Resolution of Mitochondrial NADH Dehydrogenase and Isolation of Two Iron-Sulfur Proteins†

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ABSTRACT: The low molecular weight NADH dehydrogenase which can be solubilized from the mitochondrial NADH-ubiquinone oxidoreductase complex with chaotropic agents consists of three subunits in equimolar ratio [Galante, Y. M., & Hatefi, Y. (1979) *Arch. Biochem. Biophys. 192*, 559]. The largest subunit (subunit I) can be completely separated from the other two (subunits II + III) by treatment with sodium trichloroacetate and ammonium sulfate fractionation. Both the subunit I and subunit II + III fractions contain iron and acid-labile sulfur. From visible and EPR spectroscopy and the iron and acid-labile sulfide content, we propose that the subunit II + III fraction contains a binuclear cluster. The cluster structure present in subunit I is as yet unclear. On separation of the subunits of NADH dehydrogenase, the FMN is lost.

Structural and functional analysis of the NADH-ubiquinone oxidoreductase complex (EC 1.6.5.3) of mitochondria has been greatly aided by fragmentation of the enzyme into specific subfractions. Thus, treatment of purified NADH-ubiquinone oxidoreductase (complex I of Hatefi et al., 1962) with chaotropic agents led to the separation of three distinct fractions (Hatefi & Stempel, 1967, 1969). These were (a) an insoluble hydrophobic residue containing phospholipids and low concentrations of iron and acid-labile sulfide, (b) a water-soluble iron-sulfur protein, and (c) a water-soluble iron-sulfur flavoprotein. The latter fraction (NADH dehydrogenase) is capable of catalyzing the oxidation of NADH by artificial electron acceptors and is considerably enriched in iron, acid-labile sulfide, and FMN[1] compared with the original complex I. Its properties have been extensively investigated by Hatefi & Stempel (1969) and Hatefi et al. (1969) and more recently by Galante & Hatefi (1979). The most recent work demonstrated that the dehydrogenase consists of three polypeptide subunits in equimolar ratios. The molecular weights are 51 000 (subunit I), 24 000 (subunit II), and 9000 (subunit III) as determined by $NaDodSO_4$[1] gel electrophoresis. The sum of these molecular weights (M_r 84 000) is rather higher than the value suggested by the FMN content (M_r 74 000) or by molecular exclusion chromatography of the whole dehydrogenase (M_r 69 000). Thus there is a degree of uncertainty about the exact values of the subunit molecular weights. The iron or acid-labile sulfide content of NADH dehydrogenase is 5-6-fold higher than the FMN content, strongly suggesting the presence of more than one iron-sulfur center (Galante & Hatefi, 1979).

In this report, we describe the separation of subunit I from the two smaller subunits by chaotropic agents and demonstrate that both subunits I and II contain distinct iron-sulfur centers.

Experimental Procedures

Materials

NADH dehydrogenase was prepared from bovine heart complex I by the method of Galante & Hatefi (1979). The enzyme was stored as an ammonium sulfate pellet at −50 °C.

Methods

Resolution of NADH Dehydrogenase. The standard procedure for separation of NADH dehydrogenase subunits was basically the same as that originally developed by Davis & Hatefi (1971) for separating the subunits of succinate dehydrogenase. The dehydrogenase was dissolved in 50 mM Tris-HCl, pH 7.8, containing 2 mM dithiothreitol, to give a final protein concentration of 2–4 mg/mL. A 4 M solution of $Cl_3CCOONa$ was then added to give a final concentration of 0.2 M. The solution was frozen in liquid N_2 and immediately thawed at room temperature. The freezing and thawing was repeated. The solution was then fractionated by addition of saturated and neutralized $(NH_4)_2SO_4$ to 0.12, 0.22, and 0.35 saturation. After each addition of $(NH_4)_2SO_4$, the soltion was allowed to stand for 10 min. and precipitated protein was removed by centrifugation at 90000*g* for 5 min. Except where indicated, all operations were carried out at 0–4 °C. All assays and measurements were carried out on freshly dissolved NADH dehydrogenase and on freshly prepared NADH dehydrogenase subunits.

Analytical Methods. Iron (Doeg & Ziegler, 1962), acid-labile sulfide (Fogo & Popowski, 1949), and flavin (Hatefi & Stempel, 1969) were assayed as indicated in the references. The protein was measured by the method of Bensadoun & Weinstein (1976) to avoid interference by dithiothreitol and detergents.

Preparation of Samples for EPR Spectroscopy. For EPR spectroscopy, NADH dehydrogenase was dissolved in 50 mM Tris-HCl, pH 7.8, containing 2 mM dithiothreitol. Subunit I and subunit II + III fractions were prepared as described above. The subunit I fraction was homogenized in Tris-DTT

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[1] Abbreviations: $Cl_3CCOONa$ (NaTCA in figures), sodium trichloroacetate; EPR, electron paramagnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl) aminomethane; FMN, flavin mononucleotide; DTT, dithiothreitol.

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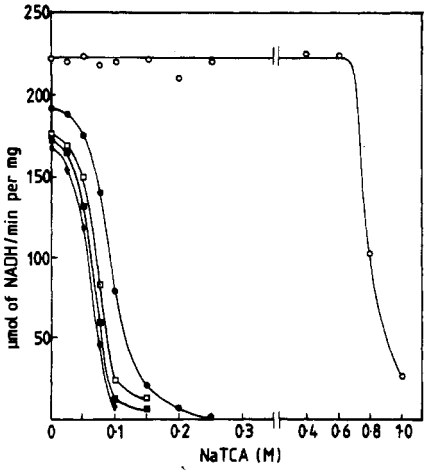

FIGURE 1: Effect of $Cl_3CCOONa$ (NaTCA) on NADH dehydrogenase activity. The enzyme (3.3 mg of protein/mL) was incubated on ice for 1 h in 50 mM Tris-HCl, pH 8.0, containing 5 mM DTT and the indicated concentrations of $Cl_3CCOONa$. Samples were assayed for NADH–menadione oxidoreductase activity before (○) and after freezing in liquid N_2 and thawing one (●), two (□), three (■), and four times (♦).

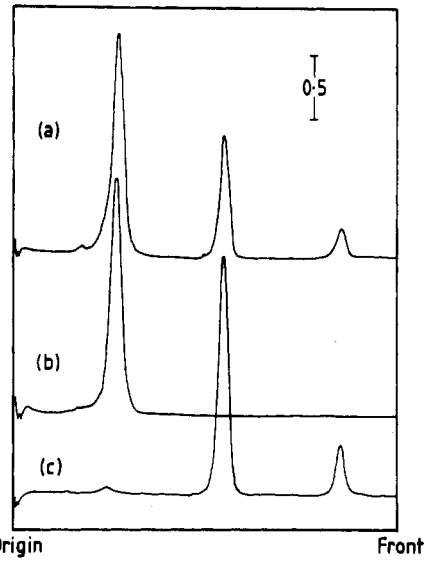

FIGURE 2: Subunit composition of NADH dehydrogenase and subfractions analyzed by $NaDodSO_4$ gel electrophoresis. (a) NADH dehydrogenase (15 μg of protein); (b) subunit I (10 μg of protein); (c) subunit II + III (10 μg of protein).

buffer and solubilized by the addition of 1 N NaOH to a final pH of 11. The subunit II + III fraction was dissolved directly in Tris–DTT buffer. Samples were reduced by addition of 0.5 M $Na_2S_2O_4$ to a final concentration of 2.5 mM, immediately transferred to EPR tubes, and frozen in liquid N_2.

EPR Spectroscopy. EPR measurements were conducted with a Varian E109 spectrometer. The magnetic field was calibrated with a proton NMR probe or on a routine basis by using a weak pitch *g*-value standard. The sample temperature was controlled by using a variable temperature cryostat (Air Products LTD-3-110). The temperature was monitored with an Allen-Bradley type carbon resistor or with a thermocouple (chromel/gold–0.07% iron) approximately 1 cm below the sample in a cold helium gas flow.

Double integration of EPR spectra was performed with a Nicolet Model 1024 computer. Cu(II)–EDTA (0.5 mM) was used as an intensity standard. The transition probability corrections were made according to Aåsa & Vänngård (1975).

Kinetic Measurements. NADH dehydrogenase activity with menadione, acetylpyridine–adenine dinucleotide, or $K_3Fe(CN)_6$ as acceptors was measured as described by Galante & Hatefi (1979).

Electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out in gels containing 12.5% (w/v) acrylamide and 0.34% bis(acrylamide) by using the conditions described by Weber & Osborn (1969). Staining and destaining were done by the method of Fairbanks et al. (1971).

Results

Resolution of NADH Dehydrogenase by Sodium Trichloroacetate. As shown in Figure 1, NADH dehydrogenase activity was inhibited by $Cl_3CCOONa$ concentrations greater than 0.6 M. Much lower concentrations were inhibitory if, after $Cl_3CCOONa$ addition, the solution was frozen in liquid nitrogen and thawed before assay of activity. Repeating the freezing and thawing caused further loss of activity. No activity remained after freezing and thawing twice in the presence of 0.2 M $Cl_3CCOONa$, and these conditions were used routinely for separation of NADH dehydrogenase subunits.

After freezing and thawing, the solution of enzyme became turbid. The degree of turbidity increased with increasing concentration of $Cl_3CCOONa$ up to 0.2 M and then decreased at concentrations of $Cl_3CCOONa$ greater than 0.4 M. The precipitated protein was separated by centrifugation at 90000*g* for 5 min and was found to consist of pure subunit I (M_r 51 000) by $NaDoDSO_4$ gel electrophoresis. A small proportion of this subunit was also precipitated by freezing and thawing the enzyme in the absence of chaotropic agents, thus accounting for the small loss of activity following such treatment (Figure1). Despite the complete loss of dehydrogenase activity following $Cl_3CCOONa$ treatment, only a portion of the largest subunit was precipitated, presumably because of the solubilizing effect of $Cl_3CCOONa$. Maximal precipitation occurred in the presence of 0.2–0.4 M $Cl_3CCOONa$ and was increased by decreasing the enzyme concentration. At a protein concentration of 1.2 mg/mL, 37% of the total protein was precipitated corresponding to 58% of subunit I. This was determined from the stain intensity of this subunit in $NaDodSO_4$ gels of the enzyme before and after centrifugation. Ammonium sulfate fractionation was used to increase the yield of subunit I and to obtain subunits II and III free from subunit I.

Purification of NADH Dehydrogenase Subunits. Addition of ammonium sulfate to 0.12 saturation precipitated most of the remaining subunit I from solutions of resolved NADH dehydrogenase. The ammonium sulfate pellet was identical with the subunit I precipitated directly by $Cl_3CCOONa$ resolution in purity (assessed by $NaDodSO_4$ gel electrophoresis) and in iron and acid-labile sulfide contents (as detailed below). Therefore, in subsequent experiments, subunit I was isolated after the addition of ammonium sulfate to 0.12 saturation directly to the thawed enzyme slution. The protein precipitating between 0.22 and 0.35 saturation with ammonium sulfate consisted of subunits II and III together. The small amount of protein precipitating between 0.12 and 0.22 saturation with ammonium sulfate contained all three subunits and was discarded. Figure 2 shows that the two major ammonium sulfate fractions were virtually free of cross-contamination. The recovery of subunit I or II in the two fractions was approximately 80%. Comparison of the integrated stain intensities of subunits II and III in Figure 2 showed that the ammonium sulfate fraction was slightly deficient in subunit III, and indeed the supernatant from treatment with ammonium sulfate to 0.35 of saturation contained small amounts of

Table I: Iron and Acid-Labile Sulfide Contents of NADH Dehydrogenase Subfractions[a]

	Fe			S		
	ng-atom/mg of protein		mol/ mol of	ng-atom/mg of protein		mol/ mol of
sample	range	av	subunit	range	av	subunit
subunit I	63-67 (6)	65	3.32	62-71 (4)	66	3.37
subunit II & III	67-71 (4)	69	2.28	60-72 (4)	68	2.24

[a] Numbers in parentheses show the number of different preparations assayed. The molecular weights of the subunit fractions were taken as 51 000 (subunit I) and 33 000 (subunits II & III).

subunit III. We tried to bring about a more complete separation of the two smaller subunits by harsher treatment, e.g., 1.0 M $Cl_3CCOONa$ and repeated freezing and thawing, but without success. The amounts of pure subunit III which we could obtain were far too small for assays of iron or acid-labile sulfide. Separation of subunit I from subunits II + III could also be achieved by ammonium sulfate fractionation of NADH dehydrogenase treated with 1.0 M $Cl_3CCOONa$ in the absence of freezing and thawing, but optimal conditions were not established.

Iron, Acid-Labile Sulfide, and Flavin Contents. Subunit I and the subunit II + III fractions both contained iron and acid-labile sulfide (Table I). The combined contents per milligram of protein were approximately 5% less than that of the original NADH dehydrogenase (Galante & Hatefi, 1979), showing that resolution by $Cl_3CCOONa$ caused only minimal further destruction of iron–sulfur centers. Subunit I contained, on average, 3.3 g-atoms of iron or acid-labile sulfide/51 000 g of protein while the subunit II + III preparation contained 2.2 g-atoms of iron or acid-labile sulfide/33 000 g of protein. The latter figure may be overestimated for two reasons. First, the molecular weight of the dehydrogenase based on gel filtration or on FMN content is some 15% smaller than the sum of the apparent subunit molecular weights derived from Na-$DodSO_4$ gel electrophoresis (Galante & Hatefi, 1979). Thus the true molecular weight of the subunit II + III preparation may be rather lower than M_r 33 000. Second, subunit III does not contain enough cysteine residues to bind an iron–sulfur center (Galante & Hatefi, 1979). The iron–sulfur center in the subunit II + III preparation is therefore probably associated with subunit II. Since the subunit II + III preparation is slightly deficient in subunit III, this would have the effect of increasing the iron content per milligram of total protein. We conclude, therefore, that subunit II contains two iron and two acid-labile sulfide atoms per molecule.

Neither of the two subunit preparations contained FMN (less than 0.5 nmol/mg of protein in each). It appears that FMN is released from the protein during the resolution process, and all attempts to keep it in protein-bound form by varying the resolution conditions were unsuccessful. We, therefore, have not been able to determine which subunit originally bound the FMN.

The iron and acid-labile sulfide assays clearly established the presence of at least two, and possibly three, iron–sulfur centers in NADH dehydrogenase. This point is dealt with in greater length in the following section.

EPR Spectroscopy of NADH Dehydrogenase and Its Subunits. EPR spectroscopy of NADH dehydrogenase reveals the presence of at least two distinct species of iron–sulfur centers which differ in their spectral line shape and spin relaxation behavior. Figure 3 shows spectra of dithionite-reduced enzyme at a microwave power setting of 1 mW and at three different sample temperatures. The spectrum of a slowly

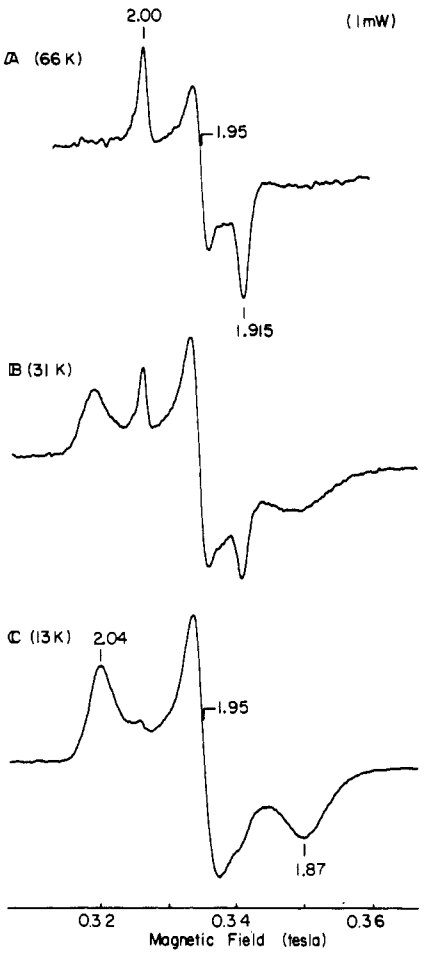

FIGURE 3: EPR spectra of NADH dehydrogenase. NADH dehydrogenase (4.47 mg of protein/mL) was reduced with dithionite, and spectra were recorded at the indicated temperatures. EPR conditions were as follows: microwave frequency 9.138 GHz; modulation amplitude 1.25×10^{-3} T; microwave power 1 mW; time constant 0.128 s; scanning rate 5×10^{-2} T/min.

relaxing species is selectively seen at 66 K, at which temperature the rapidly relaxing species is undetectable because of extreme lifetime broadening (Figure 3A). The slowly relaxing species has a spectrum of rhombic symmetry with g values of 2.00 (g_z), 1.95 (g_y), and 1.915 (g_x). At 13 K (Figure 3C) the signal from this species is mostly saturated, and a spectrum arising only from a rapidly relaxing species is seen with g values of 2.04 (g_z), 1.95 (g_y), and 1.87 (g_x). Under these EPR conditions, this signal is also partly saturated by the input microwave power. At an intermediate temperature (31 K) (Figure 3B), the spectrum shows contribution from both species. The line shapes of these iron–sulfur centers do not resemble the spectra seen in intact complex I. Double integration of the signals in a number of NADH dehydrogenase preparations gave spin concentrations of between 0.50 and 0.85 per FMN. Of this, the slowly relaxing signal accounted for approximately 20% and the rapidly relaxing signal, 80%.

Figure 4 shows EPR spectra of the purified subunit I and subunit II + III fractions reduced by dithionite. The spectra were obtained at 55 K and a microwave power of 1 mW. Unlike the EPR signals of NADH dehydrogenase (Figure 3), the spectra of the purified subunits were detectable over a much wider temperature range up to 193 K, indicating a very slow spin relaxation. Double integration gave spin concentrations of 0.2 per molecule of subunit I and 0.5 per molecule of subunit II or III. Both subunit fractions show distinct spectra which are quite different from those seen in unresolved NADH dehydrogenase.

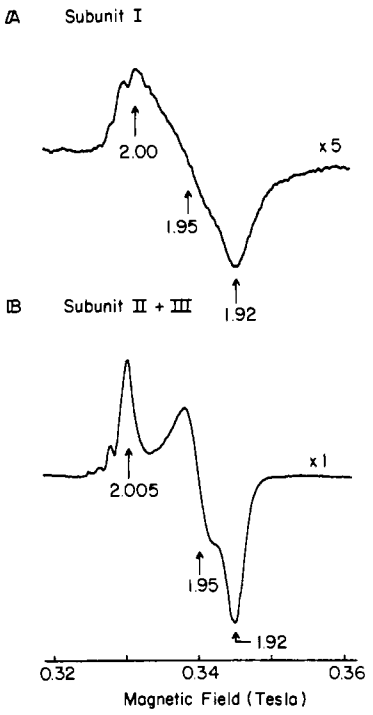

FIGURE 4: EPR spectra of subunit fractions from NADH dehydrogenase. Subunit I (5.56 mg of protein/mL) and the subunit II + III fraction (4.29 mg of protein/mL) were reduced with dithionite. EPR conditions were as follows: microwave frequency 9.271 GHz; modulation amplitude 1×10^{-3} T; microwave power 1 mW; time constant 0.25 s; scanning rate 2.5×10^{-2} T/min; sample temperature 55 K.

Visible Spectroscopy of NADH Dehydrogenase and Its Subunits. Figure 5 shows visible spectra of the dehydrogenase and the purified subunit I and subunit II + III fractions. The procedure used for analyzing the contributions of flavin and iron–sulfur chromophores to the spectrum of NADH dehydrogenase was essentially the same as those of Hatefi & Stempel (1969) and Davis & Hatefi (1971). The dehydrogenase spectra were generally similar to those reported by Hatefi & Stempel (1969) except that the iron absorption (trace 3) was considerably greater both per milligram of protein and per atom of iron. The dehydrogenase preparations of Hatefi & Stempel (1969) were isolated from urea-resolved complex I and contained only four atoms of iron or acid-labile sulfide per mole of FMN. NADH caused a bleaching of the enzyme (trace 2) which was somewhat greater than that expected from reduction of the FMN only, and the spectrum of NADH-reduced enzyme (trace 2) showed a lower absorption up to 600 nm than that of the iron–sulfur contribution (trace 3). The dithionite-reduced enzyme (trace 4) was even further bleached throughout the entire wavelength range used. Reduction of at least part of the iron–sulfur chromophores by dithionite is of course clearly indicated by the EPR results of Figure 3 and by the results of Ohnishi (1979). The FMN contribution to the spectrum is given by trace 5.

The spectra of subunit I and the subunit II + III fractions are obviously those of iron–sulfur proteins. Both were extensively bleached by sodium mersalyl, leaving no residual absorption which could be attributed to flavin, in agreement with the analyses described above. The molar extinction coefficients for the iron–sulfur chromophores at 450 nm were 3.71×10^3, 4.69×10^3, and 5.12×10^3 L mol^{-1} cm^{-1} for the dehydrogenase, subunit I, and subunits II + III, respectively. Curiously, the extinction coefficient increased on resolution of the enzyme.

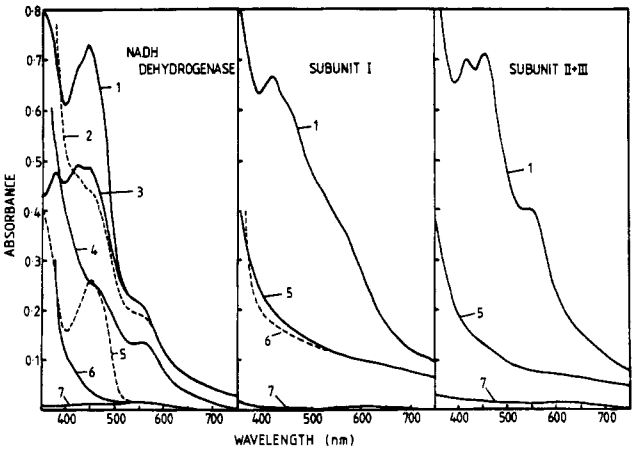

FIGURE 5: Visible spectroscopy of NADH dehydrogenase and subfractions. NADH dehydrogenase (1.62 mg of protein/mL) was dissolved in 2 M urea/50 mM Tris-HCl, pH 7.8. Subunit I was dissolved in 1.5 M urea/50 mM Tris/2 mM DTT adjusted to pH 11 with 1 N NaOH. The pH was then lowered to pH 8.9 by addition of 1.5 M urea/50 mM Tris-HCl, pH 7.8, containing 2 mM DTT. The final protein concentration was 0.71 mg/mL. Subunit II + III (0.80 mg of protein/mL) was dissolved in urea/Tris/DTT buffer at pH 7.8. Conditions were as follows: (1) oxidized minus buffer; (2) NADH (1 mM) reduced minus buffer; (3) oxidized minus mersalyl treated; (4) $Na_2S_2O_4$ reduced minus buffer; (5) mersalyl treated minus buffer; (6) mersalyl and $Na_2S_2O_4$ treated minus buffer; (7) buffer minus buffer. Sodium mersalyl and $Na_2S_2O_4$ were added in solid form.

Discussion

The presence of three distinct iron–sulfur centers in preparations of the dehydrogenase had previously been reported by Orme-Johnson et al. (1974). The enzyme they used was prepared by resolution of complex I with urea and contained only four iron or acid-labile sulfur atoms per molecule of FMN (Hatefi & Stempel, 1969). Despite this, the EPR spectrum they reported seems very similar to that of Figure 3B, even though the enzyme used in the current work contained five to six atoms of iron per molecule of FMN. In any of these preparations, not all the iron is EPR active, which may indicate some heterogeneity of the enzyme which is not reflected by the total iron and acid-labile sulfide contents or by the visible spectra.

The spectra of Figure 3 are rather different from those reported for NADH dehydrogenase by Ohnishi et al. (1981). It was found that the slowly relaxing species had g values of 2.03 (g_z), 1.94 (g_y), and 1.92 (g_x), unlike those given here, and that this spectrum strongly resembled center N-1b found in isolated complex I. We have found that the spectrum of the slowly relaxing species is extremely sensitive to handling of the enzyme under aerobic conditions or to freezing and thawing and rapidly changes from a center N-1b type to that of Figure 3A. The rapidly relaxing species is not affected. We have not yet determined whether the EPR spectra of the separated subunit fractions are dependent on the type of spectra exhibited by the parent NADH dehydrogenase, but our present conditions of resolution would clearly promote the rapid conversion of the center N-1b type spectrum to that of Figure 3A.

The properties of isolated subunit I do not allow us to positively identify the type of cluster present. The visible spectrum does not strongly resemble those of either binuclear or tetranuclear clusters (Palmer, 1973), and the iron content of 3.3 mol/mol of subunit does not distinguish between one tetranuclear or two binuclear centers in a protein which has become somewhat iron deficient or a trinuclear center. The EPR spectrum of subunit I resembles that of a binuclear

cluster when protein constraint has been eliminated by the addition of dimethylsulfoxide (Cammack, 1975). The slow relaxation behavior is also indicative of a binuclear cluster. On the other hand, the amino acid analysis of Galante & Hatefi (1979) suggests that there are insufficient numbers of cysteine residues in subunit I to accommodate two binuclear clusters which would be required to account for the observed iron content. An interesting possibility is that subunit I contains a trinuclear cluster which collapses to a binuclear cluster under the harsh conditions required for solubilization of the protein. This partial denaturation would account for the rather unusual visible and EPR spectra and the low spin concentration. Further work should clarify this point.

There is less ambiguity surrounding the subunit II + III fraction since the iron content (Galante & Hatefi, 1979), and visible and EPR spectra are all consistent with the presence of a single binuclear cluster. While we suggest that this is associated with subunit II, we cannot eliminate the possibility that the cluster is covalently attached to both subunits II and III.

In addition to the possible localization of center N-1b in the NADH dehydrogenase, the low potential of center N-1a (Ohnishi, 1979; Ohnishi et al., 1981) suggests that this center should also be close to the site of interaction of the enzyme with NADH which is known to be on subunit I (Chen & Gullory, 1981).

Despite the alterations in EPR line shape, the centers proved surprisingly stable to the methods used for purification of the subunit fractions. This prompted us to try similar procedures with the iron–sulfur protein fraction of complex I, and a partial purification of three distinct iron–sulfur proteins has been achieved (C.I. Ragan, Y.M. Galante, and Y. Hatefi, unpublished observations). The possibility of purifying most if not all of the iron–sulfur proteins of complex I has important implications for future investigations of the structure of this enzyme. Interpretation of protein-labeling studies of complex I in isolation and in the membrane (Smith & Ragan, 1980) was severely restricted by lack of knowledge of which redox centers were associated with which polypeptide. Purification of the iron–sulfur proteins not only provides this information in the most direct way but also opens up the possibility of more detailed studies on the localization of the redox centers within the polypeptides and ultimately on their localization in the intact enzyme and in the membrane.

Acknowledgments

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