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THERMODYNAMIC AND EPR CHARACTERIZATION OF MITOCHONDRIAL SUCCINATE-CYTOCHROME *c* REDUCTASE-PHOSPHOLIPID COMPLEXES

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SUMMARY

Succinate-cytochrome *c* reductase can be easily solubilized in a phospholipid mixture (1:1, lysolecithin:lecithin) in the absence of detergents. The resulting solution contains two *b* cytochromes with half-reduction potentials of 95 ± 10 mV (b_{561}), and 0 ± 10 mV (b_{566}) and cytochrome c_1 ($E_{m\,7.2} = +280\pm5$ mV). The oxidation-reduction midpoint potentials obtained by optical potentiometric titrations are identical to those determined by the EPR titrations and are 40–60 mV higher than the corresponding midpoint potentials of these cytochromes in intact mitochondria. In contrast to detergent-suspended preparations, no CO-sensitive cytochrome *b* can be detected in the phospholipid-solubilized preparation or intact mitochondria. The half-reduction potential of cytochrome b_{566} is pH-dependent above pH 7.0 (−60 mV/pH unit) while that of b_{561} is essentially pH-independent from pH 6.7–8.5, in contrast to its pH dependence in intact mitochondria. EPR characterizations show the presence of three oxidized low-spin heme-iron signals with *g* values of 3.78, 3.41 and 3.37. The identification of these signals with cytochromes b_{566} (b_T), b_{561} (b_K) and c_1 respectively is made on the basis of redox midpoint potentials. No significant amounts of oxidized high-spin heme-iron are detectable. In addition, the preparation contains four distinct types of iron-sulfur centers: S_1 and S_2 ($E_{m\,7.4} = -260$ mV and 0 mV), and two iron-sulfur proteins which are associated with the cytochrome b-c_1 complex: Rieske's iron-sulfur protein ($E_{m\,7.4} = +280$ mV) and Ohnishi's Center 5 ($E_{m\,7.4} = +35$ mV).

INTRODUCTION

The succinate-cytochrome *c* reductase isolated from pigeon breast mitochondria by the use of a mixture of ionic (deoxycholate) and non-ionic (Triton X-100) detergents, contains two *b* cytochromes and cytochrome c_1 [1]. Room-temperature spectrophotometric measurements show that one of the *b* cytochromes, b_K, exhibits a

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single symmetric α-absorption peak at 561 nm, a single β peak at 528 nm, a Soret maximum at 428 nm, and has a half-reduction potential of +55 mV at pH 7.0 (see also ref. 2). The other *b* cytochrome, b_T, has a double α peak with a maximum at 566 nm and a pronounced shoulder at 558 nm, a double β peak at 530 nm and 538 nm and a Soret band at 431 nm. Its half-reduction potential is −40 mV at pH 7.0. Cytochrome c_1 (α peak 554 nm, β peak 522 nm) has a half-reduction potential of 245 mV at pH 7.0. Since these spectral and thermodynamic properties of the reductase preparations are virtually identical to those in the intact mitochondria, it was considered a useful model system for further investigations.

The potentiometric titration technique, in combination with rapid freezing, has proven very useful in evaluating the electron paramagnetic resonance (EPR) properties of cytochromes [3] and iron-sulfur protein components [4] of the respiratory chain. Our previous efforts to resolve the oxidation-reduction potential dependence of the *b* and c_1 cytochromes of the reductase preparation were unsuccessful [2]. Orme-Johnson et al. [5, 6] however, utilizing different substrates for selective reduction of various carriers, described the EPR characteristics of the heme components of the b-c_1 segment of the electron transport chain both in the electron transport particles and in the isolated complex II plus III. They observed three low-spin iron signals with *g* values 3.33, 3.44 and 3.78 which, on the basis of their behavior during stepwise reduction, they attributed to cytochromes c_1, b_K and b_T respectively. DerVartanian et al. [7] confirmed these results and, in addition, reported antimycin A-induced changes in the EPR spectra of both *b* cytochromes. The $g = 3.44$ signal of ferricytochrome b_{561} was shifted to $g = 3.48$ and the $g = 3.8$ signal of cytochrome b_{566} "sharpened". Moreover, addition of NO caused an appearance of additional signals with $g = 2.10$, 2.07 and 2.01 values, which were attributed to the degradation products of cytochrome b_{566}.

In this paper, which is a continuation of our previous efforts [1, 8], we report a modification of the succinate-cytochrome *c* reductase preparation which has allowed us to study the complete oxidation-reduction potential dependences of the cytochromes and non-heme iron proteins in this preparation. EPR difference spectra recorded in the appropriate oxidation-reduction potential range provide relatively "clean" EPR spectra of the individual respiratory pigments without interference from other components.

MATERIALS AND METHODS

Succinate-cytochrome c reductase

Succinate-cytochrome *c* reductase was prepared from pigeon breast mitochondria essentially as described previously [1]. The final sediment obtained from the ammonium sulfate fractionation (33 %–45 %) was suspended in 50 mM phosphate buffer pH 7.2, containing a previously sonicated 0.5 % lysolecithin/0.5 % lecithin mixture, and was dialyzed for 24 h against 50 mM phosphate buffer containing no detergents. The dark red, optically clear preparation was used for further experiments at protein concentrations of 20–30 mg protein/ml (≈3 μmol heme *c*/g protein).

During purification, the antimycin A-sensitive durohydroquinone-cytochrome *c* reductase activity increases parallel to the content of cytochromes *b* and c_1 while the succinate-cytochrome *c* reductase activity increases less due to a decrease in

the succinate dehydrogenase to heme ratio (see also Table II and Fig. 5 of ref. 1).

The spectrophotometric characteristics of the *b* and c_1 cytochromes were essentially identical to those described previously [1]. The thermodynamic properties are slightly altered as reported below.

Submitochondrial particles

The thawed pigeon breast mitochondria were washed once in 10 mM phosphate buffer pH 7.2 and once in 100 mM phosphate buffer pH 7.2 and suspended in 0.25 M sucrose-0.002 M EDTA containing 20 mM Tris buffer pH 7.5. The mitochondrial suspension was sonicated for 45 s in a Branson sonifier, debris were spun down for 20 min at $10\,000 \times g$ and the resulting supernatant centrifuged at $100\,000 \times g$ for 90 min. The pellet was washed once in sucrose/EDTA/Tris medium and suspended finally in 50 mM phosphate buffer pH 7.2 at a concentration of 40–50 mg protein/ml.

The potentiometric titrations were carried out by the method of Dutton and coworkers [9–11]. The samples used for the EPR measurements were first adjusted to the desired oxidation-reduction potential in a potentiometric titration vessel, the aliquots transferred anaerobically to 3 mm internal diameter EPR tubes and frozen by immersion in isopentane-methylcyclohexane (5:1) maintained at a temperature close to its freezing point (−192 °C) by liquid nitrogen. The EPR spectra were measured with a Varian E-4 spectrometer equipped with an Air Products LTD-3-110 liquid helium cryostat. The redox mediators used are specified in the figure legends.

Egg lecithin, lysolecithin from egg lecithin, sodium deoxycholate and phenazine methosulfate were products of Sigma Chemical Company (St. Louis, Mo.); Triton X-100 of Rohm and Haas (Philadelphia, Pa.); 2-hydroxynaphthoquinone, phenazine ethosulfate and pyocyanine were purchased from K and K Laboratories (Plainview, N.Y.); and 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene) and duroquinone from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.). All other reagents were of the highest purity available.

RESULTS

Oxidation-reduction potentials of the cytochromes of succinate-cytochrome c reductase in the presence of lysolecithin-lecithin mixture

The midpoint potentials of the *b* and c_1 cytochromes have been previously measured in the preparation suspended in the presence of 0.05 % deoxycholate and 0.05 % Triton X-100 [1]. Values of 245 mV, 55 mV, and −44 mV were obtained for cytochromes c_1, b_{561} and b_{566} respectively, and compared favorably with those of Wilson et al. [2] obtained for the preparation isolated from chicken heart mitochondria. Since the change in environment caused by high concentrations of phospholipids may influence the redox behavior of the respiratory chain components, the complete oxidation-reduction potential dependence of the cytochromes *b* and c_1 in the phospholipid-suspended succinate-cytochrome *c* reductase was redetermined. The midpoint potentials obtained were 270 mV, 95 mV and −10 mV for cytochromes c_1, b_{561} and b_{566} respectively, at pH 7.0; the titration for the two *b* cytochromes is shown in Fig. 1.

The half-reduction potentials of the *b* cytochromes in intact mitochondria are pH dependent in the pH range 7.0–8.5 [2] and become more negative as the pH is made more

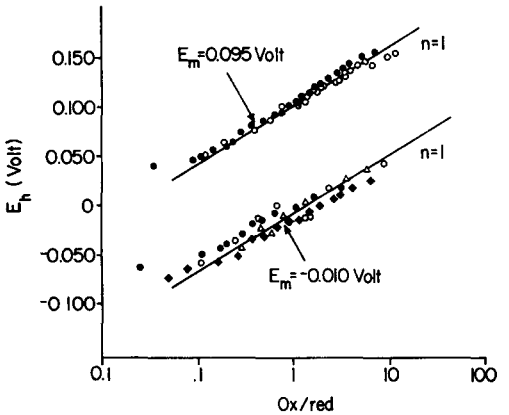

Fig. 1. Oxidation-reduction potential dependence of the *b* cytochromes in purified succinate-cytochrome *c* reductase. The purified succinate-cytochrome *c* reductase was used at a concentration corresponding to 4.1 μM cytochrome c_1 in 50 mM phosphate buffer pH 7.0. The redox mediators used were: 50 μM diaminodurene, 40 μM phenazine methosulfate, 40 μM phenazine ethosulfate, 20 μM duroquinone, 8 μM pyocyanine and 30 μM 2-hydroxynaphthoquinone. The titration was carried out as described by Dutton [11] and Dutton et al. [10] at the wavelength pair 561–575 nm. Open symbols designate the reductive titration, closed symbols the oxidative titration. The sigmoidal curve (not shown) has been resolved into two components as described by Wilson and Dutton [3].

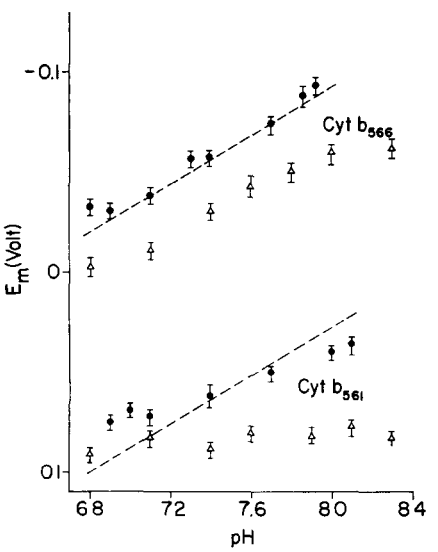

Fig. 2. The pH dependence of the half-reduction potentials of the two *b* cytochromes of succinate-cytochrome *c* reductase. The succinate-cytochrome *c* reductase was suspended either in 0.05 % deoxycholate, 0.05 % Triton X-100 (●) or in 1 % phospholipid mixture (△) at a concentration of ≈0.1 mM cytochrome c_1 (ref. 1 and Methods). For the determination of the half-reduction potentials, it was diluted 25-fold in 50 mM phosphate buffer of appropriate pH. In some experiments, 50 mM morpholinopropane sulfonate (pH 6.5–7.5) or 50 mM Tris (hydroxymethyl) aminomethane (pH 7.6–8.5) were used. The titrations were carried out as described in Fig. 1. Dashed lines represent theoretical slopes of 60 mV/pH unit. At least three determinations were made of each pH value. The error bars represent the scatter in the data.

alkaline: cytochrome b_{566} changes at a rate of -60 mV/pH unit, while cytochrome b_{561} changes at a rate of ≈ -30 mV/pH unit (cf. refs 2 and 12). The pH dependence of the *b* cytochromes of succinate-cytochrome *c* reductase suspended in (a) Triton-deoxycholate mixture and (b) lysolecithin-lecithin mixture, are shown in Fig. 2. The cytochrome b_{566} midpoint potential exhibits a pH dependence of -60 mV/pH above pH 7 in both types of preparations. On the other hand, cytochrome b_{561} is pH-dependent in the preparation of succinate-cytochrome *c* reductase suspended in Triton-deoxycholate mixture (circles in figure), but is essentially pH-independent in the pH range 6.7–8.5 in the preparation suspended in phospholipids (triangles). The pH dependence of the midpoint potential of cytochrome b_{561} in the reductase preparation suspended in Triton-deoxycholate is identical to that in the intact mitochondria, i.e., it changes less than -60 mV/pH unit.

The EPR spectra of the cytochromes of succinate-cytochrome c reductase

The EPR spectrum of succinate-cytochrome *c* reductase at an oxidation-reduction potential of 350 mV is shown in Fig. 3. Two low-spin iron signals are visible with *g* values of ≈ 3.8 and 3.4, in agreement with Orme-Johnson et al. [5, 6] and DerVartanian et al. [7] for the fully oxidized preparation. Broader scans taken at this potential value (not shown) reveal the presence of a signal with a *g* value of 4.3, of similar amplitude to the *g* 3.4 signal. In the best preparations, virtually no *g* 6 signal is detected. The appearance of high-spin heme signals at $g = 6.0$ parallels modification of the cytochromes *b*, e.g., conversion of b_{566} to a form spectrophotometrically indistinguishable from that of b_{561} [1], accompanied by the formation of carbon

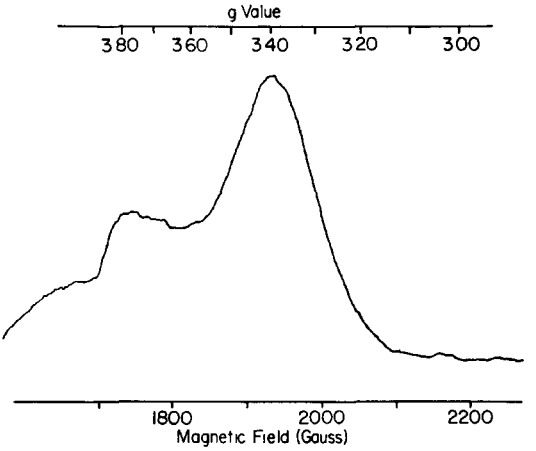

Fig. 3. The EPR spectrum of the succinate-cytochrome *c* reductase at the oxidation-reduction potential of +345 mV. Purified succinate-cytochrome *c* reductase was suspended in 50 mM phosphate buffer pH 7.0 containing 1 % phospholipids at a concentration of 85 μM cytochrome c_1. The redox mediators used were: 80 μM diaminodurene, 60 μM phenazine methosulfate, 60 μM phenazine ethosulfate, 50 μM duroquinone, 12 μM pyocyanine, 40 μM 2-hydroxynaphthoquinone, 6 μM resorufin, 77 μM phenosafranine, 80 μM benzyl viologen and 120 μM methylviologen. The oxidation-reduction potential was adjusted to desired values by the addition of aliquots of freshly prepared solution of sodium dithionite. EPR frequency 9.20 GHz, temperature 10 °K, microwave power 5 mW. Scanning time 4 min. Time constant 1 s.

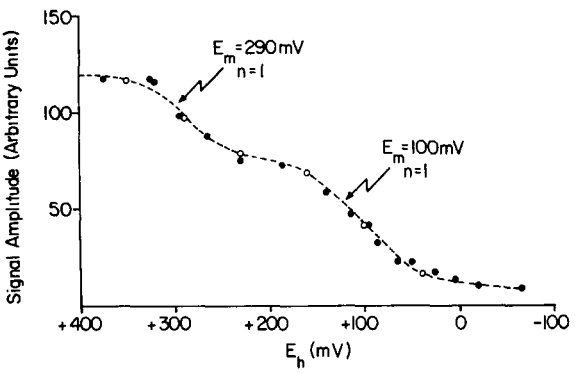

Fig. 4. The oxidation-reduction potential dependence of the $g = 3.4$ signal of succinate-cytochrome *c* reductase. The conditions are as given in Fig. 3.

monoxide sensitivity of the preparation. For instance, treatment with 10 mM KSCN [1] or 0.1 % Triton X-100 for 1 h at 25 °C induced spectral changes in both *b* cytochromes, and caused pronounced sensitivity which paralleled the appearance of the $g = 6$ iron signal (Leigh and Erecińska, unpublished observations).

A complete potentiometric titration in the potential range between +350 mV and −100 mV allows the assignment of the various EPR signals to the individual cytochromes of the succinate-cytochrome *c* reductase. The $g = 3.4$ signal is de facto composed of two EPR signals with slightly differing *g* values. As seen from the plot of Fig. 4, the titration curve can be resolved into two components with *n* values of 1 and midpoint potentials of 295 mV and 100 mV, corresponding to cytochromes c_1 and b_{561} respectively. These values for the midpoint potentials are nearly identical to those obtained by optical measurements. The $g = 3.8$ signal is reduced in the range +50 mV−−50 mV with an effective *n* value of 1 and a midpoint potential of 5 mV (see Fig. 5) indicative of cytochrome b_{566}. The midpoint of cytochrome b_{566} in the optical measurements is −5 mV (±10 mV) for the same pH value.

Since the midpoint potentials of the three cytochromes are at least 100 mV apart, difference spectra taken between samples poised at appropriate potentials can be used to describe the characteristics of the individual components. Typical results

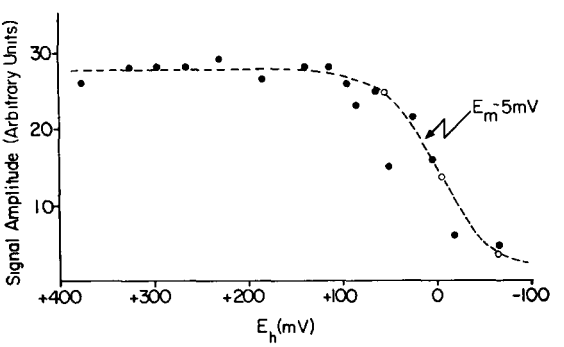

Fig. 5. The oxidation-reduction potential dependence of the $g = 3.8$ signal of succinate-cytochrome *c* reductase. Experimental conditions are the same as those of Fig. 3.

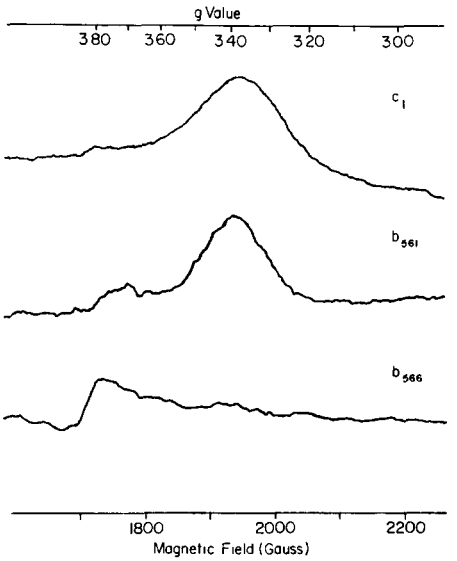

Fig. 6. EPR difference spectra of the individual cytochromes of succinate cytochrome *c* reductase. Conditions are the same as those of Fig. 3. Cytochrome c_1 difference spectrum was taken between samples with potentials of 345 mV and 150 mV, cytochrome b_{561} 150 mV minus +50 mV, and cytochrome b_{566} +50 mV minus −100 mV.

of potentiometric difference spectra are shown in Fig. 6. These results indicate that cytochromes c_1, b_{561} and b_{566} are characterized by g_z values of 3.37, 3.41 and 3.78 respectively.

The EPR spectra of the b and c_1 cytochromes in pigeon breast submitochondrial particles

In order to compare the EPR characteristics of the cytochrome signals in succinate-cytochrome *c* reductase to those in their more "natural" environment, the oxidation-reduction potential dependence of the low-spin heme signals in submitochondrial particles prepared from breast mitochondria were studied. As seen in Fig. 7, at an oxidation-reduction potential of 295 mV, in addition to the low-spin iron signals at $g = \approx 3.8$ and 3.4, an asymmetric peak with g value of 3.0 is visible, attributed to the presence of cytochromes *c* and *a* in the submitochondrial particles (the preparation is partially depleted of cytochrome *c*). Redox titration in the potential range 300 mV−−100 mV indicates the presence of three components with half-reduction potentials and EPR characteristics essentially identical to those identified as cytochromes c_1, b_{561} and b_{566} in the isolated succinate-cytochrome *c* reductase.

The EPR spectra of the non-heme iron components of the succinate-cytochrome c reductase

Results of potentiometric titrations of the iron-sulfur components in isolated succinate-cytochrome *c* reductase over the potential range +350 mV−−400 mV are shown in Fig. 8. Peak-to-peak amplitudes of the principal EPR absorption bands at $g = 1.94$ and 1.90 are plotted as a function of the oxidation-reduction potential.

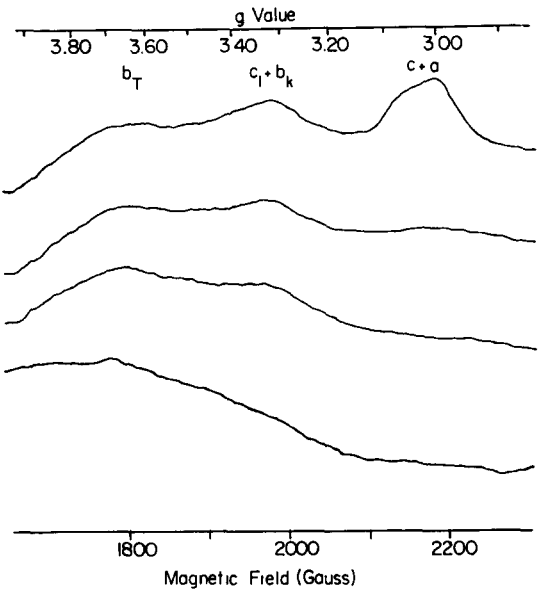

Fig. 7. EPR spectra of the *b* and *c* cytochromes in pigeon breast submitochondrial particles. Pigeon breast submitochondrial particles were suspended in 0.1 M phosphate buffer, pH 7.0 at a protein concentration of 42 mg/ml and anaerobic potentiometric titration was carried out as described in the legend to Fig. 3, Redox potentials for the four curves are (from top to bottom): +295 mV, +145 mV, +85 mV, and −25 mV. EPR spectra obtained with microwave frequency 9.11 GHz, microwave power 10 mW and temperature 8.7 °K. Scanning time 4 min. Time constant 1 s.

The spectrum shown in Fig. 9 is identified as Rieske's iron-sulfur protein [13] and titrates as a one-electron donor/acceptor with half-reduction potential of +300 mV at pH 7.0, in good agreement with a previous estimate from submitochondrial particle studies [3]. At $g = 1.94$ and the same pH value, two components are seen with half-reduction potentials of +35 mV and −255 mV.

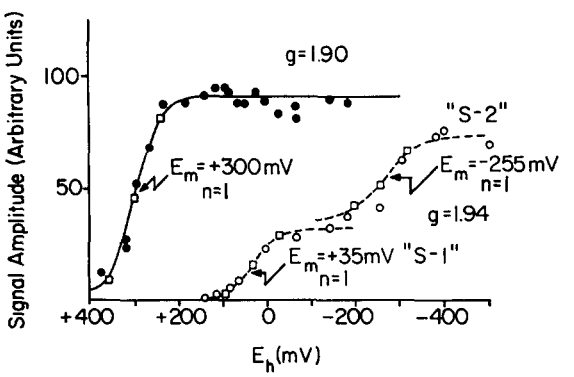

Fig. 8. The oxidation-reduction potential dependence of the non-heme iron signals of succinate-cytochrome *c* reductase. Conditions are the same as those of Fig. 3. Spectra were run with 1.0 mW of microwave power at a temperature of 8.0 °K. Scanning time 2 min. Time constant 1 s.

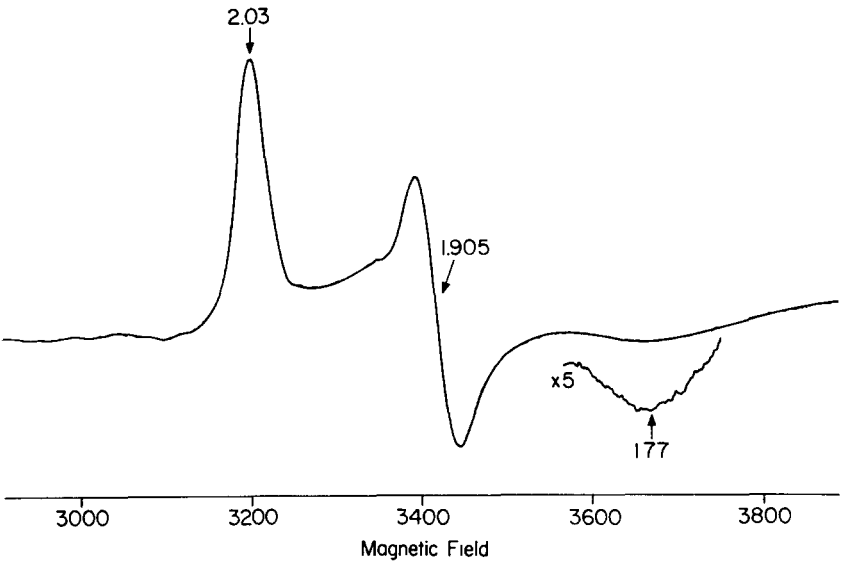

Fig. 9. EPR spectrum of Rieske's non-heme iron protein. Experimental conditions are the same as those of Fig. 3. EPR frequency 9.11 GHz, power 10 mW, at a sample temperature of 15 °K. Scanning time 2 min. Time constant 1 s.

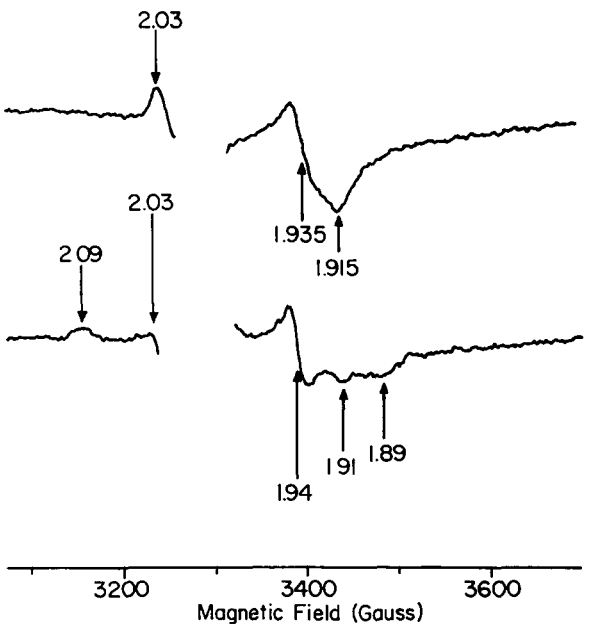

Fig. 10. EPR difference spectra of non-heme iron signals of succinate-cytochrome *c* reductase. Experimental conditions are the same as those given in Fig. 3. The upper spectrum is the difference spectrum between samples with potentials of −350 mV and −150 mV; the lower one is the difference spectrum between samples with potentials of +50 mV and −100 mV. EPR frequency 9.18 GHz, power 10 mW, temperature, 6.3 °K. Scanning time 2 min. Time constant 1 s.

EPR difference spectra of individual iron-sulfur protein components were recorded at 6.3 °K from samples with appropriate redox potentials. Fig. 10A shows the EPR difference spectrum obtained by subtracting the spectrum of a sample poised at a redox potential of −150 mV from a similar sample poised at −350 mV. These potential values were chosen to give the spectrum appropriate for the −255 mV component. This component is seen to be characterized by g_z, g_y and g_x values of 2.03, 1. 945 and 1.91 respectively, and is identified as iron-sulfur protein S_2 from succinic dehydrogenase [4]. Fig. 10B shows an analogous EPR difference spectrum between samples with potentials of −50 mV and 100 mV. Two components are seen in this potential range (−50 mV minus +100 mV) which, although they have virtually identical midpoint potentials, are distinguished by differences in peak positions and temperature dependences. These two components are identified as iron-sulfur protein S_1 with peaks at $g = 2.03$, 1.94 and 1.91; and Ohnishi's center 5 [14] with peaks at $g = 2.09$, 1.94 and 1.89. Comparison of these EPR spectra with those of isolated succinate dehydrogenase and submitochondrial particles indicate a succinate-dehydrogenase/cytochrome c_1 ratio of approximately 1:10.

On the basis of the half-reduction potential values and characteristic EPR spectra, we can identify in the succinate-cytochrome *c* reductase preparation four different iron-sulfur components. Two are associated with succinate dehydrogenase: iron-sulfur centers S_1 and S_2 ($E_{m\,7.4} = -260$ mV and 0 mV; cf. also ref. 4 and 15) and two are associated with cytochromes *b* and c_1: center 5 ($E_{m\,7.4} = +35$ mV; cf. also ref. 14 and 15) and Rieske's iron-sulfur protein ($E_{m\,7.0} = +280$ mV; cf. also ref. 13 and 3).

On the high-spin iron signal of the succinate-cytochrome c reductase

Succinate-cytochrome *c* reductase suspended in the mixture of phospholipids contains practically no high-spin iron signal at $g = 6$ and the absorption spectrum of the reduced preparation is not affected by the addition of carbon monoxide. On the other hand, frozen-thawed preparations suspended in 0.05 % deoxycholate-0.05 % Triton X-100 mixture, exhibit a $g = 6$ signal which increases with increasing storage time. Simultaneously, the disappearance of the absorbance on the long wavelength side of the α absorption band of the reduced cytochrome *b* is noted, in agreement with our previous findings on the "instability" of the *b* cytochromes [1].

DISCUSSION

Our previous studies on isolated succinate-cytochrome *c* reductase [1] resulted in delineation of the spectral and thermodynamic properties of the enzyme. In continuation of these studies, we find that the EPR properties of the preparation are very similar to those of the intact membranes. In agreement with other workers [5–7], we observe three low-spin heme-iron signals with *g* values of 3.78, 3.41 and 3.37, characteristic of cytochromes b_{566}, b_{561} and c_1. Parallel potentiometric optical and EPR titrations indicate that the half-reduction potentials are +280 mV for cytochrome c_1, +100 mV for cytochrome b_{561} and 0 mV for cytochrome b_{566}, identical at 298 ° and 5 °K.

It is perhaps interesting to note that the addition of phospholipids to the succinate-cytochrome *c* reductase causes a shift in the half-reduction potentials of all

the three cytochromes towards more positive values as compared to those in the intact mitochondria and Triton-deoxycholate preparation. Moreover, no pH dependence of the half-reduction potential of cytochrome b_{561} in the pH range 7.0–8.5 could be observed. This indicates that a particular membrane environment may determine the response of a protein to external pH changes either by shielding the active groups or by changing their pK values. Similar differences in the response of half-reduction potentials with respect to pH in the membrane-bound and free form have been noted recently in photoredoxin in photosynthetic reaction centers by Dutton et al. [16]. In contrast to cytochrome b_{561}, cytochrome b_{566} has a pH-dependent half-reduction potential in both types of succinate-cytochrome c reductase preparations.

The EPR signals of the iron-sulfur proteins and their half-reduction potential values are in agreement with those reported by Ohnishi and coworkers [14, 4]. Thus, the iron-sulfur proteins of various preparations exhibit essentially the same paramagnetic properties, a fact worth noting in view of the variety of methods used for their isolation.

Finally, the possible significance of the high-spin iron signal deserves some comment. Since our best preparations contain no g 6 signal, we are inclined to think that its appearance is indicative of modified cytochromes in the preparation. On the basis of our observations on the spectral characteristics of the succinate-cytochrome c reductase [1] and the differences in sensitivity of both b cytochromes to various treatments, we suggest that the high-spin $g = 6$ signal is characteristic of modified cytochrome b_{561}. The basis of our suggestion is the fact that cytochrome b_{566} is modified to a form with a very broad absorption band (liberation of heme from the protein?), mimicking the net disappearance of the absorbance of its reduced form. In contrast, modification of cytochrome b_{561} which accompanies b_{566} modification occurs without major changes in its spectral characteristics. It is manifested in the lowering of the half-reduction potential value of the protein and its carbon monoxide sensitivity; thus, an open 6th ligand position is a reasonable possibility.

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