LOW TEMPERATURE ELECTRON PARAMAGNETIC RESONANCE STUDIES ON IRON-SULFUR CENTERS IN CARDIAC NADH DEHYDROGENASE

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SUMMARY:

EPR signals arising from at least seven iron-sulfur centers were resolved in both reconstitutively active and inactive NADH dehydrogenases, as well as in particulate NADH-UQ reductase (Complex I). EPR lineshapes of individual iron-sulfur centers in the active dehydrogenase are almost unchanged from that in Complex I. Iron-sulfur centers in the inactive dehydrogenase give broadened EPR spectra, suggesting that modification of iron-sulfur active centers is associated with loss of the reconstitutive activity of the dehydrogenase. With the reconstitutively active dehydrogenase, the $E_{m8.0}$ value of Center N-2 (iron-sulfur centers associated with NADH dehydrogenase are designated with prefix N) was shifted to a more negative value than in Complex I and restored to the original value on reconstitution of the enzyme with purified phospholipids.

Ragan and Racker (1) have recently reported preparation of NADH dehydrogenase from NADH-UQ reductase [Complex I (2)], which retains full activity with $K_3Fe(CN)_6$ as an electron acceptor, but lacks rotenone-sensitive NADH-UQ reductase activity. Recombination with purified phospholipids restores rotenone sensitive NADH-UQ reductase activity. The resulting preparation combines with Complex III to reconstitute antimycin A-sensitive NADH-cytochrome c reductase (3). This reconstitutively active NADH dehydrogenase is obtained only when the extraction of the enzyme by cholate is performed in the presence of dithionite and dithiothreitol. If these reducing reagents are omitted during the

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extraction, the resulting dehydrogenase gives very low rates of rotenone-sensitive UQ reductase when combined with phospholipids. Reconstitutively active and inactive NADH dehydrogenases contain approximately the same concentrations of non-heme iron and acid labile sulfide, and spectrophotometric analysis does not reveal any differences (1). Recently, the EPR characteristics of multiple [at least seven (4)] iron-sulfur centers in the NADH dehydrogenase region of the respiratory chain have been studied using mitochondria, submitochondrial particles (SMP) (5,6) or Complex I (7,8). In the present investigation, characteristics of individual iron-sulfur centers in both reconstitutively active and inactive purified NADH dehydrogenases were investigated, using low temperature (< 77°K) EPR spectrometry and the redox titration technique of Dutton (9).

MATERIALS AND METHODS

Reconstitutively active and inactive NADH dehydrogenases were purified from Complex I, as reported by Ragan and Racker (1). Reconstitution of the dehydrogenase with phospholipids was performed as described previously (1). Samples were kept frozen in liquid nitrogen and thawed before the experiments. The half-reduction potentials at pH 8.0 ($E_{m8.0}$) were determined potentiometrically, according to Dutton (9) and Wilson *et al.* (10). EPR measurements were performed as previously reported (11).

RESULTS AND DISCUSSION

In the present communication, iron-sulfur centers associated with the NADH dehydrogenase are designated with prefix N, as proposed by Ohnishi and Pring (4). In a similar fashion, iron-sulfur centers associated with succinate dehydrogenase were previously designated as Centers S-1 and S-2 (11). EPR signals arising from at least 7 iron-sulfur centers; namely, Center N-2 (Fig. 1), Center N-1a plus N-1b (Fig. 2), Center N-3 plus N-4 (Fig. 3) and Center N-5 plus N-6 (Fig. 4); can be resolved in both reconstitutively active (Spectra A) and inactive (Spectra B) NADH dehydrogenases.

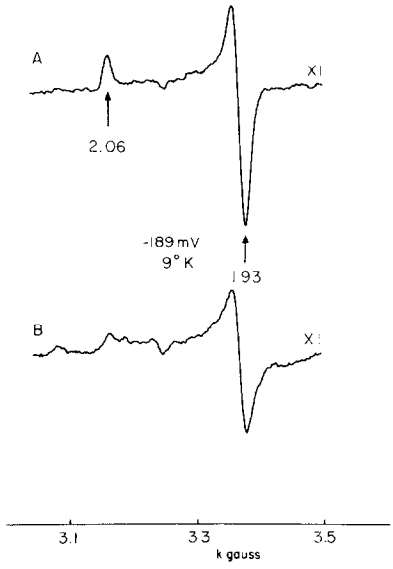

Figure 1: EPR spectra of iron-sulfur center N-2 in the purified, reconstitutively active and inactive NADH dehydrogenases, respectively.

Reconstitutively active (Fig. 1A) or inactive (Fig. 1B) NADH dehydrogenase (each at protein concentrations of 10.6 mg per ml) was stirred in 0.6 M sucrose, 1 mM histidine, and 50 mM Tris-HCl (pH 8.0) under a continuous flow of argon. The redox potential of the suspension was measured according to Dutton (9). The following redox mediators were added; 62.5 μM phenazine ethosulfate, 62.5 μM phenazine methosulfate, 6.3 μM duroquinone, 6.3 μM pyocyanine, 7.5 μM resorufin, 30 μM 2 OH-naphtoquinone, 77.5 μM phenosafranine, 73.5 μM benzyl viologen, and 133 μM methyl viologen. The desired redox potential of the suspension, as shown in the Figure (-189 mV) was attained by the addition of aliquots of freshly prepared dilute solution of dithionite. Aliquots (about 0.3 ml) were transferred anaerobically to quartz EPR tubes and frozen rapidly by immersion in isopentane at its freezing point (113°K). EPR measurements were performed with a Varian E-4 spectrometer. Samples were cooled to 9°K with a fast stream of cold helium gas derived from boiling liquid helium. Temperature was measured with a thermocouple Au/Co versus Pt. EPR operating conditions; microwave frequency, 9.09 GHz; modulation amplitude, 12.5 gauss; microwave power, 10 mW; time constant, 0.3 sec; scanning time, 500 gauss/min. Spectra A and B were recorded consecutively, using samples in EPR tubes of the same diameter.

In order to obtain EPR spectra of individual iron-sulfur centers with minimum interference from other iron-sulfur centers, appropriate redox potentials (E_h) of the enzyme suspension and the temperature of EPR measurements

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Footnote 1: Using pigeon heart mitochondria, only one of two iron-sulfur N-1 components was found to show an ATP-dependent midpoint potential value. This component was designated as Center N-1a, the other as Center N-1b, before individual EPR signals were resolved potentiometrically (13). More recently, the $E_{m7.2}$ values of Center N-1a and N-1b in pigeon heart mitochondria were determined as -380 ±15 mV and -250 ± 15 mV, respectively (4).

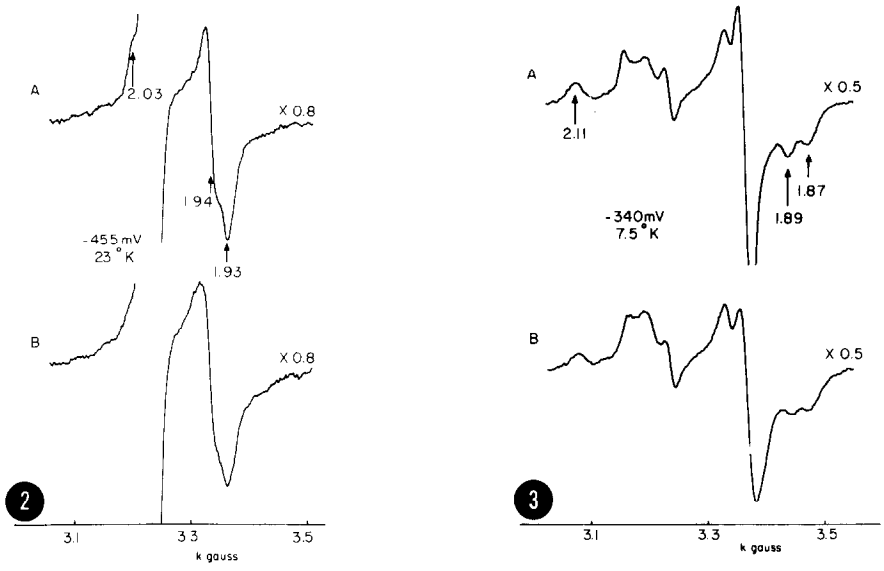

Figure 2: EPR spectra of iron-sulfur center N-1a plus N-1b in the reconstitutively active (A) and inactive (B) NADH dehydrogenases, respectively.
Experimental conditions were the same as described in Figure 1, except for the E_h value of the enzyme suspension (-455 mV) and the sample temperature of EPR measurements (23°K).

Figure 3: EPR spectra of reconstitutively active (A) and inactive (B) NADH dehydrogenases, showing signals arising from Center N-3 plus N-4
Experimental conditions are the same as described in the legend of Figure 1, except for the E_h value (-340 mV) and sample temperature of the EPR recording (7.5°K).

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were chosen, as illustrated in the Figures. The line shape of EPR spectra of individual iron-sulfur centers in the reconstitutively active dehydrogenase (Fig. 1-4, Spectra A) shows no significant differences from that of the iron-sulfur centers in Complex I (7,12) or in beef heart SMP (12). Most of the iron-sulfur centers in the reconstitutively inactive NADH dehydrogenase show broadened EPR spectra (Fig. 1 - Fig. 4, Spectra B), in comparison with those of the corresponding iron-sulfur centers in the reconstitutively active dehydrogenase, although general EPR characteristics such as the g value of the signals and temperature and power dependence of the EPR spectra are quite similar. These observations suggest that some modification of molecular environoment around the iron-sulfide active centers is associated with the loss of reconstitutive activity.

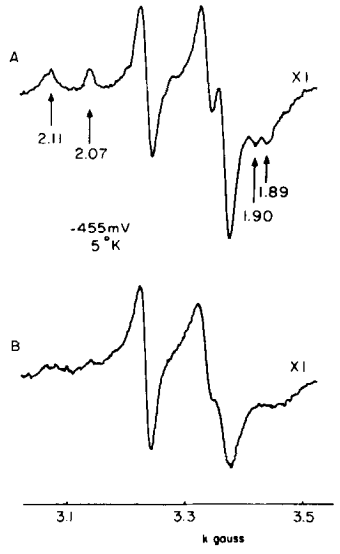

Figure 4: EPR spectra of reconstitutively active (A) and inactive (B) NADH dehydrogenases, showing signals arising from Center N-5 plus N-6. Experimental conditions are the same as described in the legend of Figure 1, except for the E_h value (-455 mV), sample temperature of the EPR recording (5°K), and the EPR power setting (5 mW).

In Complex I, the measured $E_{m8.0}$ value of Center N-2 is approximately -135 ± 15 mV, which is considerably more electronegative than the $E_{m8.0}$ value obtained for Center N-2 in beef heart SMP ($E_{m8.0}$ = -80 mV ± 15 mV). Upon extraction of the dehydrogenase from Complex I, the $E_{m8.0}$ value of Center N-2 becomes further electronegative, as in the case of iron-sulfur Center S-2 in the succinate dehydrogenase (11). The $E_{m8.0}$ value of Center N-2 in the reconstitutively active dehydrogenase is -210 ± 15 mV, while the $E_{m8.0}$ value of Center N-2 in the inactive dehydrogenase shows a tendency to go further electro negative (-265 ± 15 mV).

Iron-sulfur Center N-1, contributing to the so-called "g = 1.94" signal, was resolved potentiometrically into two components[1] [Center N-1a and N-1b], using a computer program devised by Martin Pring (14). $E_{m8.0}$ values of Center N-1a and N-1b are -385 ± 15 mV and -260 ± 15 mV, respectively. Midpoint

potentials of these Centers seem to remain almost unchanged during the extraction of the enzyme from Complex I, which differs from the characteristics of Center N-2.

So far, EPR signals resulting from Center N-3 and N-4 have not been resolved potentiometrically. Redox titrations of the combined signals give an $E_{m8.0}$ value of -245 ± 15 mV in either reconstitutively active or inactive dehydrogenase, which is similar to the $E_{m8.0}$ value obtained from Complex I. More recently, additional EPR signals at g values of 2.11, 2.07, 1.90 and 1.89 have been detected [c.f. ref. (4,15)] at extremely low temperatures close to that of liquid helium (Figure 4). Under these EPR conditions, the g = 1.87 signal which arises from both Centers N-3 and N-4, is almost undetectable. Thus, g = 2.11 and 1.89 signals obtained at such low temperatures arise from neither Center N-3 nor N-4. These four signals appear to arise from at least two different iron-sulfur centers, which are designated as Centers N-5[2] and N-6 (4), but signals responsible for these two centers cannot be sorted out from the difference in their midpoint potentials. $E_{m8.0}$ values of the combined spectra of Centers N-5 and N-6 were in the region of -260 mV in both reconstitutively active and inactive dehydrogenases, and also in Complex I.

Upon recombination of reconstitutively active NADH dehydrogenase with purified phospholipids, the $E_{m8.0}$ value of about 60% of Center N-2 is raised to -135 ± 15 mV (the $E_{m8.0}$ value obtained originally in Complex I before the extraction of the enzyme). Other iron-sulfur centers do not show such a dramatic midpoint potential change upon reconstitution.

These data suggest that iron-sulfur center N-2 is probably surrounded by a phospholipid layer in the mitochondrial membrane, and exposure of the center to a more aqueous environment by removal of phospholipid with cholate tends to shift the midpoint potential of Center N-2 to a more electronegative value. Upon recombination of the active dehydrogenase with phospholipids, the shift

Footnote 2: Center N-5 is not related to the previously reported "Center 5". "Center 5", which shows EPR signals with g values of 2.09 and 1.89, was detected in cytochrome bc_1 particles (16), but not in NADH dehydrogenases.

in midpoint potential is partially reversed, suggesting that during the reconstitution procedure, part of the Center N-2 is restored to its original environment. The fraction which is restored (60%) is in reasonable agreement with the extent of restoration of rotenone-sensitive NADH-UQ reductase. Detailed data on these iron-sulfur centers in Complex I, in purified dehydrogenases and in the reconstituted systems, will be reported elsewhere (12).

Recently, Baugh and King (17) reported a reconstitutively active NADH dehydrogenase preparation which was extracted from mitochondrial membranes with Triton X-100. This enzyme contains almost no phospholipids and reacts with either $K_3Fe(CN)_6$ or UQ as electron acceptors. The latter reaction is inhibited by rotenone or Amytal.

It is interesting that about 30% of Center N-2 in the NADH dehydrogenase solubilized with Triton X-100 has an $E_{m8.0}$ value almost the same as in Complex I (-135 ± 15 mV), although it shows quite broad EPR spectra (18,19) similar to that of Center N-2 in the reconstitutively inactive NADH dehydrogenase described in this communication (Figure 1, spectrum B).

Modifications of the active center of iron-sulfur proteins or of their environments, give rise to either changes in midpoint potentials, or broadening of EPR spectra of active centers; however, these two parameters appear to be quite independent and the molecular mechanism for these changes remains to be elucidated.

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