EPR BEHAVIOR OF A SOLUBLE CARDIAC NADH DEHYDROGENASE

by

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SUMMARY--A soluble, lipid free, NADH dehydrogenase isolated from cardiac muscle shows the characteristic iron-sulfide centers similar to those found in submitochondrial particles and mitochondria. The g-values obtained by low temperature EPR spectroscopy are 2.026, 1.943 and 1.927 for temperature "insensitive" center 1; 2.058 and 1.943 for center 2; and 2.111, 1.885 and 1.866 for centers 3 plus 4. The behavior of the EPR resonances suggests that iron may be coordinated not only with the labile-sulfide resulting in the iron-sulfide centers, but that iron is also liganded in complexes and spin-states as yet unidentified.

Recently a soluble NADH dehydrogenase which contains negligible amounts of lipid has been isolated and purified from cardiac submitochondrial particles (1, 2). Unlike soluble preparations previously reported which react with only ferricyanide or ubiquinone but not both, this preparation shows high catalytic activities toward both electron acceptors. The inhibitory behavior of the dehydrogenase toward rotenone and Amytal is the same as that of mitochondria. The dehydrogenase is also reconstitutively active, i.e. when it reacts with particles devoid of NADH dehydrogenase, it is able to reincorporate with the particles resulting in an active and integral NADH-cytochrome c reductase or NADH oxidase system sensitive to antimycin A (1, 2). It is therefore of considerable importance to examine this enzyme with respect to its redox functionally active iron-sulfide centers by low-temperature EPR spectroscopy to ascertain whether the same centers are present as in Complex I (3), mitochondria, and submitochondrial particles (4). Our studies reveal that the same three to four iron-sulfide centers exist in this dehydrogenase.

EXPERIMENTAL--The NADH dehydrogenase used was prepared as reported recently (2). It catalyzed the oxidation of about 1700 μmoles of NADH per minute per mg of protein (biuret method) and showed reconstitutive activity of approxi-

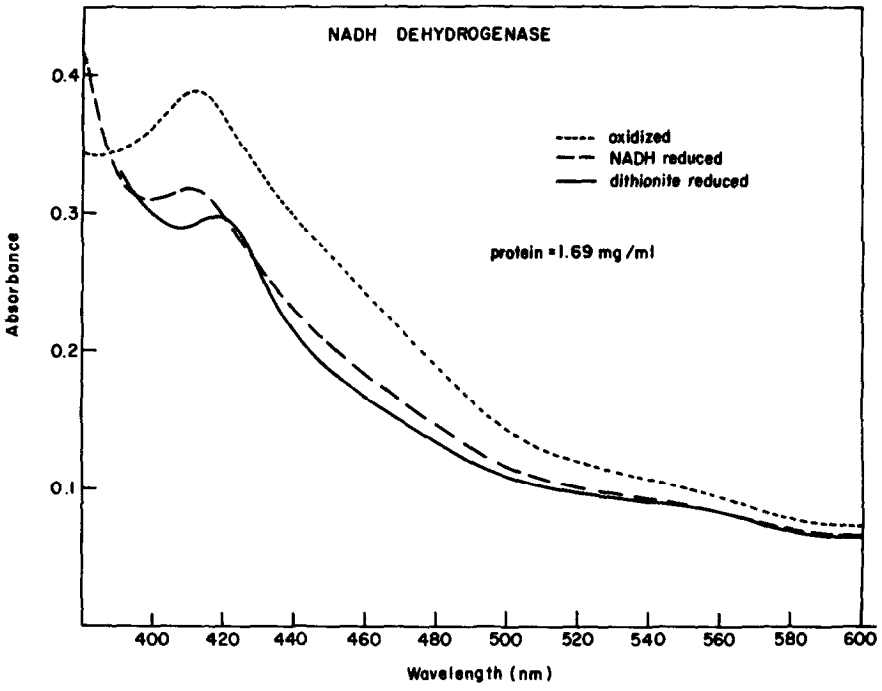

Fig. 1. Absorption spectrum of NADH dehydrogenase in air.

mately 10,000 moles of NADH oxidized per minute per mole of dehydrogenase FMN at 23° in a reconstituted NADH-cytochrome c reductase system sensitive to antimycin A. The latter activity was higher than that reported previously (2) because of the improved conditions in the reconstitution used. Other operational details are presented in the legends of the figures.

RESULTS--This dehydrogenase exhibits typical iron-flavoprotein spectra as shown in Fig. 1. At least 85% reduction of the dithionite reducible absorption is achieved by NADH in air.

Figure 2 depicts the resonances observed when NADH dehydrogenase is reduced with NADH under aerobic conditions. Curve A of the figure shows the center 2-type reduced iron-sulfide signal when NADH dehydrogenase is reacted with a low concentration of NADH (approximately -30 mV). The g-values of this center 2 absorption have been found to be 2.058 and 1.927. Orme-Johnson et al. (3) have reported center 2 signals at 2.054 and 1.922, while Ohnishi

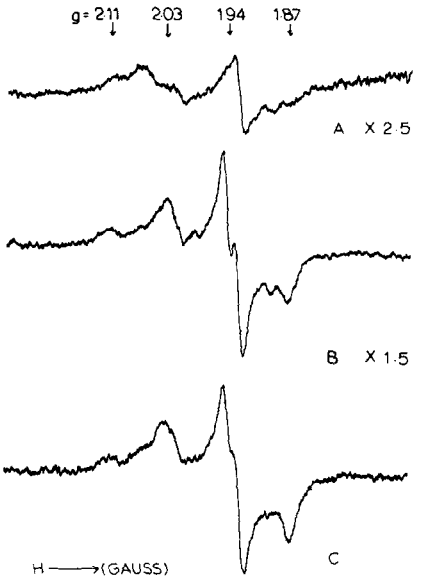

Fig. 2. EPR spectra of reduced NADH dehydrogenase. A, after reduction of the enzyme (12.2 mg/ml) with NADH (concentration adjusted to approximately -30 mV) for 1 min at 25° under aerobic conditions; B, after reduction with NADH (1 mM) for 1 min at 25° under aerobic conditions; C, as B but measured at 20 mW microwave power. The conditions for EPR experiments were essentially as those described in Ref. 8 with the following exceptions: frequency, 9.173 GHz; temperature, 12°K; time constant, 0.3 sec; modulation amplitude, 5.9 G; microwave power in A and B, 1.5 mW; scanning rate, 400 gauss per min.

et al. (4) found the positions at 2.05 and 1.92 in pigeon heart mitochondria. When the dehydrogenase is reduced with a large excess of NADH (approximately 1 mM), Fig. 3A is obtained. The signals observed arise from a mixture of centers 1, 2, and 3 plus 4. The g-values of center 1 have been found to occur at 2.026, 1.943 and 1.927 and those of centers 3 plus 4 at g = 2.111, 1.885 and 1,866. The observed spectrum is very similar to that observed by other workers. By differential titration in pigeon heart mitochondria, Ohnishi et al. (4) estimate the signals of center 1 at 2.02, 1.94, and 1.92, whereas those of centers 3 plus 4 at 2.10, 1.89, and 1.86 at 8.8°K. Similar values were reported earlier by Orme-Johnson et al. (3).

Quantitation of the reduced iron-sulfide centers by double integration

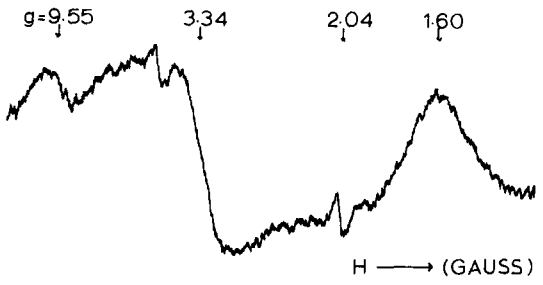

Fig. 3. EPR spectrum of isolated NADH dehydrogenase (12.4 mg/ml). The conditions for EPR experiments were the same as in Fig. 2 except that the frequency was 9.174 GHz; scanning rate, 1000 gauss per min; and microwave power 25 mW.

and comparison to the cupric-EDTA standard has been made on the assumption that the temperature "insensitive" center 1 signal arises from a binuclear iron-sulfide complex, while those of centers 2 and 3 may each be attributable to a four-iron cluster. On this basis approximately 36% of the chemically determined iron content can be accounted for. A complete recovery of spin intensity may be unlikely since the resonances observed in the oxidized state (vide infra) of NADH dehydrogenase involve a significant amount of spin intensity not directly attributable to the iron-sulfide centers. This value is actually in good agreement with that reported by Orme-Johnson et al. for Complex I (3). It might be pointed out that our soluble dehydrogenase contains 28 iron and labile sulfide atoms each per mole of acid extractable flavin. If the assumption of a ferredoxin type structure is used, 7 iron centers may be expected. But overlap of resonances could prevent discernible signals even at very low temperatures. However, the suggestion that center 5 may be the "iron-sulfur protein on the oxygen side of the rotenone inhibition site in Complex I" (4, and more importantly, see reference 5) is not precise and indeed may be incorrect as also shown by the data presented by Ohnishi et al. (4). Moreover, the separation of Complex I into structural protein, iron-sulfur protein and flavoprotein has been made only by simple ammonium sulfate fractionation after urea treatment of the Complex (6). We

have found that Complex I prepared according to the latest method (6) shows at least 14 bands on SDS (sodium dodecylsulfate) gel electrophoresis.

Figure 2C depicts the effect of high microwave power on the reduced iron-sulfide resonances detected on reduction with excess NADH of NADH dehydrogenase. It is clear that the use of excess power has resulted in partial saturation of center 3 and a general broadening of important line shape parameters from the other centers.

Figure 3 shows a 5000 gauss field scan of the oxidized preparation of NADH dehydrogenase at 12°K. Several complex resonances are clearly discernible. Signals are seen at g = 9.55, 4.22, and 2.04. In addition rather broad resonances appear at approximately g = 3.34 and 1.60. The signals at g = 9.55, 3.34, 2.04, and 1.60 are especially temperature dependent, essentially disappearing above 40°K and increase with high microwave power (greater than 10 mW). The signal at g = 9.55 as well as part of the signal at g = 4.22 has been reported in oxidized rubridoxin which contains high-spin ferric ion liganded with four cysteine residues (7). However, the intensity of this resonance in oxidized NADH dehydrogenase may not be sufficient to consider that it represents an important redox component. The remainder of the signal at g = 4.22 is also weak and can be attributed to high-spin ferric ion and as in the case of a large number of iron-containing proteins may be a minor contaminant which is redox active in a non-enzymatic manner. The nature of the signals at g = 3.34 and 1.60 is less clear although it is likely that these signals represent iron but in a symmetry and spin state yet to be identified. The rather weak signal at g = 2.04 may not be involved in the main redox path because of its small intensity in comparison to that of the reduced absorptions arising from the iron-sulfide centers on reduction of the dehydrogenase with excess NADH. Ohnishi *et al.* (4) have also observed an unidentified signal at g = 2.04 in pigeon heart mitochondria.

At any rate the EPR resonances observed in the oxidized and reduced states of NADH dehydrogenase indicate that iron atoms are coordinated not

only with sulfide resulting in the iron-sulfide centers observed in the reduced state but also bound with as yet unidentified ligands and spin states. The similarity and number of iron-sulfide centers observed in the soluble NADH dehydrogenase as compared to those reported in the intact respiratory particle adds an important line of evidence to support the conclusion that the dehydrogenase has not been structurally altered during the solubilization and purification, and thus resembles the membrane-bound NADH oxidation apparatus in mitochondria in active site functional capabilities, therefore the dehydrogenase is reconstitutively active.

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References

1. Baugh, R. F., and King, T. E., 164th ACS Meeting, New York, Abstracts #196 and 197, Biol. Chem. Div. (1972).
2. Baugh, R. F., and King, T. E., Biochem. Biophys. Res. Commun., 49, 1165 (1972).
3. Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E., and Beinert, H., Biochem. Biophys. Res. Comm., 44, 446 (1971); see also in Proceedings of Second International Symposium on Oxidases and Related Systems (T. E. King, H. S. Mason, and M. Morrison, Eds.), University Park Press, Baltimore, 1972.
4. Ohnishi, T., Wilson, D. F., Asakura, T., and Chance, B., Biochem. Biophys. Res. Commun., 46, 1631 (1972); see also in Proceedings of Second International Symposium on Oxidases and Related Systems (T. E. King, H. S. Mason, and M. Morrison, Eds.), University Park Press, Baltimore, 1972.
5. Hatefi, Y., Proc. Nat. Acad. Sci. (U. S.), 60, 733 (1968).
6. Hatefi, Y., and Stempel, K. E., J. Biol. Chem., 244, 2350 (1969); also Hatefi, Y., Stempel, K. E., and Hanstein, W. G., J. Biol. Chem., 244, 2358 (1969).
7. Peisach, J., Blumberg, W. E., Lode, E. T., and Coon, M. J., J. Biol. Chem., 246, 5877 (1971).
8. Forget, P., and DerVartanian, D. V., Biochim. Biophys. Acta, 256, 600 (1972).