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THERMODYNAMIC AND EPR CHARACTERIZATION OF IRON-SULFUR CENTERS IN THE NADH-UBIQUINONE SEGMENT OF THE MITOCHONDRIAL RESPIRATORY CHAIN IN PIGEON HEART

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SUMMARY

Several iron-sulfur centers in the NADH-ubiquinone segment of the respiratory chain in pigeon heart mitochondria and in submitochondrial particles were analyzed by the combined application of cryogenic EPR (between 30 and 4.2 °K) and potentiometric titration.

Center N-1 (iron-sulfur centers associated with NADH dehydrogenase are designated with the prefix "N") resolves into two single electron titrations with $E_{m7.2}$ values of -380 ± 20 mV and -240 ± 20 mV (Centers N-1a and N-1b, respectively). Center N-1a exhibits an EPR spectrum of nearly axial symmetry with $g_{//} = 2.03$, $g_{\perp} = 1.94$, while that of Center N-1b shows more apparent rhombic symmetry with $g_z = 2.03$, $g_y = 1.94$ and $g_x = 1.91$. Center N-2 also reveals EPR signals of axial symmetry at $g_{//} = 2.05$ and $g_{\perp} = 1.93$ and its principal signal overlaps with those of Centers N-1a and N-1b. Center N-2 can be easily resolved from N-1a and N-1b because of its high $E_{m7.2}$ value (-20 ± 20 mV).

Resolution of Centers N-3 and N-4 was achieved potentiometrically in submitochondrial particles. The component with $E_{m7.2} = -240\pm20$ mV is defined as Center N-3 ($g_z = 2.10$, ($g_y = 1.93$?), $g_x = 1.87$); the -405 ± 20 mV component as Center N-4 ($g_z = 2.11$, ($g_y = 1.93$?), $g_x = 1.88$). At temperatures close to 4.2 °K, EPR signals at $g = 2.11$, 2.06, 2.03, 1.93, 1.90 and 1.88 titrate with $E_{m7.2} = -260\pm20$ mV. The multiplicity of peaks suggests the presence of at least two different iron-sulfur centers having similar $E_{m7.2}$ values (-260 ± 20 mV); hence, tentatively assigned as N-5 and N-6.

Consistent with the individual $E_{m7.2}$ values obtained, addition of succinate results in the partial reduction of Center N-2, but does not reduce any other centers in the NADH-ubiquinone segment of the respiratory chain. Centers N-2, N-1b, N-3, N-5 and N-6 become almost completely reduced in the presence of NADH, while Centers N-1a and N-4 are only slightly reduced in pigeon heart submitochondrial particles. In pigeon heart mitochondria, the $E_{m7.2}$ of Center N-4 lies much closer to

Abbreviation: FCCP, trifluoromethyl oxycarbonylphenylhydrazone.

that of Center N-3, so that resolution of the Center N-3 and N-4 spectra is not feasible in mitochondrial preparations. $E_{m7.2}$ values and EPR lineshapes for the other iron-sulfur centers of the NADH-ubiquinone segment in the respiratory chain of intact mitochondria are similar to those obtained in submitochondrial particle preparations. Thus, it can be concluded that, in intact pigeon heart mitochondria, at least five iron-sulfur centers show $E_{m7.2}$ values around -250 mV; Center N-2 exhibits a high $E_{m7.2}$ (-20 ± 20 mV), while Center N-1a shows a very low $E_{m7.2}$ (-380 ± 20 mV).

INTRODUCTION

Since the first detection of EPR signals due to iron-sulfur centers in the mitochondrial membrane at temperatures below 77 °K [1, 2], a number of iron-sulfur centers in mitochondria have been detected. Orme-Johnson et al. [3] resolved EPR signals in NADH-ubiquinone reductase (Complex I) [4], using the reductive titration technique of Orme-Johnson and Beinert [5], and assigned them to four different iron-sulfur centers which they designated as Center 1 to Center 4. Ohnishi et al. [6, 7] determined potentiometrically the half-reduction potentials at pH 7.2 ($E_{m7.2}$) of these iron-sulfur centers in yeast [6] and pigeon heart [7] submitochondrial particles, and demonstrated a large potential gap between Center 1 and Center 2. They also pointed out that the Center 1 signal (the "$g = 1.94$" signal associated with NADH dehydrogenase) is composed of two different iron-sulfur centers which were called Center 1a and Center 1b at that time. Center 1a was distinguished from Center 1b by its energy-dependent $E_{m7.2}$ value [8]. More recently, EPR signals which probably arise from two additional iron-sulfur centers have been detected by EPR measurements at temperatures slightly above 4.2 °K in pigeon heart submitochondrial particles [9, 10], NADH-ubiquinone reductase [10, 11], and in the purified NADH dehydrogenase preparations from beef heart [11]. In the present paper, the prefix N- is used to denote those iron-sulfur centers associated with NADH dehydrogenase, and the prefix S- for those associated with succinate dehydrogenase, as proposed recently by Ohnishi et al. [9, 12]. The present report describes the potentiometric resolution and thermodynamic characterization of several iron-sulfur centers in the NADH-ubiquinone segments of the respiratory chain in pigeon heart submitochondrial particles, and presents their resolved EPR spectra. Studies on the functional role of iron-sulfur centers in Site I energy coupling will be reported in the following paper.

MATERIALS AND METHODS

Pigeon heart mitochondrial were prepared by the method of Chance and Hagihara [13]. Pigeon heart submitochondrial particles were prepared according to Lindsay et al. [14] using the Yeda press. $E_{m7.2}$ values of iron-sulfur centers were measured potentiometrically according to Dutton [15]. Pigeon heart submitochondrial particles were stirred under Ar in 0.2 M mannitol, 0.05 M sucrose and 50 mM morpholinopropane sulfonate buffer (pH 7.2) in the presence of appropriate redox mediators. The desired redox potential of the suspension was attained by the addition of aliquots of a freshly prepared dilute solution of dithionite disssolved in oxygen-free 100 mM morpholinopropane sulfonate buffer (pH 7.2). Aliquots of the submitochondrial

particle suspension (about 0.3 ml) were transferred anaerobically into quartz EPR tubes as described by Wilson et al. [16], and frozen rapidly by immersion into a 5 : 1 (v/v) solution of isopentane and methylcyclohexane at 81 °K. Peak to peak amplitudes or peak heights of the same species, measured under the same EPR conditions, were used as the parameters proportional to the concentration of their respective iron-sulfur centers in the reduced form. In the present investigation, no quantitative evaluation of EPR spectra was attempted. In order to attain intensified signals, rather high power settings were adopted and most signals are slightly saturated. However, no line shape modifications due to power saturation were observed within the power range used.

A computer program devised by Martin Pring [17] for the resolution of the redox titration into single-electron transfer components of unknown $E_{m\,7.2}$ and unknown relative signal amplitude by standard non-linear optimization techniques was used. The computer analysis was performed with a Digital Equipment Corporation PDP-10 computer (N.I.H. Biotechnology Resources RR 15).

EPR measurements were performed with a Varian E-4 spectrometer. The temperature of the samples for EPR measurements was controlled by means of a variable temperature cryostat (Air Products Model LTD-3-110). Temperature was measured with a carbon register installed just below the bottom of the EPR sample tube. The g values were corrected using the free radical signals (with known g values) of crystalline diphenylpicrylhydrazyl and also by a commercial weak pitch standard; these were measured separately after recording the sample spectra, under the same EPR settings. The difference between two EPR spectra was obtained using a Nicolet Signal Averager (NIC-1074). EPR sample tubes with matched diameters were used for the performance of difference spectra and redox titrations.

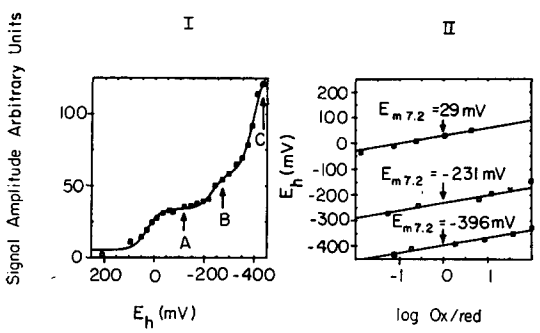

Fig. 1. Computer analysis of the redox titration of the "$g = 1.94$" signal using pigeon heart submitochondrial particles. Pigeon heart submitochondrial particles (26 mg protein/ml) pretreated with trifluoromethyl oxycarbonylphenylhydrazone (FCCP) (0.5 nmol/mg protein) were titrated anaerobically in 0.2 M mannitol, 0.05 M sucrose and 70 mM morpholinopropane sulfonate buffer (pH 7.2). Present as redox mediators were: 63 μM phenazine methosulfate, 63 μM phenazine ethosulfate, 38 μM duroquinone, 9 μM pyocyanine, 8 μM resorufin, 20 μM 2-hydroxy-naphtoquinone, 78 μM phenosafranine, 74 μM benzyl viologen, and 133 μM methyl viologen. EPR operating conditions were: modulation frequency, 100 kHz; modulation amplitude, 12.5 G; microwave power, 20 mW; microwave frequency, 9.05 GHz; time constant, 0.3 s; scanning rate, 500 G/min. The EPR sample temperature was 22.5 °K. (I) Peak-to-peak amplitude of "$g = 1.94$" signal as a function of E_h. The arrows at A, B and C refer to the E_h at which the spectra of Fig. 2 were taken. (II) Resolution of curve I into three $n = 1$ components.

RESULTS

Fig. 1 shows a redox titration of the "$g = 1.94$" signal in pigeon heart submitochondrial particles, in order to resolve the EPR spectra of Centers N-1a and N-1b and to determine their $E_{m7.2}$ values. The titration clearly gives three component curves (Fig. 1-I). Amplitudes of the "$g = 1.94$" signal plotted as a function of E_h show a good fit to the computer-simulated titration curve (Fig. 1-I) which is composed of three single-electron transfer components with $E_{m7.2}$ values of $+29$, -231, and -396 mV, respectively (Figs 1-I and 1-II). The iron-sulfur center with $E_{m7.2} = +29$ mV corresponds to Center S-1 [12, 16] which is associated with succinate dehydrogenase in agreement with previously reported $E_{m7.2}$ values [6, 16]. The "$g = 1.94$" signal associated with NADH dehydrogenase resolves into two components having $E_{m7.2}$ values of -231 and -396 mV. As will be discussed in the follow-

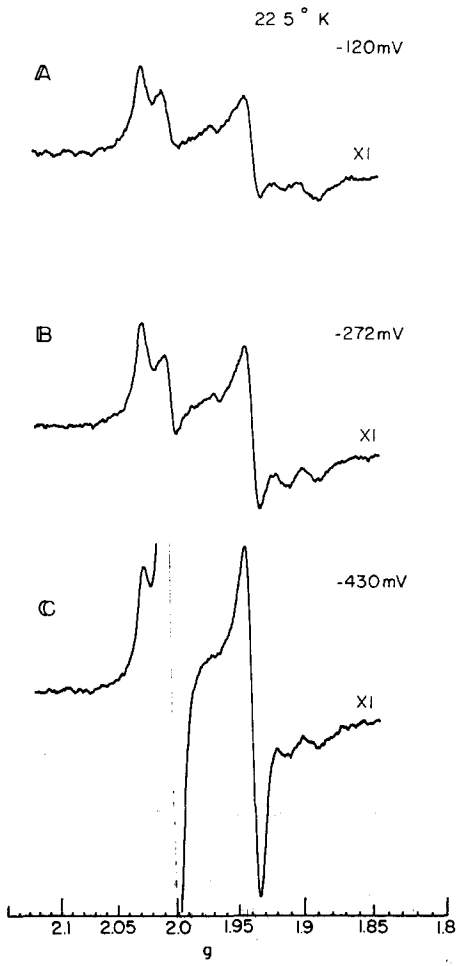

Fig. 2. EPR spectra of iron-sulfur centers observed at three different redox potentials. Experimental conditions are described in the legend of Fig. 1, A, B and C correspond to the same data in Fig. 1.

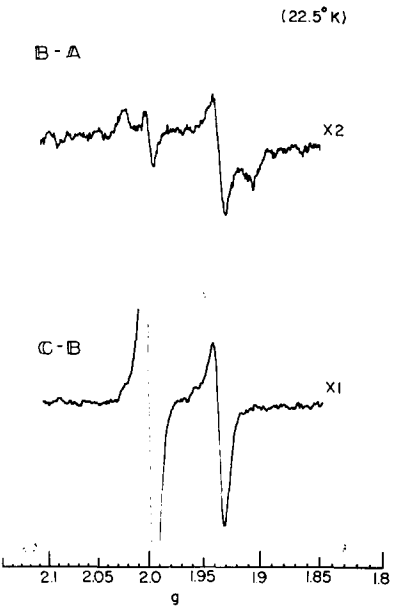

Fig. 3. EPR spectra of iron-sulfur Center N-1b and Center N-1a. Spectra were obtained as difference spectra of B-A and C-B, respectively, from Fig. 2. After input of spectra A, B, and C into Nicolet Signal Averager at the same gain and computing subtractions, difference spectrum B-A was transferred back to the recorder with two times higher gain than that of C-B as illustrated in the figure.

ing paper, Center N-1a is the lower potential species with an ATP-dependent midpoint potential, while the higher $E_{m7.2}$ of Center N-1b does not vary with ATP.

Because of the large difference in their $E_{m7.2}$ values, it is now possible to obtain individual EPR spectra of Centers N-1a and N-1b. The EPR spectra of submitochondrial particle suspensions equilibrated at −120, −272 and −430 mV (illustrated with A, B and C, respectively in Fig. 1-I) are presented in Fig. 2. At −120 mV, Center S-1 and Rieske's iron-sulfur center [19] are almost completely reduced, giving signals at $g_z = 2.03$, $g_y = 1.94$, $g_x = 1.91$; and $g_z = 2.03$, $g_y = 1.90$, $g_x = 1.79$, respectively. In addition to these two iron-sulfur centers, Center N-1b is about 80 % reduced at −272 mV, while Center N-1a is almost completely in the oxidized form. At −430 mV, Center N-1a is also about 80 % reduced. As given in Fig. 3, the

TABLE I

RELATIVE CONTRIBUTION OF THREE SPECIES OF IRON-SULFUR CENTERS TO SIGNAL AMPLITUDE AT "$g = 1.94$" IN PIGEON HEART SUBMITOCHONDRIAL PARTICLES

EPR conditions are the same as described in the legend of Fig. 1.

Iron-sulfur centers	Contribution (%)
$(Fe\text{-}S)_{S-1}$	30±5
$(Fe\text{-}S)_{N-1b}$	25±5
$(Fe\text{-}S)_{N-1a}$	45±5

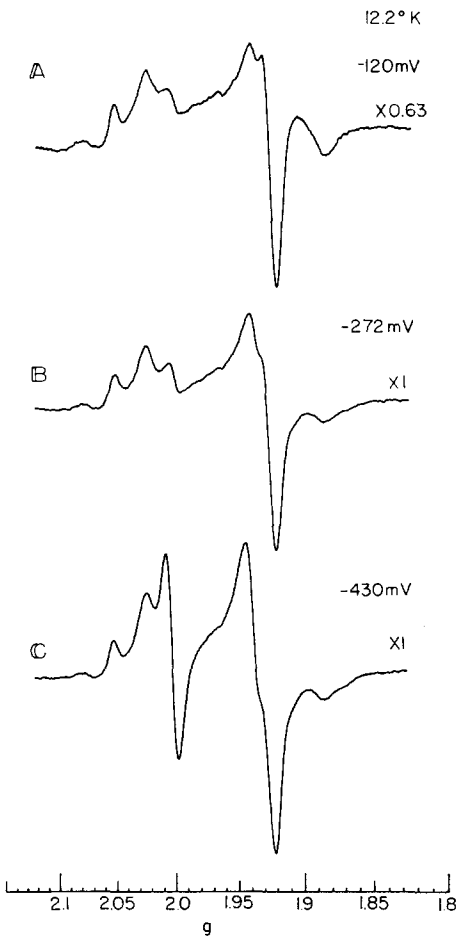

Fig. 4. EPR spectra of iron-sulfur centers observed at three different potentials. Same samples as in Fig.2, measured at a lower temperature (12.2 °K). Experimental conditions as in Fig. 1.

individual spectra of Centers N-1b and N-1a are obtained as the difference spectra of B-A and C-B, respectively. Center N-1b has a spectrum characteristic of rhombic symmetry with $g_z = 2.03$, $g_y = 1.94$ and $g_x = 1.91$, while the spectrum of Center N-1a is reminiscent of axial symmetry with $g_{//} = 2.03$, $g_{\perp} = 1.94$. Although Center N-1b contributes slightly to the spectrum C-B, the lineshape of Center N-1a is not significantly affected. Signals from both Centers N-1a and N-1b can be detected over a wide range of temperatures (< 77 °K). However, temperature profiles of these centers in the lower temperature range (< 20 °K) have not been rigorously studied, because of overlapping signals arising from other centers. The relative contribution of Centers S-1, N-1b, and N-1a to the peak-to-peak amplitude of the "$g = 1.94$" signal at 23 °K and 20 mW power is shown in Table I.

If one examines the same EPR samples as those in Figs 1–3 at 12.2 °K instead of 22.5 °K, the EPR spectra shown in Fig. 4 are obtained in place of those in Fig. 2. Resonance signals from Center N-2 occur at $g_{//} = 2.05$, $g_{\perp} = 1.93$ (Fig. 4A),

approximating axial symmetry. Center N-2 signals start to saturate around 3 mW at 12.2 °K. Signals from "Center 5" [7] in the cytochrome b-c_1 complex can also be seen at $g_z = 2.08$ and $g_x = 1.89$, and overlap with a signal at $g = 1.89$, due to Rieske's center. Under these conditions, EPR resonances of Center S-1 saturate considerably [12] and do not interfere with the titration of Center N-2 monitored by the signal height at $g = 1.92$ (the minimum peak position), relative to the high field base line. The resonance of Center N-2 overlaps with signals from Centers N-1b and N-1a in the $g = 1.93$ region as presented in spectra B and C. However, titration of the Center N-2 signal can be easily separated from that of Centers N-1a and N-1b because of the large difference in $E_{m\,7.2}$ value (see Fig. 5). While the $E_{m\,7.2}$ value for Center N-1a (Fig. 5) is the same as that obtained in Fig. 1, the $E_{m\,7.2}$ values for Center N-1b in the two experiments differ significantly. This discrepancy may be due to the uncertainty of both end-points inherent in the titration curve of any middle component.

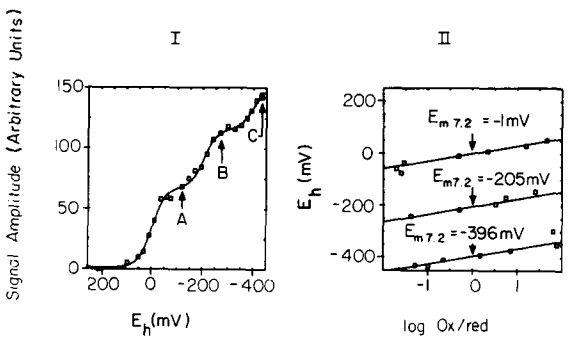

Fig. 5. Computer analysis of the redox titration of the signal amplitude of the principal absorbance. Same samples as in Figs 1–4, but measured at 12.2 °K. Conditions otherwise as in Fig. 1.

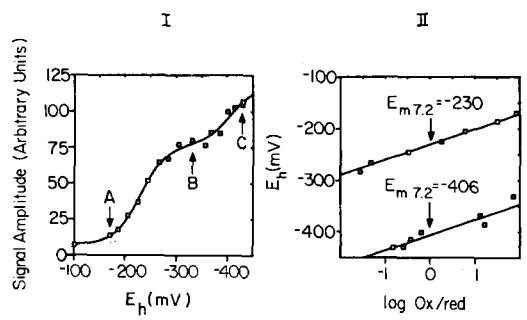

Fig. 6. Computer analysis of the redox titration of Centers N-3 and N-4. Pigeon heart submitochondrial particles at a higher protein concentration (43.6 mg/ml) were used in this and the following titrations. Particle suspension pretreated with FCCP. Redox dyes added: 81 μM phenazine methosulfate, 65 μM duroquinone, 16 μM pyocyanine, 10 μM resorufin, 32 μM 2-hydroxy-naphtoquinone, 150 μM phenosafranine, 142 μM benzyl viologen, 257 μM methyl viologen. EPR operating conditions were the same as in Fig. 1, except the EPR sample temperature was 8.4 °K. (I) Peak heights of $g = 1.87$ signal as a function of E_h. The arrows at A, B and C refer to E_h at which the spectra of Fig. 7 were taken. (II) Resolution of Curve II into two $n = 1$ components.

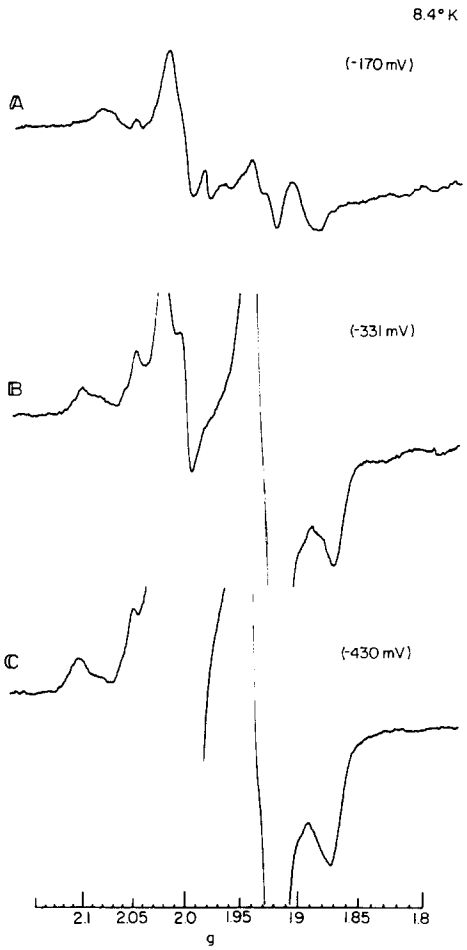

Fig. 7. EPR spectra of iron-sulfur centers observed at three different redox potentials. The same samples as shown in Fig. 6 with arrows A, B and C. Conditions as in Fig. 6.

EPR measurements at 8.4 °K reveal signals due to Centers N-3 and N-4 in addition to the iron-sulfur centers described above. Resolution of the Center N-3 and N-4 resonances has been difficult for the following reasons: (1) The EPR peak positions of these centers lie very close to each other; (2) Their signal amplitudes are small in comparison with those of Centers N-1 or N-2; and (3) Previous methods of temperature regulation using variations in the flow rate of cold He gas and a thermocouple as the temperature detector were not sufficiently sensitive and stable at temperatures below 10 °K. Potentiometric resolution of Centers N-3 and N-4 is described in Fig. 6. Titration of the $g = 1.87$ signal amplitude from the high field base line results in a biphasic redox curve for two single-electron transfer components having $E_{m7,2}$ values of −230 and −406 mV. In Fig. 7, EPR spectra of pigeon heart submitochondrial particle suspension equilibrated at −170, −331 and −430 mV (illustrated as A, B and C, respectively in Fig. 6) are presented. In order to isolate

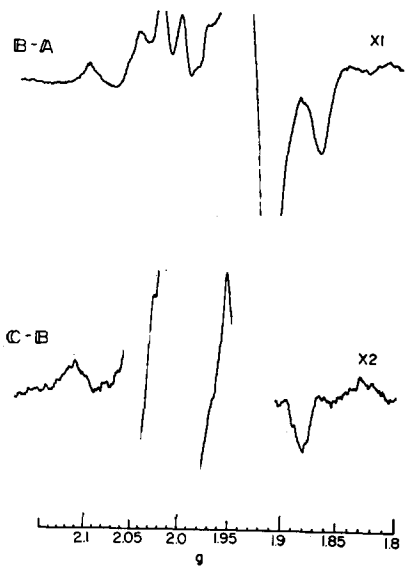

Fig. 8. EPR spectra of iron-sulfur Centers N-3 and N-4. Spectra were obtained as difference spectra of B-A and C-B, respectively, from Fig. 7. Conditions as in Fig. 6.

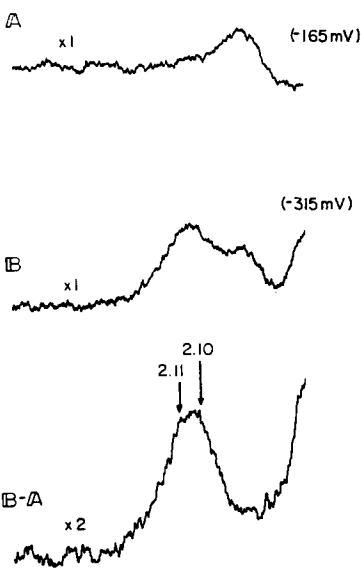

Fig. 9. Low field EPR spectra of Center N-3 plus N-4 in intact mitochodria. System poised at two different redox potentials and the difference spectrum is given. The mitochondrial suspension (40 mg protein/ml) was titrated without FCCP treatment. All other experimental conditions are the same as those in Fig. 6.

the EPR spectra of Centers N-3 and N-4, respective difference spectra B-A and C-B, as in Fig. 8, are obtained. The -230 mV species (N-3) exhibits resonances at $g_z = 2.10$ ($g_y = 1.93$?), and $g_x = 1.87$. Center N-4, whose $E_{m\,7.2} = -406$ mV, shows resonances at $g_z = 2.11$ ($g_y = 1.93$?) and $g_x = 1.88$. Central signal position of

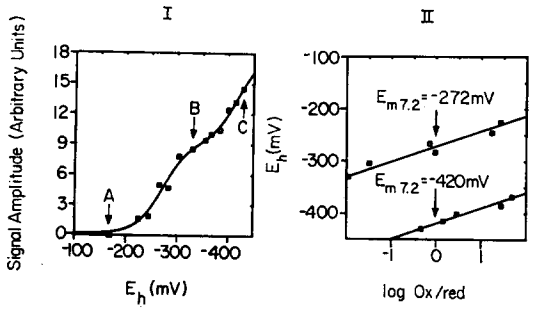

Fig. 10. Computer analysis of the redox titration of Centers N-5 and N-6. Same redox samples of pigeon heart submitochondrial particles as in Fig. 6, here monitored at 4.7 °K. (I) Signal amplitude at $g = 2.11$ was plotted as a function of redox potential, E_h. All other conditions are the same as those described in Fig. 6.

Centers N-3 and N-4 could not be resolved due to overlapping signals from Centers N-1 and N-2. In intact pigeon heart mitochondria, Centers N-3 and N-4 have spectra similar to their spectra in submitochondrial particles. However, both iron-sulfur centers have indistinguishable $E_{m\,7.2}$ values (-245 ± 20 mV) in whole mitochondria; thus, the g_z signal obtained with mitochondria gives double peaks at $g = 2.10$ and 2.11 which arise from Centers N-3 and N-4, respectively (Fig. 9).

Upon lowering the temperature to 4.7 °K, EPR signals at 2.11, 2.06, 1.93, 1.90 and 1.88 emerge. Redox titration of the amplitude of the $g = 2.11$ resonance from the low field base line results in the biphasic curve shown in Fig. 10. This titration curve resolves two single-electron transfer components with $E_{m\,7.2}$ values of -272 and -420 mV. Similar results are obtained when the redox titration is performed by measuring peak-to-peak amplitudes of the major signal in the $g = 1.93$ region or

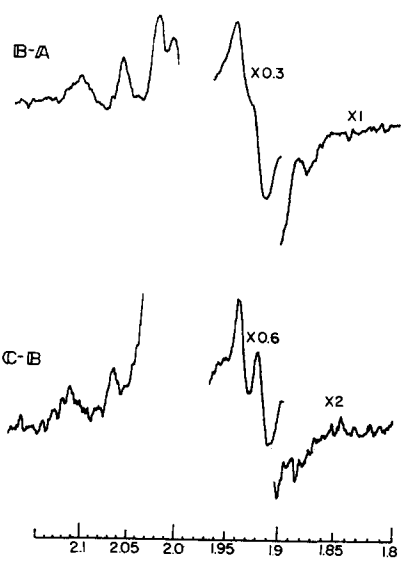

Fig. 11. Resolved EPR spectra of Centers N-5 and N-6. Spectra obtained as the difference spectra of B-A and C-B, in Fig. 10 (I). Conditions as in Fig. 9.

peak heights at $g = 1.88$ from the high field base line. EPR spectra of the -272 and -420 mV centers (difference spectra B-A and C-B) are shown in Fig. 11. As seen in spectrum B-A, the amplitudes of the $g = 2.11$, 2.06, 2.03, 1.93, 1.88 peaks and the $g = 1.90$ shoulder are almost maximal at -331 mV. Therefore, these signals cannot be sorted out in the basis of different midpoint potentials. Signals detected at $g = 2.11$ and 1.88 at this temperature appear not to originate from Center N-4, because of their difference in $E_{m7.2}$ values. $g = 2.11$ and 1.88 signals exhibit slightly different temperature and power dependence; at 4.7 °K, $g = 2.11$ signal starts to saturate around 5 mW while $g = 2.06$ signal saturates at somewhat higher microwave power. Thus it appears probable that at least two additional iron-sulfur centers (tentatively designated as N-5 and N-6) with similar $E_{m7.2}$ values of -260 ± 20 mV are present in submitochondrial particles. In spectrum B-A (Fig. 11), signals arising from Center S-2 can also be recognized. This center exhibits resonances at $g_z = 2.03$, $g_y = 1.94$ and $g_x = 1.91$, and its $E_{m7.4}$ value measured in succinate-cytochrome *c* reductase is around -260 mV. Neither NADH nor succinate can reduce Center S-2 in submitochondrial particles; S-2 can be reduced only by dithionite [12]. In spectrum C-B of Fig. 11, additional resonances are seen at $g = 2.12$, 2.07 and 1.88, together with large signals in the $g = 1.93$ region. These signals suggest the presence of additional iron-sulfur centers; however, the noise level in this spectrum is too great to allow a definite characterization. The average $E_{m7.2}$ values and signal positions of iron-sulfur centers associated with NADH dehydrogenase in pigeon heart submitochondrial particles are summarized in Table II.

TABLE II

$E_{m7.2}$ VALUES AND SIGNAL POSITIONS OF IRON-SULFUR CENTERS IN THE NADH-UBIQUINONE SEGMENT OF THE RESPIRATORY CHAIN IN PIGEON HEART SUBMITOCHONDRIAL PARTICLES

Iron-sulfur centers	$E_{m7.2}$ (mV)	Field positions
N-2	-20 ± 20	2.05 1.93
N-1b	-240 ± 20	2.03 1.94 1.91
N-3	-240 ± 20	2.10 (1.93?) 1.87
N-5,6	-260 ± 20	(2.11, 2.06, 2.03, 1.93, 1.90, 1.88)
N-1a	-380 ± 20	2.03 1.94
N-4	-410 ± 20	2.11 (1.93?) 1.88

Fig. 12 presents EPR spectra of reduced iron-sulfur centers in one uncoupled pigeon heart submitochondrial particle suspension which was brought to anaerobiosis by incubation with either 4 mM NADH ($E_{m7.0} = -320$ mV) or 10 mM succinate ($E_{m7.0} = +31$ mV). Due to the varying spin relaxation rates of different iron-sulfur centers, EPR signals arising from various iron-sulfur centers can be partly resolved simply by changing the temperature of the sample at a constant EPR power setting. Comparison of spectrum A with spectrum E (Fig. 12) reveals that about 30 % of Center N-1a is reduced, which is consistent with the -380 mV $E_{m7.2}$ value of Center N-1a, assuming that Centers S-1 and N-1b are almost completely reduced by 3.5 mM NADH in the presence of 0.5 mM NAD. The low reduction level of Center

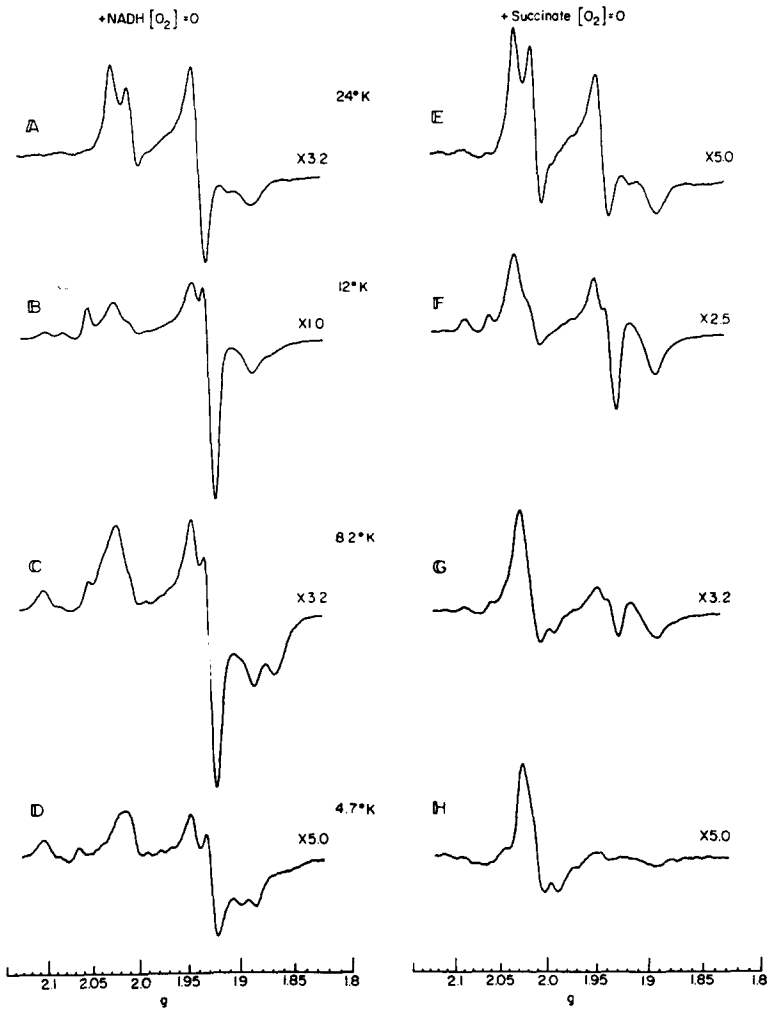

Fig. 12. EPR spectra of multiple iron-sulfur centers in uncoupled pigeon heart submitochondrial particles at different temperatures. Submitochondrial particles at protein concentration of 73 mg per ml were preincubated with FCCP (0.3 nmol/mg protein) and incubated with either 4 mM NADH or with 10 mM succinate for 5 min at room temperature. EPR spectra were recorded at the temperatures indicated in the figure. All other conditions are as described in Fig. 1.

N-1a is also recognized if one compares the line shape of the principal absorbance of Fig. 12B with that of Fig. 4C. Spectra B and F (Fig. 12) illustrate that about 25 % of Center N-2 is reduced by succinate. No other iron-sulfur centers in the NADH-ubiquinone segment of the respiratory chain undergo reduction in the presence of succinate. Signals at $g = 2.10$ and 1.87 observed at 8.2 °K in NADH-reduced pigeon heart submitochondrial particles (spectrum C) suggest that only Center N-3, and not N-4, is reduced with NADH. Neither Center N-3 and N-4 can be reduced by succinate (Fig. 12G). The $g = 1.90$ shoulder (Fig. 11B-A) can be seen as a separate peak in pigeon heart submitochondrial particles reduced with NADH under anaerobic conditions (Fig. 12D), because Center S-2 can be reduced by dithionite, but not by

NADH. These observations again agree with the $E_{m7.2}$ values obtained potentiometrically, which show that both Centers N-5 and N-6 are reduced by NADH, but not by succinate.

DISCUSSION

The present investigation provides evidence indicating that several distinct iron-sulfur centers are located in the NADH-ubiquinone segment of the mitochondrial respiratory chain. These iron-sulfur centers are resolved on the basis of individual midpoint potentials and EPR characteristics: line shape, temperature and power dependence. The midpoint potentials of these centers cover a span between −380 mV (Center N-1a) and −20 mV (Center N-2). All other centers show $E_{m7.2}$ values in the range of −240 to −260 mV. Chemical analysis of non-heme iron and flavin in NADH-ubiquinone reductase (Complex I) [4] reveals that 16–22 non-heme iron atoms per FMN molecule [4, 20] are present in this segment of the respiratory chain. In fact, EPR spectra of iron-sulfur centers N-1a, N-1b, N-2–N-6 have also been detected in Complex I and in the NADH dehydrogenase [11] prepared by the procedure of Ragan and Racker [20].

The existence and function of Center N-1a in the mitochondrial respiratory chain have been a subject of skepticism in the past. Because its midpoint potential is at least 60 mV more negative than that of NADH, Center N-1a becomes only partly reduced upon addition of NADH (Fig. 12) under usual experimental conditions. Center N-1a cannot be reduced by dithionite unless a trace of redox mediator such as methyl viologen or benzyl viologen is present. Similar observations have been reported for ferrodoxins in photosynthetic bacteria [21], for reasons not yet clarified. Thus, only the potentiometric technique of Dutton [15] has so far resolved Center N-1a and determined the value of its midpoint potential. According to this method, all membrane-bound electron carriers equilibrate with the Pt electrode via mediator dyes because of the phase separation which the membrane introduces. However, consistent with present observations, Albracht [10] recently suggested the presence of two different types of iron-sulfur centers which give rise to a "$g = 1.94$" signal in Complex I, using a system not involving redox mediating dyes. He was able to distinguish the two forms by their differential stability in the presence of NADH [10] although both centers exhibit similar spectra of rhombic symmetry. In pigeon heart submitochondrial particles, it is possible to distinguish spectra of Centers N-1a from that of N-1b based on their different line shapes, so that the partial reduction of Center N-1a by NADH is observable. Therefore, Center N-1a appears to be a genuine species whose physiological $E_{m7.2}$ value is below that of NADH. Quantitation of its spin concentration, as well as that for Center N-1b, is presently being investigated.

Center N-2 is the only component in the NADH-ubiquinone segment of the respiratory chain in pigeon heart mitochondria which has a midpoint potential close to that of ubiquinone or cytochrome *b*. Hence, succinate can partly reduce Center N-2, but none of the other centers in the NADH dehydrogenase region. Center N-2 determined in Complex I exhibits $E_{m8.0} = -135 \pm 20$ mV [11], which is lower than that obtained in pigeon heart submitochondrial particles. Still, this is the highest among iron-sulfur centers in Complex I (ref. 11 and Ohnishi, T. and Ragan, I. C., unpublished). These observations conflict with the relative midpoint potentials report-

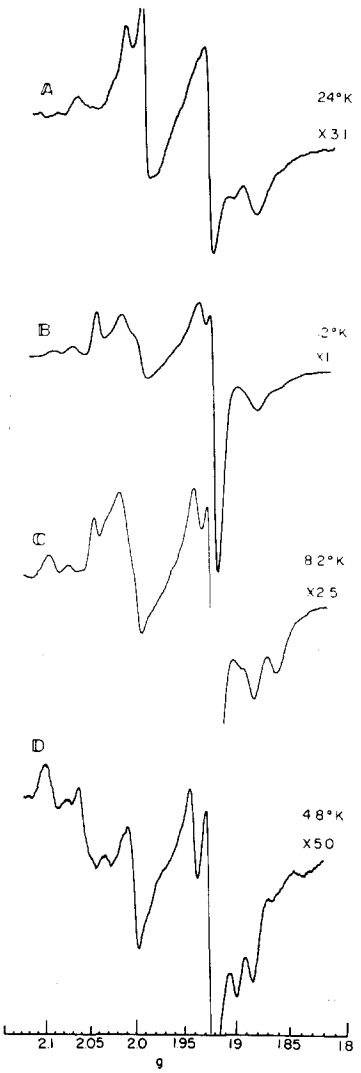

Fig. 13. EPR spectra of pigeon heart tissue slices at different temperatures. Pigeon heart tissue slices were spun down to the bottom of EPR tubes and incubated for 5 min at room temperature in order to achieve anaerobiosis. EPR operating conditions are the same as in Fig. 12.

ed by Orme-Johnson et al. [22], namely, N-3 $\geqq$ N-2 > N-4 > N-1. However, their reductive titration of Center N-2 (Fig. 7 in ref. 22) gave an anomalous triphasic curve, although the reduction level of Center N-2 was determined from the $g = 2.05$ signal which is free of interference from the other centers. These observations point out the difficulty in achieving redox equilibrium between Center N-2 and low-potential components when NADH is used as a reducing agent in the absence of mediators.

Disruption of the mitochondrial membranes during the preparation of submitochondrial particles results in a negative shift in the midpoint potential of Center N-4 (from -245 ± 20 mV to -410 ± 20 mV). Such a change, in the absence of a

concomitant change in the EPR spectrum, may suggest that the molecular environment in the vicinity of Center N-4 is modified during the preparation of submitochondrial particles. The question arises as to whether the low midpoint potential of Center N-1a could be due to artifacts of the mitochondrial preparation. In order to examine this possibility, EPR spectra of the iron-sulfur centers were recorded for anaerobic pigeon heart tissue slices (Fig. 13). The line shape of the central absorption band (spectra A–C) shows that Center N-1a remains largely oxidized under anaerobiosis in vivo. This observation is consistent with a very low midpoint potential for Center N-1a in intact organs. Other iron-sulfur centers are present in the reduced form, as in isolated mitochondria. Consequently, with the exception of Center N-4, the iron-sulfur centers observed in isolated mitochondria and submitochondrial particles remain in their physiological state. In addition, EPR spectra of Fig. 13 show that the line shape of individual iron-sulfur centers in vivo is very similar to that in mitochondrial or submitochondrial preparations. EPR signals detectable only at extremely low temperatures are tentatively attributed to two additional iron-sulfur centers (Centers N-5 and N-6) due to multiplicity of distinct peaks. However, it does not exclude the possibility that these signals derive from spin-spin interactions between iron-sulfur centers as in the case of two iron-sulfur centers in bacterial ferredoxin molecules [23].

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REFERENCES

1 Ohnishi, T., Asakura, T., Wohlrab, H., Yonetani, T. and Chance, B. (1970) J. Biol. Chem. 245, 901–902
2 Ohnishi, T., Asakura, T., Yonetani, T. and Chance, B. (1971) J. Biol. Chem. 246, 5960–5964
3 Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E., Beinert, H. and Hatefi, Y. (1971) Biochem. Biophys. Res. Commun. 44, 446–452
4 Hatefi, Y., Haavik, A. G. and Griffiths, D. E. (1962) J. Biol. Chem. 237, 1676–1680
5 Orme-Johnson, W. H. and Beinert, H. (1969) J. Biol. Chem. 244, 6143–6148
6 Ohnishi, T., Asakura, T., Wilson, D. F. and Chance, B. (1972) FEBS Lett. 21, 59–62
7 Ohnishi, T., Wilson, D. F., Asakura, T. and Chance, B. (1972) Biochem. Biophys. Res. Commun. 46, 1631–1638
8 Ohnishi, T., Wilson, D. F. and Chance, B. (1973) Biochem. Biophys. Res. Commun. 49, 1087–1092
9 Ohnishi, T. and Pring, M. (1974) in Dynamics of Energy Transducing Membrane (Ernster, L., Estabrook, R. W. and Slater, E. C., eds), pp. 169–180, Elsevier Publishing Co., Amsterdam
10 Albracht, S. P. J. (1974) Biochim. Biophys. Acta 347, 183–192
11 Ohnishi, T., Leigh, J. S., Ragan, C. I. and Racker, E. (1974) Biochem. Biophys. Res. Commun. 56, 775–782
12 Ohnishi, T., Winter, D. B., Lim, J. and King, T. E. (1973) Biochem. Biophys. Res. Commun. 53, 231–237

13 Chance, B. and Hagihara, B. (1963) Proceedings of the Fifth International Congress on Biochemistry, Moscow, 1961 (Sissakian, A. N. M., ed.), Vol. 5, p. 3, Pergamon Press, New York
14 Lindsay, J. G., Dutton, P. L. and Wilson, D. F. (1972) Biochemistry 11, 1937–1943
15 Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80
16 Wilson, D. F., Erecińska, M., Dutton, P. L. and Tsuzuki, T. (1970) Biochem. Biophys. Res. Commun. 41, 1273–1278
17 Dutton, P. L., Erecińska, M., Mukai, Y., Sato, N., Pring, M. and Wilson, D. F. (1972) Biochim. Biophys. Acta 267, 15–24
18 Beinert, H. and Sands, R. H. (1960) Biochem. Biophys. Res. Commun. 3, 41–46
19 Rieske, J. S., Hansen, R. E. and Zaugg, W. S. (1964) J. Biol. Chem. 239, 3017–3022
20 Ragan, C. I. and Racker, E. (1973) J. Biol. Chem. 248, 6876–6884
21 Hall, D. O. and Evans, M. C. W. (1969) Nature 223, 1342–1348
22 Orme-Johnson, N. R., Hansen, R. E. and Beinert, H. (1974) J. Biol. Chem. 249, 1922–1927
23 Mathews, R., Charlton, S., Sands, R. H. and Palmer, G. (1974) J. Biol. Chem. 249, 4326–4328