymically active units of the Complexes I (NADH : Q oxidoreductase, EC 1.6.99.3), III (QH_2: ferricytochrome *c* oxidoreductase, EC 1.10.2.2) and IV (ferrocytochrome *c* : O_2 oxidoreductase, EC 1.9.3.1) contain only one molecule of FMN, cytochrome c_1 or cytochrome *a*, respectively.

It has been shown that NADH : Q oxidoreductase, both as present in beef heart submitochondrial particles and in the purified form, contains only one Fe-S cluster 1 per two Fe-S clusters 2 and per two FMN molecules [1,2]. All Fe-S clusters are needed, however, for a proper functioning of the enzyme [3,4]. For beef-heart mitochondria this situation is further complicated by the

proposed heterogeneity of cluster 1 [1,2], that is not detectable in mitochondria from pigeon heart or yeast [5,6].

An analysis of the EPR signals of QH_2: ferricytochrome *c* oxidoreductase has led De Vries et al. [7] to propose that two separate Fe-S clusters and the cytochromes *b*-566 and *b*-558 are present in a concentration equal to one half that of cytochrome c_1.

A similar situation exists for the ferrocytochrome *c* : O_2 oxidoreductase. Aasa et al. [8] have demonstrated that the EPR signal of one of the copper atoms in the purified enzyme is a superposition of two signals with different line shapes and belonging to paramagnets with different redox potentials. Hagen and Albracht [9] have also observed this heterogeneity.

It is known that the plasma membrane of aerobically grown cells of *Paracoccus denitrificans* is remarkably similar to the inner membrane of mitochondria [10—16], especially with respect to the composition of the respiratory chain. EPR spectra of the NADH : Q oxidoreductase in plasma membranes have been published [12] but nothing is known about the stoichiometry of the Fe-S clusters 1 and 2 in this complex. A signal of a possible Fe-S cluster in the region of the antimycin-sensitive site has not yet been reported. There is no information on the EPR spectrum of cytochrome *c* oxidase except that cytochrome *a* is a low-spin heme with $g_z = 2.87$ [17]. In one case [12] the copper signal was reported absent although enough copper was present to allow EPR detection. Also the relative concentration of the different redox complexes is not known. Other redox components, viz. hydrogenase, formate and lactate dehydrogenases and nitrate and nitrite reductase may be present dependent upon the growth conditions [11].

Recently Ludwig and Schatz [18] have purified a cytochrome *c* oxidase from this bacterium that contains only two subunits of apparent molecular weights 45 000 and 28 000. One mole heme *a* and one mole Cu were detected per 36.5 kg of protein, suggesting that the minimal unit consists of two heme *a* groups and two Cu atoms embedded in the two subunits. The molecule is thus much simpler than the enzyme from mitochondria that contains at least six subunits [19]. If this simplification would also hold for the two other redox complexes of the respiratory chain of *Pa. denitrificans*, these bacterial complexes become attractive for studies on the mechanism of energy-coupled electron transfer.

Before undertaking isolation procedures to test this possibility it is worthwhile to know whether all prosthetic groups of the respiratory chain of mitochondria are present in membrane particles of the bacterium. In addition the EPR line shapes of the Fe-S clusters and the cytochromes are of interest since this very sensitively reflects differences in the direct environment of the paramagnet. This paper deals with these questions. The results extend and refine the earlier conclusion from other studies [10—12] that the prosthetic group composition of the respiratory chain of *Pa. denitrificans* is practically identical to that of beef-heart submitochondrial particles. Only minor differences in the environment of some paramagnets have been observed.

Materials and Methods

Organism and growth conditions

Paracoccus denitrificans NCIB 8944 was grown aerobically at 30°C in the liquid medium described by Chang and Morris [20] with methanol (50 or 100 mM), mannitol (20 or 50 mM) or succinate (50 mM) as carbon and energy source and NH_4Cl as nitrogen source. The nutrient for growth on methanol was supplemented with $NaHCO_3$ (0.05%) plus Difco yeast extract (0.01%) or plus a mixture of trace elements (80 nM $CuSO_4$, 1 μM $CoCl_2$, 2.3 μM NaB_4O_7 and 1.5 μM $ZnSO_4$).

Preparation of membrane fragments

Beef-heart submitochondrial particles were prepared by disruption of mitochondria with ultrasound and subsequent differential centrifugation.

Pa. denitrificans cells were harvested by centrifugation (15 min, 23 000 × *g*). Efficient conversion of cells to spheroplasts was obtained by the method of Witholt et al. [21]. Membrane fragments were prepared from the spheroplasts via an osmotic shock procedure [22] and finally suspended in 50 mM Tris-HCl buffer (pH 7.8).

Spectroscopy

Recording of, manipulations with and simulations of EPR spectra were as previously described [2,23]. Optical spectra were recorded using an Aminco DW-2 spectrophotometer in the dual wavelength mode.

Results

The oxidized state

At T = 45—50 K a signal in the g = 2 region can be clearly detected in mannitol- or methanol-grown cells (Fig. 1). The signal from cells grown on mannitol (trace B) is indistinguishable from that obtained with beef-heart submitochondrial particles (trace C) except for the line at g = 2.3 in trace B which is the g_y line of cytochrome *a*. Membrane particles of methanol-grown cells, trace A, show a number of additional lines in the g = 2.4—2.2 and g = 1.95 region, which may originate from adaptive components that are supposed to function in organisms growing on methanol. If trace B is compared with the simulation in trace D no differences in line shape can be observed (trace F) within the limits of the signal/noise ratio, except for the line at g = 2.3. This means that the EPR-detectable Cu atoms present in the bacterial and in the mitochondrial respiratory chain have the same magnetic (and protein) environment.

At T = 45—50 K additional absorption lines could be observed at g = 6, 4.3 and 2.8 whether or not 0.3 mM $K_3Fe(CN)_6$ was present. These signals are due to a high-spin d^5-ion in a strong axial field, probably a heme, a high-spin d^5-ion in a rhombic field, probably aspecifically bound Fe^{3+}, and cytochrome *a* of cytochrome *c* oxidase, respectively. The g_z peak of cytochrome *a* has been reported previously [17]. Since for a quantification of the complete signal of cytochrome *a*, using the area under the g = 2.8 peak, knowledge of all the three *g*-values is required, we optimalized the condition for detection (Fig. 2). The

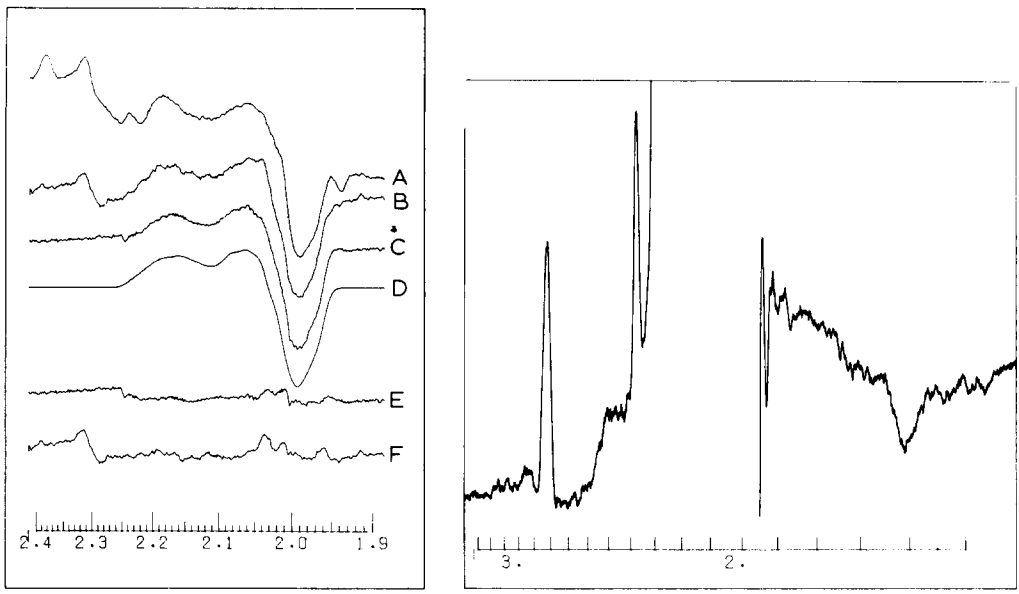


Fig. 1. Comparison of the EPR signals of the copper in cytochrome *c* oxidase as present in membrane particles from *Pa. denitrificans* and in beef-heart submitochondrial particles. A. Membranes particles from *Pa. denitrificans* cells grown on methanol. The spectrum was corrected for a base line recorded with a water-filled EPR tube under the same conditions. B. Membrane particles from *Pa. denitrificans* cells grown on mannitol. The spectrum was corrected for a base line as in A. C. Spectrum of beef-heart submitochondrial particles corrected for a water-base line. D. Line-shape simulation of a spectrum of purified beef-heart cytochrome *c* oxidase recorded at 46 K. Detailed information on this simulation will be given elsewhere (Hagen, W.R. and Albracht, S.P.J., in preparation). All spectra were converted to the same frequency. Their amplitudes were adjusted such that a difference spectrum with trace D gave a minimal residual signal. E. Difference spectrum C minus D. F. Difference spectrum B minus D. The modulation frequency for this and other spectra was 100 KHz. The *x*-axis scale refers to *g*-values. EPR conditions: microwave frequency (F), 9315.0 MHz; temperature (T), 49 K for A and B, 46 K for C; incident microwave power (P), 102 mW for A and B, 65 mW for C; modulation amplitude (MA), 1.25 mT for A and B, 0.63 mT for C; scanning rate (SR), 50 mT/min.

Fig. 2. EPR spectrum of cytochrome *a* in cytochrome *c* oxidase as present in membrane particles of *Pa. denitrificans* grown on methanol. EPR conditions: F, 9332.6 MHz; T, 19 K; P, 25.7 mW; MA, 1.25 mT; SR, 25 mT/min.

approximate *g*-values are 1.61, 2.36 and 2.84 for g_x, g_y and g_z, respectively. The observed widths at half height were 12.3 mT, 5 mT and 4.0 mT for the *x*, *y* and *z* direction, respectively. These parameters are different from those of mitochondrial cytochrome *a* (g_x = 1.446, g_y = 2.213, g_z = 3.034 and widths of 20.5 mT, 7.0 mT and 4.8 mT for the *x*, *y* and *z* direction, respectively [8]). The difference in *g*-values is a direct indication that the ligand field of the Fe^{3+}-ion [24,25] is slightly different in the two proteins. The difference in line widths is merely a consequence of the absolute *g*-values, since the line width in such systems is governed by *g*-strain [26,9]. The mean value for the ratio of the concentrations of Cu and cytochrome *a* is somewhat greater than 1 (Table I) but the accuracy of the present quantifications suggests that the deviation from 1 is not significant. The concentrations are about half the concentration of the total heme *a* determined optically, using the extinction coefficient given in

TABLE I

RELATIVE CONCENTRATIONS OF SOME EPR DETECTABLE GROUPS IN THE RESPIRATORY CHAINS OF BEEF-HEART SUBMITOCHONDRIAL PARTICLES AND MEMBRANE PARTICLES FROM *PARACOCCUS DENITRIFICANS*

Sample	Complex I			Complex II			Complex III	Complex IV		
	Fe-S cluster		Ratio 1/2	Fe-S cluster [j]		Ratio 1/2	Fe-S clusters 1 + 2	Cu	Cyt. *a*	Ratio Cu/ cyt. *a*
	1 [i]	2		1	2					
Submitochondrial particles [a]	0.34	0.69	0.50	1	1.02	0.98	2.44	4.40	4.27	1.03
[σ] [b]	0.04	0.06	0.05	—	0.09	0.08	0.22	0.34	0.34	0.06
Submitochondrial particles [c]		0.45 (FMN)			1 (FAD)		2.32 (cyt. c_1)			
Pa. denitrificans grown on:										
mannitol [d,e]	1.00	0.76	1.32	1	0.73	1.37	2.36	3.05	2.68	1.14
mannitol [e,f]	0.82	0.52	1.58	1	1.57	0.64	6.28	2.40	2.23	1.08
mannitol [f,g]	0.48	0.50	0.96	1	0.99	1.01	1.91	—	1.66	—
succinate [e,f]	0.41	0.40	1.03	1	1.33	0.75	2.83	2.55	1.69	1.51
succinate [e,g]	0.22	0.30	0.73	1	2.06	0.49	1.39	2.77	1.95	1.42
methanol [e,h]	0.77	0.75	1.03	1	—	—	1.03	5.23	3.31	1.58
methanol [e,f]	1.03	0.64	1.61	1	1.29	0.78	5.77	9.61	9.08	1.06
methanol [e,g]	0.65	0.30	2.17	1	0.58	1.72	4.58	8.89	7.04	1.26
mean	0.67	0.52	1.30	1	1.22	0.96	3.27	— [k]	— [k]	1.29
[σ] [b]	0.29	0.18	0.46	—	0.51	0.44	2.02	—	—	0.21

[a] Values are mean of measurements on 10 batches of submitochondrial particles. Data for the Complexes I and II are taken from Refs. 2 and 23.
[b] Standard deviation using N-1 weighting.
[c] Values computed from Ref. 35.
[d] 20 mM nutrient present in growth medium.
[e] 0.01% yeast extract present in growth medium.
[f] 50 mM nutrient present in growth medium.
[g] Mixture of Cu, Co, Zn and B present in growth medium instead of yeast extract.
[h] 100 mM nutrient present in growth medium.
[i] For submitochondrial particles this is the sum of cluster 1a and cluster 1b.
[j] Cluster 1 is defined as the [2Fe-2S] cluster, cluster 2 as the [4Fe-4S] cluster [23].
[k] Mean values are not relevant in these columns since the relative concentration of Complex IV is significantly greater with methanol as a substrate than with mannitol or succinate.

[18]. Since the intensity of the $g = 6$ signal is not altered by reduction of the samples with ascorbate in the presence of 5 mM NaN_3, conditions under which the signals of Cu and cytochrome *a* nearly completely disappear, it does probably not belong to cytochrome *c* oxidase.

At 11 K the signal of the [4 Fe-4 S]$^{3+(3+,2+)}$ cluster of succinate dehydrogenase was clearly observed, together with a readily saturable radical signal (Fig. 3). Addition of $K_3Fe(CN)_6$, that in submitochondrial particles of beef heart causes the appearance of a relatively intense radical signal, due to the semiquinone of Q-10 [23], had no effect. The line shape of the [4 Fe-4 S] cluster (Fig. 3, trace A) is comparable to that of the cluster in purified Com-

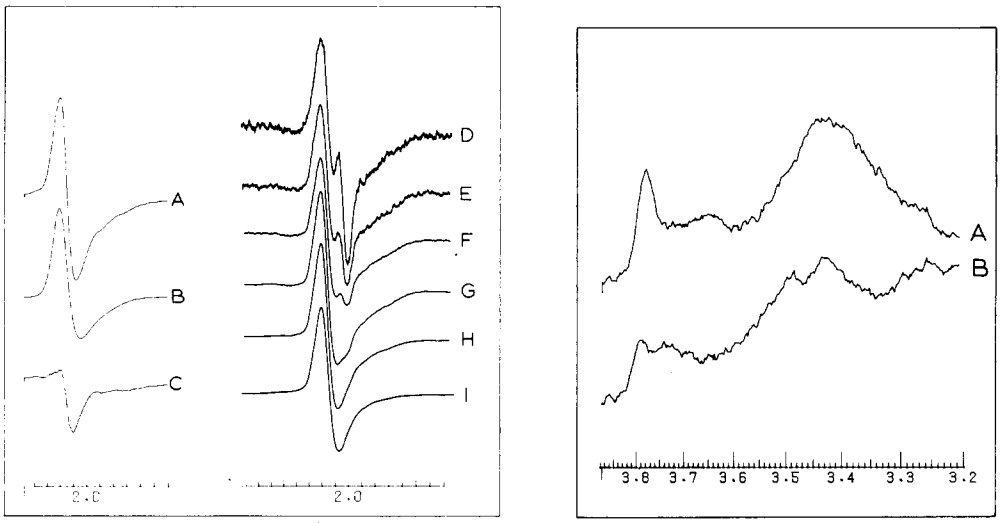


Fig. 3. EPR spectrum of the [4 Fe-4 S] cluster of succinate dehydrogenase as present in *Pa. denitrificans*. A. Membrane particles of cells grown on mannitol. B. Simulation of the [4 Fe-4 S] cluster as present in Complex II isolated from beef-heart mitochondria. For details see [23]. C. Difference spectrum A minus B. The disappearance of the peak at $g = 2.02$ was taken as a criterium for a good subtraction. D—I. Membrane particles of mannitol-grown cells, mixed with 0.3 mM $K_3Fe(CN)_6$ for 30 s at 0° C. The microwave power was increased from D to I, whereas the gain was decreased. For a non-saturating signal, the intensity is proportional to the product of the gain and the square root of the power. The normalized gains calculated accordingly are for D—I, respectively, 1, 1.26, 1.30, 1.12, 1.18 and 1.23. EPR conditions: F, 9317 MHz for A and 9332 for D—I; T, 11 K; P, —10 dB for A and —50, —40, —30, —20, —10 and 0 dB for D-I. The maximal incident microwave power was 257 mW; MA, 1.25 mT; SR, 25 mT/min.

Fig. 4. Comparison of the g_z peaks of the EPR spectra of the cytochrome *b* and c_1 of *Pa. denitrificans* and submitochondrial particles. A. Submitochondrial particles Mg-ATP particles [36]. B. Membrane particles of *Pa. denitrificans* cells grown on mannitol, and mixed with 0.3 mM $K_3Fe(CN)_6$ for 30 s at 0° C. Both spectra are an average of 16 scans corrected for an 16 scan-averaged water-base line. The spectra were converted to the same frequency. EPR conditions: F, 9331.5 MHz; T, 10 K; P, 102 mW; MA, 1.25 mT, SR, 40 mT/min.

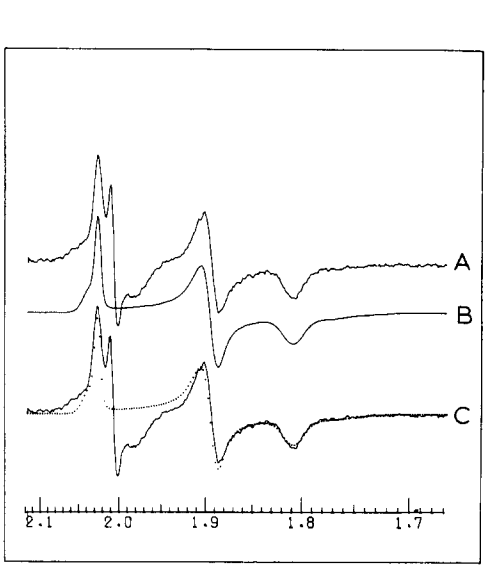
plex II of beef heart (trace B) [23], although no detailed comparison is possible for the broad high-field portion of the signal, due to overlapping signals of Cu, cytochrome *a* and the strongly saturated radical signal (residual signal in trace C). Under none of the conditions used in this study was the Q˙-Q˙ biradical signal [27] observed in paracoccal particles. Submitochondrial particles of beef heart always show this signal especially after reduction with ascorbate.

In the $g = 3$—4 region the g_z lines of the cytochromes *b*-566, *b*-558, *b*-562 and c_1 [28,7] can be detected in submitochondrial particles (Fig. 4, trace A). As these lines are very weak due to the large *g*-anisotropy [29] detection in the present samples was only possible at partly saturating microwave powers, so the relative intensities of the peaks at $g = 3.78$ and 3.43 do not reflect the true concentrations [7] of the several cytochromes. Similar peaks are detectable with membrane preparations from mannitol-grown cells. The concentration of the cytochromes in the present samples are only just within the detection limit of the EPR spectrometer used in combination with a signal averager.

The reduced states

When membrane particles of the bacterium are reduced with ascorbate, the signals of cytochrome *c* oxidase nearly completely disappear and a signal of the Fe-S clusters of the cytochrome b-c_1 complex appears (Fig. 5, trace A). A comparison of the signal with that obtained with beef-heart submitochondrial particles [7] (Fig. 5, trace B) is given in Fig. 5, trace C. Taking into account the signal to noise ratio, the resemblance is surprisingly good. The conclusion must be that the [2 Fe-2 S] clusters 1 and 2 in the beef-heart complex [7] are also present in the respiratory chain of the bacterium in the same 1 : 1 ratio and that the local protein environment is exactly the same in both chains. In addition the same line shape changes in the g_x-region were observed as in submitochondrial particles, when the redox potential was lowered with succinate, NADH or dithionite (not shown).

When membrane particles were incubated with succinate for 15 min at 30°C to ensure full activation of succinate dehydrogenase most components of the respiratory chain were reduced, the resultant spectra being the same as with



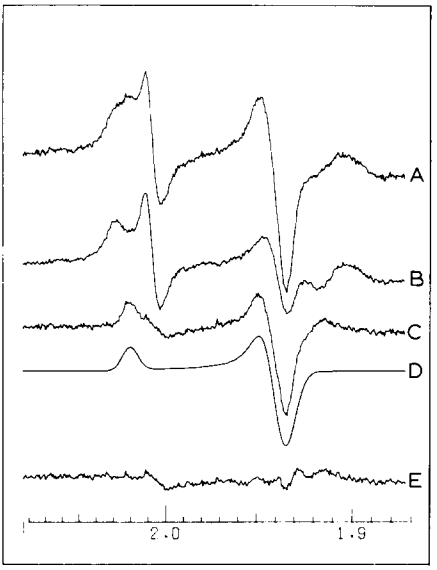


Fig. 5. EPR spectrum of the Fe-S clusters in QH_2 : ferricytochrome *c* oxidoreductase as present in membrane particles of *Pa. denitrificans* cells grown on mannitol. A. Membrane particles treated with 5 mM ascorbate and 5 mM NaN_3 for 90 s at 0°C. The spectrum is an average of 16 scans corrected for a 9 scan-averaged water-base line. EPR conditions: F, 9332.2 MHz; T, 25 K; P, 2.6 mW; MA, 1.25 mT; SR, 50 mT/min. B. Simulation of the corresponding signal in beef-heart submitochondrial particles. For parameters see Fig. 3 or Ref. 7. C. Superposition of A (solid line) and B (dotted line).

Fig. 6. The EPR line shape of Fe-S cluster 1 of NADH : Q oxidoreductase of *Pa. denitrificans*. A. Membrane particles of cells grown on mannitol were mixed with 5 mM NaN_3 and 16 mM succinate and incubated for 15 min at 30°C, after which NADH (3 mM) was added. After 1 min at 30°C the suspensions was frozen in liquid nitrogen. B. Membrane particles mixed with a few grains of solid $Na_2S_2O_4$ for 45 s at 0°C. Both spectra were corrected for a water-base line. C. Difference spectrum A minus B corrected for the difference in tube-calibration factors. D. Computer simulation on basis of the apparent *g*-values ($g_{x\text{-}y}$ = 1.9375, g_z = 2.019) and line widths (widths x-y = 2.2 mT, z = 1.5 mT). E. Difference spectrum C minus D. EPR conditions for A and B: F, 9331 MHz; T, 44 K; P, 2.6 mW; MA, 1.25 mT; SR, 10 mT/min.

samples reduced with NADH plus succinate. This suggests that the membrane particles isolated by the present method still contain enzymes involved in the citric-acid cycle. In beef-heart submitochondrial particles succinate can only reduce about half of cluster 2 of NADH dehydrogenase, and does not reduce clusters 1, 3 or 4. The EPR spectrum at 44 K of reduced membrane particles of mannitol-grown cells (Fig. 6, trace A), which is similar to that published earlier [12], is a superposition of the signals of Fe-S cluster 1 of NADH dehydrogenase, cluster 1 of succinate dehydrogenase, cluster 1 and 2 of the cytochrome b-c_1 complex and a free radical. It differs from that of submitochondrial particles (see e.g. Fig. 2 of Ref. 2) only in that no signal is present from the Fe-S clusters of the mitochondrial outer membranes [30—31].

Although the lineshape of cluster 1 of beef-heart NADH dehydrogenase [1,2] strongly suggests that it is a superposition of two signals, in submitochondrial particles from the yeast *Candida utilis* [6] or in pigeon-heart mitochondria [5] the shape is axial and gives no reason to suppose heterogeneity. It is of interest, therefore, to obtain a line shape of this cluster in the bacterium. Normally this can be easily obtained as a difference spectrum of the respiratory chain reduced with NADH and reduced with succinate [2], but in the bacterial

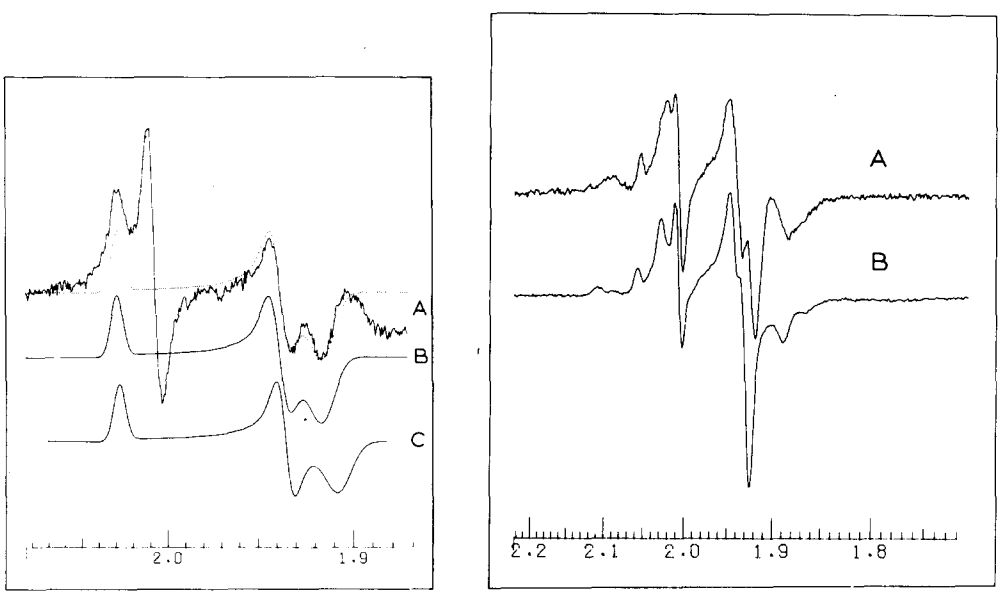


Fig. 7. The estimated EPR line shape of the [2 Fe-2 S] cluster of succinate dehydrogenase of *Pa. denitrificans*. A. (solid line) Difference spectrum of trace B of Fig. 6 minus trace F of Fig. 6. The subtraction factor was estimated such as to obtain a line shape in the g = 1.90—1.95 region resembling that of the mitochondrial enzyme. A (dotted line) and B: approximate simulation. Parameters used: g_x = 1.9155, g_y = 1.9381, g_z = 2.0277 and widths 22.7 mT, 1.6 mT and 1.16 mT for the x, y and z direction, respectively. C. Simulation of the line shape of the beef-heart enzyme. Parameters (from Ref. 23): g_x = 1.9074, g_y = 1.9351, g_z = 2.0264 and widths 24.3 mT, 1.4 mT and 1.16 mT for the x, y and z direction, respectively.

Fig. 8. Comparison of EPR spectra at 8 K of fully reduced membrane particles of *Pa. denitrificans* and beef-heart submitochondrial particles. A. Same sample as used for Fig. 6, trace A. B. Beef-heart submitochondrial particles were incubated with 50 mM succinate for 15 min at 30°C, after which NADH (4 mM) was added and after 30 s at 0°C the suspension was frozen in liquid nitrogen. The spectra were converted to the same frequency. EPR conditions: F, 9331.6 MHz; T, 8 K; P, 60 μW; MA, 1.25 mT; SR, 25 mT/min.

preparations the two substrates give the same degree of reduction. However, the membrane preparation of the bacterium behaved towards incubation with $Na_2S_2O_4$ in the same way as submitochondrial particles in that reduction of the clusters 1, 3 and 4 of NADH dehydrogenase is much slower than that of the other components. A spectrum is displayed in Fig. 6, trace B, where much less of cluster 1 of NADH dehydrogenase is detected. A difference spectrum then gives the line shape of this cluster (Fig. 6, trace C). Its shape is axial as can be confirmed by comparison (trace E) with a simulation (trace D). The simulation was used to estimate the concentration of the cluster in spectra such as Fig. 6, trace A.

The line shape of cluster 1 of succinate dehydrogenase (EC 1.3.99.1) can now be estimated by subtracting the shape of cluster 1 of NADH dehydrogenase (Fig. 6, trace C) from Fig. 6, trace B. This is shown in Fig. 7, trace A. When simulated (Fig. 7, trace A, dotted line and trace B) it was found that the g_z- and g_y-values were nearly identical to those of the cluster from beef heart, but that the g_x-value is considerably shifted, indicating that the shape is less rhombic. For comparison the line shape of cluster 1 of beef-heart succinate dehydrogenase [23] is given in Fig. 7, trace C. The protein environment in the cluster of the bacterium is thus slightly different from that of beef heart. The simulation in trace B is not optimal, which means that the intensity of the signal as determined with the help of the simulation is not very accurate. The g-values are, however, reliable. The overlapping signal of the Fe-S clusters of QH_2 : ferricytochrome c oxidoreductase makes a further refinement at this stage impossible. A purified preparation of succinate dehydrogenase would overcome these problems.

The line shape of cluster 1 of succinate dehydrogenase, as obtained from Fig. 6, trace A, by appropriate subtraction of the shape of cluster 1 of NADH

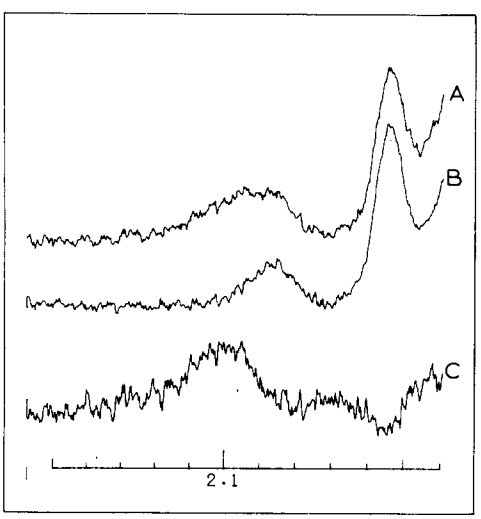


Fig. 9. The g_z lines of Fe-S cluster 3 of NADH : Q oxidoreductase and electron-transferring flavoprotein : Q oxidoreductase of *Pa. denitrificans*. A. Same sample as used for Fig. 6, trace A. B. Same sample as used for Fig. 6, trace B. C. Two times enlarged difference spectrum A minus B. Both spectra are corrected for a water-base line. EPR conditions: F, 9326 MHz; T, 8 K; P, 0.26 mW; MA, 1.25 mT; SR, 5 mT/min.

dehydrogenase (Fig. 6, trace C), is identical to that displayed in Fig. 7, trace A within the signal to noise limits. This means that the position of the g_x line is independent of the nature of the reductant, succinate or dithionite. This resembles cluster 1 in the respiratory chain of *Candida utilis* [23,6], but is unlike the cluster in the membrane-bound beef-heart enzyme, where the g_x shifts noticeably to lower field when dithionite is added to succinate-reduced particles [23]. We have not examined whether the temperature dependence of the spin-lattice relaxation is different with succinate and dithionite, as is the case in submitochondrial particles of *C. utilis* and beef heart [23].

Spectra of reduced particles from the bacterial plasma membrane and from beef-heart mitochondria at 8 K and non-saturating power are shown in Fig. 8. The difference in the overall shape is partly due to a shift to higher field of the *g*-values of cluster 2 of the bacterial NADH dehydrogenase. The main reason is, however, that the relative intensities of the several signals contributing to the spectra are different. In the bacterium there is more cluster 1 of NADH dehydrogenase relative to cluster 2 than in the beef-heart system. Also there is more electron-transferring flavoprotein : Q oxidoreductase [32,33] in the bacterium and this adds to the difference in shape in the regions at $g = 2.08$, 1.94 and 1.89. Thus the broad peak around $g = 2.09$ is a superposition of the g_z peak of the Fe-S cluster in electron-transferring flavoprotein : Q oxidoreductase at $g = 2.086$ and that of cluster 3 of NADH dehydrogenase at $g = 2.10$ as can be seen in detail in Fig. 9. A short incubation with $Na_2S_2O_4$ reduces the cluster of the former enzyme but not cluster 3 of the latter.

At greater microwave powers a difference is observed in the relaxation behaviour of the clusters 3 and 4 of NADH dehydrogenase of *Pa. denitrificans* and mitochondria (not shown). Whereas in the beef-heart enzyme the signal of cluster 3 ($g_x = 1.88$, $g_y = 1.92$—1.94, $g_z = 2.10$) remains visible at high power and the signal of cluster 4 ($g_x = 1.86$, $g_y = 1.92$—1.94, $g_z = 2.04$) disappears [34,1], the opposite is true for the enzyme in *Pa. denitrificans*. Thus although both clusters are present without a noticeable difference in their *g*-values (at the present resolution), the spin-lattice relaxation is quite different, indicating that although the protein structure in the vicinity of the cluster has the same magnetic symmetry, the heat transport to the protein surface must be different.

We could confirm the optical observations of John and Whatley [11] concerning the close similarity in optical spectra of membrane particles from *Pa. denitrificans* and submitochondrial particles (spectra not shown).

Stoichiometry of the paramagnets

The concentration of the paramagnets was estimated by the methods described earlier [2,23]. For cluster 1 of NADH dehydrogenase a comparison of experimental spectra under the proper conditions with the simulated line shape (Fig. 6, trace D) was used. The same method was applied for both the [2 Fe-2 S] and [4 Fe-4 S] clusters (Fig. 7, trace B and Fig. 3, trace B, respectively) of succinate dehydrogenase, for the clusters 1 and 2 of the cytochrome b-c_1 complex (Fig. 5, trace B) and for the copper of cytochrome *c* oxidase (Fig. 1, trace D). For cluster 2 of NADH dehydrogenase the area of the low-field half of the g_z line was used [2]. The cytochrome *a* concentration was

determined using the area under the g_z line. The quantifications on the beef-heart mitochondrial respiratory chain were carried out in the same way using the appropriate simulated line shapes. In this case ten different batches of sub-mitochondrial particles, that have also been used in [2,23], were examined. For *Pa. denitrificans* each membrane particle preparation was obtained from batch cultures grown under different conditions to see if the concentration of the various paramagnets is sensitive to growth condition. For comparison data estimated by Beinert [35] for beef-heart submitochondrial particles, based on the amounts of flavin and cytochrome c_1 are included.

It can be seen in Table I that relative concentrations of the Complexes I, II and III, calculated from the EPR data on the beef-heart system agree quite well with those calculated from the flavin and cytochrome c_1 contents [35]. The internal stoichiometry of NADH- and succinate dehydrogenase have been presented in more detail in previous work [2,23]. The relative concentrations of the complexes in the bacterium are comparable to those in submitochondrial particles. The oxidase content seems to be nutrient dependent, being greater in methanol-grown cells. Although the amount of Complex I relative to that of Complex II is quite constant, the concentration of Complex III scatters considerably irrespective of the nutrient. The reason for this is not clear. Under all conditions there is more electron-transferring flavoprotein : Q oxidoreductase in the bacterium than in beef-heart mitochondria (not shown).

In considering the internal composition of the complexes it is striking that the concentration ratio of the clusters 1 and 2 in NADH dehydrogenase in the bacterium is considerably greater than in the beef-heart enzyme [1,2]. The prosthetic group composition of succinate dehydrogenase in the bacterium is not significantly different from the enzyme in beef heart (Table I). The same holds for the cytochrome b-c_1 complex (Fig. 5) and cytochrome c oxidase (Table I).

Discussion

NADH : Q oxidoreductase

As already reported by Meijer et al. [12] the EPR spectrum at $T \leqslant 20$ K of reduced membrane particles of the bacterium qualitatively resembles that of submitochondrial particles from beef heart. The present study shows, however, that many details differ significantly.

The signal of cluster 1 is of an axial type (Fig. 6) with no suggestion of heterogeneity, in contrast to the cluster in beef-heart submitochondrial particles [1,2], but in agreement with some other mitochondrial respiratory chains such as those from the yeast *Candida utilis* [6] or pigeon heart [5]. The signal shape and peak positions of the clusters 2, 3 and presumably also 4 are similar to those of the beef-heart enzyme [1], but the relaxation behaviour of the clusters 3 and 4 differ. The EPR line shapes and g-values of these clusters suggest that the immediate protein environment of the respective clusters in the enzymes from both sources is precisely the same, forming a ligand field of the same strength and symmetry. The difference in relaxation behaviour suggests that the protein structure of the molecule as a whole is, however, different in the two enzymes, resulting in an overall change in the drain of excess spin

energy absorbed by the Fe-S clusters to the cooling bath on the periphery of the molecule. A different subunit composition might well be the reason. This possibility is strengthened by the major difference between the two enzymes, namely the stoichiometry of the clusters 1 and 2, which is significantly higher in the bacterial enzyme (Table I). In addition, the concentration of cluster 3, estimated from the area of the g_z peak at $g = 2.10$ (Fig. 9, trace C) is similar to that of both the clusters 1 and 2. These findings suggest that the enzyme in *Pa. denitrificans* might indeed be of a basically different architecture than the beef-heart mitochondrial enzyme.

Succinate : Q oxidoreductase

The succinate dehydrogenase in the bacterial enzyme is very similar to that in beef heart so far as the EPR spectra are concerned. In contrast to enzymes bound to beef-heart mitochondrial inner membranes [23] but in agreement with that in *Candida utilis* [6] the line shape is the same with succinate as with dithionite as reductant. It is not certain whether the acceptor site of the enzyme is the same as in beef heart. We could not detect a $Q^{\cdot}$-$Q^{\cdot}$-biradical signal in the paracoccal particles under any of the conditions used in this study. In beef-heart particles this signal is specifically dependent on the presence of Q-10 and Complex II [27].

QH_2 : ferricytochrome c oxidoreductase

The cytochromes *b* and c_1 in the membrane particles of *Pa. denitrificans* are only just detectable in EPR spectra (Fig. 4), but their appearance seems to be similar to those of beef heart. The signal to noise ratio is too low for an estimate of their concentration. The overall EPR line shape of the Fe-S clusters is indistinguishable from that of beef-heart submitochondrial particles under the same conditions (Fig. 5), except for a minimal difference in the value of g_y. The bacterium contains two different [2 Fe-2 S] clusters in equal amounts with a protein environment that is exactly the same as in the mitochondrial enzyme.

Ferrocytochrome c : O_2 oxidoreductase

The copper signal is indistinguishable from that of beef-heart submitochondrial particles (Fig. 1). The cytochrome *a* signal (Fig. 2) reveals less anisotropy, the line widths are smaller and the relaxation is less rapid. The signal intensity of the copper and the cytochrome *a* are about the same (Table I). The heme *a* concentration estimated from optical spectra, using the extinction coefficient determined in [18] is at least 1.6 times greater than that calculated from the EPR spectra, suggesting that, as with the mitochondrial enzyme, only part of the heme *a* and Cu is EPR detectable. The purified enzyme is needed for more definite quantitative results.

Summarizing it can be said that all the EPR-detectable prosthetic groups of the respiratory chain of beef-heart submitochondrial particles are present in *Pa. denitrificans*. The two systems are thus very similar, although detailed magnetical properties sometimes differ significantly, suggesting differences in the protein part of the different complexes. The signal of the $Q^{\cdot}$-$Q^{\cdot}$-biradical [27] was not observed.

Ludwig and Schatz [18] reported that the purified cytochrome c oxidase of

the bacterium contains only 2 subunits, in contrast to the beef-heart enzyme that contains at least 6 [19]. The present study shows that this has hardly any effect upon the immediate protein environment of the EPR-detectable groups in the enzyme. Together with the fact that microorganisms are much better amenable for many purposes, this makes the bacterial enzyme highly attractive for further studies of cytochrome oxidase. The same possibly holds for the other complexes of the respiratory chain.

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