DNA polymerases $\beta$ and $\gamma$. Consequently, the relation of DNA polymerase $\delta$ from bone marrow to the $\alpha$-type DNA polymerase is an important issue. It is conceivable that $\delta$ is derived from $\alpha$ by the loss of a 13000 molecular weight polypeptide. Proteolytic peptide analyses to explore a possible primary structure relationship between $\delta$ and $\alpha$ are necessary to explore such a possible derivative relationship. However, it is now clear that $\delta$ is distinct from $\alpha$ in the integral presence of a 3′–5′-exonuclease and in molecular structure. The roles of DNA polymerases $\alpha$ and $\delta$ in cellular DNA replication remain an unanswered question.

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# Purification of Three Iron–Sulfur Proteins from the Iron–Protein Fragment of Mitochondrial NADH–Ubiquinone Oxidoreductase†

C. Ian Ragan,* Yves M. Galante, and Youssef Hatefi

ABSTRACT: A fragment containing non-heme iron and acid-labile sulfide but little flavin can be solubilized from the mitochondrial NADH–ubiquinone oxidoreductase complex with chaotropic agents. This iron–protein fragment [Hatefi, Y., & Stempel, K. E. (1969) *J. Biol. Chem. 244*, 2350] has been resolved with detergents and ammonium sulfate fractionation into iron and acid-labile sulfide containing fractions, here called ISP-I and ISP-(II + III). ISP-I consists predominantly of a single polypeptide of molecular weight 75000. ISP-(II + III) consists predominantly of three polypeptides in equimolar concentrations with molecular weights of 49000, 30000, and 13000. Treatment of the latter with sodium trichloroacetate followed by ammonium sulfate fractionation results in separation of the 49000 molecular weight polypeptide from the two smaller subunits. Both of these subfractions (ISP-II and ISP-III, respectively) contain non-heme iron. The three iron–sulfur proteins have been characterized by their absorption spectra and iron and acid-labile sulfide contents. On the basis of the distribution of iron among the fractions obtained from chaotropic resolution of the NADH–ubiquinone oxidoreductase complex, a minimum of six or seven iron–sulfur centers are present in this enzyme.

There is still considerable uncertainty as to the number and cluster structure of the iron–sulfur centers of mitochondrial NADH–ubiquinone oxidoreductase (e.g., Ohnishi, 1979). While such uncertainty exists, it is extremely difficult to propose plausible pathways of electron transfer within the enzyme and to suggest mechanisms by which this process is coupled to proton translocation across the inner mitochondrial membrane. EPR[1] spectroscopy has been invaluable in providing information on the iron–sulfur clusters of the enzyme, but the technique suffers from several drawbacks. First, the line shape and intensity of the EPR signals are extremely sensitive to apparently trivial modification of the enzyme during purification. Second, the midpoint potentials of certain centers are also prone to extensive alterations, making their detection difficult in certain instances (e.g., Ohnishi et al., 1981). Third, the spin–spin interaction between neighboring clusters can render them "EPR silent".

Despite these problems, it is widely agreed that there are at least four centers in the enzyme (designated N-1, N-2, N-3, and N-4 by Ohnishi) as originally put forward by Orme-

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[1] Abbreviations: EPR, electron paramagnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; ISP, iron–sulfur protein; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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Johnson et al. (1974). The existence of at least two N-1 type centers is also agreed (Hearschen et al., 1981), but their spin concentration and also that of the putative center N-5 (e.g., Ohnishi, 1979) are less than anticipated on the basis of the flavin content of the NADH–ubiquinone reductase complex. When EPR is used, the N-1 centers are reported to be binuclear (Albracht & Subramanian, 1977; Salerno et al., 1977) while N-2, -3, and -4 were found to be tetranuclear (Albracht & Subramanian, 1977). However, core extrusion on a soluble, high molecular weight NADH dehydrogenase preparation revealed four binuclear and only two tetranuclear centers per flavin (Paech et al., 1981).

The approach adopted by us in a previous paper (Ragan et al., 1982) is to separate and purify individual iron–sulfur proteins of the enzyme. The advantages of this method are that the existence of a cluster can be unambiguously demonstrated, its cluster structure can be determined by core extrusion without interference from other centers, and the separation may allow previously EPR-silent centers to become visible. In addition, the identification of a center with a particular polypeptide enables us to relate information on the structure of the enzyme (e.g., Smith & Ragan, 1980) to its function.

The basis of the purification method is the use of chaotropic agents to separate the NADH–ubiquinone oxidoreductase complex [complex I of Hatefi et al. (1962)] into three fragments (Hatefi & Stempel, 1969): a soluble iron–sulfur flavoprotein with NADH dehydrogenase activity, a soluble iron–sulfur protein fraction, and an insoluble hydrophobic protein fraction. The "NADH dehydrogenase" (Galante & Hatefi, 1979) or "flavoprotein" fragment (Heron et al., 1979) consists of three subunits and contains the NADH binding site (Chen & Guillory, 1981) and the FMN of the original enzyme (Galante & Hatefi, 1979). Further fragmentation (Ragan et al., 1982) revealed that the two largest subunits were both iron–sulfur proteins. The "iron–protein" fragment, which accounts for approximately 20% of complex I protein, is the subject of the present paper. We show that three distinct iron–sulfur proteins can be purified from this material and describe their properties in isolation. In addition, we provide evidence that the third fragment obtained from chaotropic resolution of complex I contains at least two additional iron–sulfur centers.

## Materials and Methods

*Preparation of the Iron–Protein Fragment.* Complex I was isolated from beef heart mitochondria by the method of Hatefi & Rieske (1967) and stored as ammonium sulfate pellets at −70 °C. When required, the enzyme was thawed, dissolved in 0.25 M sucrose/50 mM Tris-HCl, pH 8.0, containing 2 mM DTT, and dialyzed against 10 volumes of the same buffer for 1 h at 4 °C. We have found that dialysis of the enzyme in this way improves the purity of the iron–protein fragment. Moreover, when it is intended to purify the dehydrogenase as well, dialysis helps to prevent floating of the final ammonium sulfate precipitate with consequent improvement to the yield. The slightly turbid solution of complex I was then resolved with $NaClO_4$ and the iron–protein fragment isolated by ammonium sulfate fractionation as described by Galante & Hatefi (1979). The pellets were dissolved in sucrose–Tris–DTT buffer at a protein concentration of approximately 10 mg/mL and centrifuged at 90000*g* for 15 min to remove a small amount of insoluble material. The solution was then stored in 1- or 2-mL portions at −70 °C.

*Preparation of ISP-I.* All operations were carried out at 0–4 °C. Small scale purifications using 10–15 mg of the iron–protein fragment were performed as follows. The iron–protein fragment was diluted to 5 mL with 50 mM Tris-HCl, pH 7.8, containing 2 mM DTT (the final protein concentration was 2–3 mg/mL). A 10% (w/v) solution of sodium deoxycholate was added to a final concentration of 0.3% (w/v), and the solution was fractionated with cold, neutralized and saturated ammonium sulfate. After each addition of ammonium sulfate, the solution was allowed to stand for 10 min, and precipitated material was pelleted by centrifugation at 40000*g* for 15 min. Protein precipitated between 0 and 0.040 saturation was discarded while the fraction obtained between 0.040 and 0.0725 saturation was dissolved in Tris–DTT buffer to give a clear solution. Urea (120 mg) was dissolved in the solution and the volume adjusted to 1 mL. Sodium deoxycholate (10% w/v) was added to give a final concentration of 0.3% (w/v), and ammonium sulfate fractionation was carried out as described above. The material precipitated between 0.0525 and 0.0825 saturation (ISP-I) was dispersed in 0.5 mL of Tris–DTT buffer to give a somewhat turbid solution. Solubility could be improved by brief exposure to ultrasonic irradiation.

*Preparation of ISP-II and ISP-III.* All operations were carried out at 0–4 °C. Small scale purifications were carried out as follows. The iron–protein fragment (10–15 mg of protein) was diluted to a final volume of 5 mL with Tris–DTT buffer containing a final concentration of 1% (w/v) octyl glucoside. Saturated, neutralized ammonium sulfate was added to 0.35 saturation and the precipitated material collected by centrifugation as described above. The precipitate was thoroughly homogenized by hand in 0.8 mL of Tris–DTT buffer and allowed to stand for 30 min. The suspension was then centrifuged at 40000*g* for 30 min, and the supernatant, containing ISP-(II + III), was removed.

For separation of the two iron–sulfur proteins, 4 M $Cl_3C$-COONa was added to a final concentration of 1 M, and the clear solution was then frozen in liquid $N_2$. After thawing, the turbid solution was centrifuged at 40000*g* for 15 min. The residue (ISP-II) was homogenized in 0.3 mL of Tris–DTT buffer. To the supernatant, containing ISP-III, was added ammonium sulfate to 0.30 saturation, and the precipitate (ISP-III) was collected by centrifugation as above and homogenized in 0.3 mL of Tris–DTT buffer.

*Analytical Methods.* Iron (Doeg & Ziegler, 1962), acid-labile sulfide (Fogo & Popowski, 1949), and protein (Bensadoun & Weinstein, 1976) were assayed as described in the references. For flavin analysis, samples were diluted to 1.3 mL with 50 mM Tris-HCl, pH 7.8, and heated to 100 °C for 3 min in foil-wrapped tubes. Precipitated protein was removed by centrifugation, and the supernatants were assayed for flavin by measuring the change in extinction at 450 nm minus 550 nm following addition of dithionite. Results obtained by this method were identical with those obtained by using trichloroacetic acid to precipitate the protein (Hatefi & Stempel, 1969). All flavin was assumed to be FMN.

*Gel Electrophoresis.* $NaDodSO_4$ gel electrophoresis was carried out in cylindrical gels containing 12.5% (w/v) acrylamide and 0.34% bis(acrylamide) using the conditions described by Weber & Osborn (1969). Alternatively, samples were run on 1-mm thick slab gels containing a concave gradient of acrylamide between 13% (w/v) and 16% (w/v) (Earley & Ragan, 1981). Gels were stained and destained by the method of Weber & Osborn (1969). Molecular weights were determined by comparison with standard markers obtained from British Drug Houses (Poole, United Kingdom).

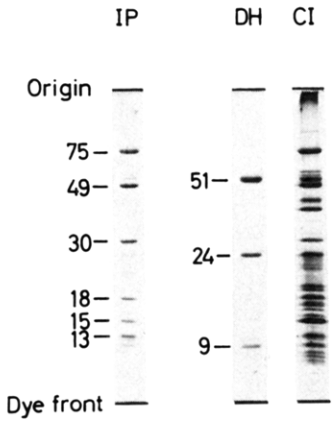


FIGURE 1: Polypeptide composition of complex I and its subfractions. Samples were analyzed by electrophoresis on gradient gels as described under Materials and Methods. IP, iron–protein fragment (9 μg of protein); DH, NADH dehydrogenase (3.6 μg of protein); CI, complex I (45 μg of protein). Molecular weights in thousands are indicated to the left of the tracks.

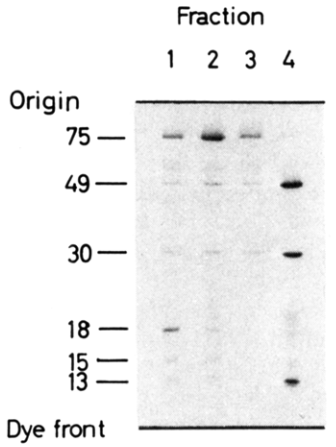


FIGURE 2: Polypeptide composition of fractions obtained from deoxycholate/ammonium sulfate treatment of the iron–protein fragment. The iron–protein fragment was treated with deoxycholate and fractionated with ammonium sulfate as described under Materials and Methods. Samples were analyzed on gradient gels. (Fraction 1) Material precipitated at 0.040 saturation with ammonium sulfate (1.9 μg of protein); (fraction 2) material precipitated between 0.040 and 0.0725 saturation (2.1 μg of protein); (fraction 3) material precipitated between 0.0725 and 0.0875 saturation (1.0 μg of protein); (fraction 4) material precipitated between 0.0875 and 0.140 saturation (4.9 μg of protein). The relative amounts of protein added are in proportion to the yields obtained. Molecular weights in thousands are indicated.

## Results

*Properties of the Iron–Protein Fragment.* The resolution of complex I by chaotropic agents was originally described by Hatefi & Stempel (1967), who showed that approximately 20–30% of the protein can be rendered water soluble. This soluble material can be separated by ammonium sulfate precipitation into two distinct and well-defined fractions, the iron–protein fragment and the NADH dehydrogenase (Hatefi & Stempel, 1969; Galante & Hatefi, 1979) or "flavoprotein fragment" (Heron et al., 1979). The iron–protein fragment isolated from $NaClO_4$-resolved complex I contains 37–48 nmol of Fe/mg of protein and similar concentrations of acid-labile sulfide (Hatefi & Stempel, 1967). The polypeptide composition is shown in Figure 1 alongside that of NADH dehydrogenase and complex I. As reported previously (Heron et al., 1979; Earley & Ragan, 1981), the iron–protein fragment contains subunits of molecular weights 75 000, 49 000, 30 000, 18 000, 15 000, and 13 000. The last band probably contains more than one polypeptide (Heron et al., 1979). Also present are traces of the NADH dehydrogenase subunits ($M_r$ 51 000, 24 000, and 9000; Galante & Hatefi, 1979) and minor amounts of several polypeptides with molecular weights in the region of 20 000. The iron–protein fraction is soluble in the absence of added detergents and chaotropic agents and appears to form a single structural entity since no separation of the subunits can be achieved by gel filtration or ammonium sulfate fractionation, and the three largest polypeptides at least are present in a 1:1:1 molar ratio (Ragan, 1976). From the sum of the subunit molecular weights and the iron content, it is clear that more than one iron–sulfur center must be present in this fragment.

*Fractionation with Detergents and Ammonium Sulfate.* In the presence of detergents such as Triton X-100, deoxycholate, cholate, or octyl glucoside, ammonium sulfate fractionation of the iron–protein fragment causes separation of the subunits into distinct fractions. With increasing ammonium sulfate concentration, fractions enriched in the 18-, 75-, and (49 + 30 + 13)-kdalton subunits are sequentially precipitated. The 15-kdalton polypeptide is distributed throughout these fractions while the NADH dehydrogenase impurity remains soluble. All the above detergents give the same kind of fractionation pattern, but deoxycholate gives the cleanest separations (Figure 2). Fraction 1, enriched in the 18-kdalton polypeptide, contains less iron (30–35 nmol/mg of protein) than the starting material, while fraction 2, enriched in the 75-kdalton subunit, contains more iron (40–51 nmol/mg of protein) than the starting material. Thus we assume that the 75-kdalton subunit, unlike the 18-kdalton subunit, is an iron–sulfur protein. The other fraction with substantial amounts of iron (fraction 4) contains the 49-, 30-, and 13-kdalton subunits.

Higher concentrations of ammonium sulfate are required to precipitate the iron–protein subunits when octyl glucoside is used. The 75-kdalton subunit does not separate well from the 18- and 15-kdalton subunits, but the (49 + 30 + 13)-kdalton fraction routinely contains approximately 25% more iron per milligram of protein than the corresponding fraction obtained with deoxycholate. These three subunits are the only ones which are freely soluble after precipitation with ammonium sulfate, and they could therefore be easily purified by precipitating all subunits and extracting the pellet with buffer. We have therefore used octyl glucoside to purify this fraction and deoxycholate to purify the 75-kdalton subunit. Improvements in the purity of the latter subunit can be achieved by a second fractionation by ammonium sulfate in the presence of deoxycholate and urea.

*Fractionation with $Cl_3CCOONa$ and Ammonium Sulfate.* Treatment of the iron–protein fraction with high concentrations of $Cl_3CCOONa$ followed by freezing in liquid $N_2$ and thawing causes dissociation of the subunits, some of which are precipitated. Further separation can be achieved by ammonium sulfate fractionation of the soluble material. While we were not able to purify any single iron–sulfur protein directly by this method, we did find that the 49-kdalton subunit could be separated from the 30- and 13-kdalton subunits. We therefore applied the same procedure to the (49 + 30 + 13)-kdalton fraction obtained from octyl glucoside treatment. After a solution of this fraction was frozen and thawed in the presence of 1 M $Cl_3CCOONa$, the 49-kdalton subunit precipitated while the 30- and 13-kdalton subunits could be recovered from the supernatant by precipitation with ammonium sulfate (Figure 3). Lower concentrations of $Cl_3CCOONa$ did not give such clean separation of the subunits.

In a subsequent description of the properties of the subunits, we refer to the 75-, 49-, and (30 + 13)-kdalton fractions as ISP-I, -II, and -III, respectively. The unresolved (49 + 30

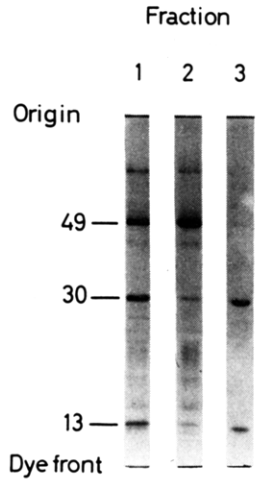


FIGURE 3: Polypeptide composition of fractions obtained from octyl glucoside/ammonium sulfate treatment of the iron–protein fragment and further resolution by $Cl_3CCOONa$. The iron–protein fragment was precipitated by ammonium sulfate in the presence of octyl glucoside and the soluble fraction [ISP-(II + III)] extracted as described under Materials and Methods. This was then resolved with $Cl_3CCOONa$ into ISP-II and ISP-III. Samples were analyzed on gradient gels. (Fraction 1) 2.8 μg of ISP-(II + III) protein; (fraction 2) 4.2 μg of ISP-II protein; (fraction 3) 2.0 μg of ISP-III protein. Molecular weights in thousands are indicated.

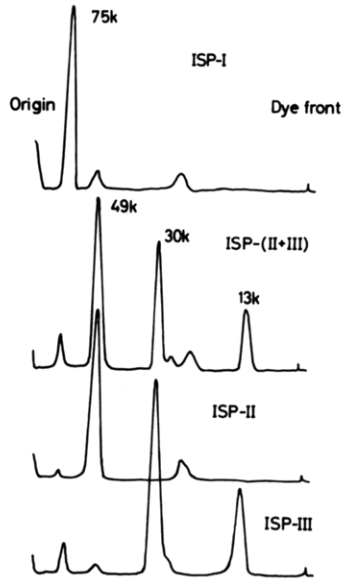


FIGURE 4: Polypeptide composition and purity of isolated iron–sulfur proteins. Samples were analyzed on cylindrical gels using the Weber & Osborn (1969) system. Amounts of protein applied to the gels were as follows: ISP-I, 15 μg; ISP-(II + III), 25 μg; ISP-II, 10 μg; ISP-III, 15 μg. Molecular weights in thousands are indicated.

Table I: Iron and Acid-Labile Sulfide Contents of Isolated Iron–Sulfur Proteins[a]

| | Fe | | S | |
|---|---|---|---|---|
| | ng-atom/mg of protein | mol/ mol of protein | ng-atom/mg of protein | mol/ mol of protein |
| ISP-I | 45 ± 5 (4) | 3.4 | 43 ± 3 (4) | 3.2 |
| ISP-(II + III) | 39 ± 5 (7) | 3.6 | 36 ± 4 (4) | 3.3 |
| ISP-II | 38 ± 4 (4) | 1.9 | 35 ± 3 (3) | 1.7 |
| ISP-III | 29 ± 2 (5) | 1.25 | 30 ± 3 (3) | 1.3 |

[a] Figures are given as the mean ± the standard error. Values in parentheses show the number of different preparations assayed. The molecular weights of the proteins were taken as 75 (ISP-I), 92 [ISP-(II + III)], 49 (ISP-II), and 43 (ISP-III) kdaltons.

+ 13)-kdalton fraction is ISP-(II + III).

*Solubility Properties.* The final preparations of ISP-I, -II, and -III are all insoluble at neutral pH. Solutions may be obtained by increasing the pH to, e.g., 11, but the iron–sulfur centers decolorize rather rapidly under these conditions. The rather less pure ISP-I obtained after the first fractionation with deoxycholate and ammonium sulfate is, however, quite soluble probably because of bound detergent. ISP-(II + III) is also soluble for the same reason, while ISP-III is soluble in the presence of 1 M $Cl_3CCOONa$ and before precipitation with ammonium sulfate.

*Purity of the Iron–Sulfur Proteins.* $NaDodSO_4$ gels of the iron–sulfur proteins are shown in Figure 4. All contained minor amounts of contaminating polypeptides but attempts to remove these by further fractionation with detergents or chaotropic agents did not meet with any success. From staining intensities, we estimate the purities of the samples shown in Figure 4 to be 80% (ISP-I), 84% (ISP-II + III), 84% (ISP-II), and 90% (ISP-III). The three major subunits of ISP-(II + III) seem to be present in a 1:1:1 molar ratio.

*Iron and Acid-Labile Sulfide Contents.* Table I shows the iron and acid-labile sulfide contents of several preparations of the iron–sulfur proteins. ISP-I contains, on average, 3.3 mol of Fe or S/mol of subunit. This value is consistent with the presence of a trinuclear center or if the protein had lost some iron during purification with a tetranuclear or two binuclear centers. Similar amounts of iron and acid-labile sulfide are also present in ISP-(II + III), but subsequent separation of ISP-II and ISP-III leads to some losses. These two proteins contain, on average, 1.8 (ISP-II) and 1.25 (ISP-III) mol of Fe or S/mol of protein. These values are consistent with the presence of binuclear centers in each of these proteins. Recovery of iron during fractionation of the iron–protein fragment with deoxycholate or octyl glucoside is quite high. The averaged iron content per milligram of protein of all the recovered fractions is routinely 80–90% of that of the starting material. Thus we do not think that any of the separated iron–sulfur proteins have become substantially iron deficient during purification from the iron–protein fragment. The possibility remains that the iron–protein fragment itself loses iron–sulfur centers during its purification from complex I. This possibility is dealt with later.

*Visible Spectra of the Iron–Sulfur Proteins.* The purest forms of ISP-I, -II, and -III are insoluble except at very high pH under which conditions the iron–sulfur chromophore may be modified. The spectra shown in Figure 5 were obtained, where possible, from somewhat less pure samples which were soluble at neutral pH. For example, the spectrum of ISP-I was obtained from protein which had been through only one cycle of deoxycholate/salt fractionation, while that of ISP-III was obtained from the supernatant remaining after removal of ISP-II. ISP-(II + III) is soluble as prepared while ISP-II was solubilized at alkaline pH. Urea was also present in the samples to minimize the development of turbidity during addition of mersalyl and dithionite (Hatefi & Stempel, 1969). All spectra are those of iron–sulfur proteins. Dithionite caused almost as much bleaching as mersalyl, indicating that the iron–sulfur centers can still be reduced. Residual absorbance in the presence of mersalyl can be attributed to turbidity. This is particularly evident in the spectrum of ISP-II which proved difficult to solubilize completely without loss of the iron–sulfur center. The molar extinction coefficients for the iron–sulfur chromophore at 450 nm (oxidized minus reduced) were 3970 (ISP-I), 3010 (ISP-II + III), 3250 (ISP-II), and 4960 (IS-

Table II: Recovery of Iron, Flavin, and Protein during Chaotropic Resolution of Complex I[a]

| fraction | protein (mg) | | Fe (ng-atom) | | FMN (nmol) | |
|---|---|---|---|---|---|---|
| | a | b | a | b | a | b |
| complex I | 100 | 100 | 2210 | 2210 | 97.6 | 97.6 |
| P | 74.1 | 74.1 | 532 | 532 | 3.0 | 3.0 |
| S1 | 27.6 | 29.7 | 1435 | 1544 | 87.0 | 93.9 |
| S2 | 1.9 | | 192 | | 5.8 | |
| S1 + S2 | 29.5 | 29.7 | 1627 | 1544 | 92.8 | 93.9 |
| S1 + S2 + P | 103.6 | 103.8 | 2159 | 2076 | 95.8 | 96.9 |
| IP | | 19.2 | | 925 | | 10.0 |
| MAS | | 1.41 | | 61 | | 2.7 |
| DH | | 4.89 | | 381 | | 61.1 |
| S3 | | 0.74 | | 149 | | 16.5 |
| P + IP + MAS + FP + S3 | | 100.3 | | 2144 | | 93.3 |
| % recovery | | 100.3 | | 97.0 | | 95.6 |

[a] Complex I (50 mg) was dialyzed and resolved with 0.5 M $NaClO_4$ at 35 °C for 10 min as described by Galante & Hatefi (1979). A sample was taken for assays. Soluble and particulate fractions were separated by centrifugation at 70000*g* for 30 min. The supernatant, S1, was retained for assays and for further fractionation. The pellet was homogenized by hand in sucrose–Tris–DTT buffer to a final volume of 4.0 mL and further treated with 0.5 M $NaClO_4$ at 35 °C for 10 min. Centrifugation as above was used to separate the soluble (S2) and particulate (P) fractions. The latter was homogenized in buffer to a final volume of 2.1 mL. The first soluble fraction, S1, was further treated with saturated and neutralized ammonium sulfate as described by Hatefi & Stempel (1969). The iron–protein fraction (IP) was obtained between 0 and 0.275 saturation, the middle ammonium sulfate fraction (MAS) between 0.275 and 0.364 saturation, and the NADH dehydrogenase fraction (DH) between 0.364 and 0.529 saturation. These precipitates were dissolved in buffer to give final volumes of approximately 1 mL. S3 is the supernatant remaining after removal of the DH fraction. All fractions were assayed for protein, iron, and flavin as described under Materials and Methods. Corrections were made for sampling losses, and all figures are expressed per 100 mg of complex I protein for convenience. Figures in columns a refer to the actual recovery of S1, a portion of whose volume is trapped in the pellet. This trapped material should appear in S2, and P should be free of all but traces of solubilized material.. Figures for S1 in columns b are obtained by multiplying the protein, iron, or flavin content per milliliter of S1 by the total, not recovered, volume, thus correcting for the trapped material. If S2 contains only the trapped portion of S1, S1 + S2 in columns a should equal S1 in columns b, and S1 + S2 + P in columns a or S1 + P in columns b should equal complex I. Figures for the ammonium sulfate fractions are based on columns b. As discussed in the text, additional iron, over and above that expected from residual S1, is present in S2. The final recovery of iron (last line) is corrected for this additional loss.

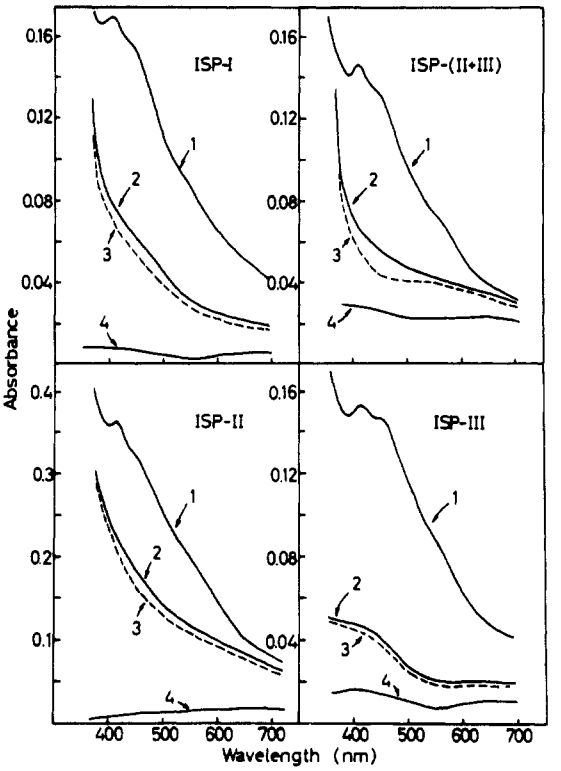


FIGURE 5: Absorption spectra of isolated iron–sulfur proteins. ISP-I was obtained after only one cycle of deoxycholate/ammonium sulfate precipitation while ISP-III was not precipitated by ammonium sulfate after removal of ISP-II as explained in the text. All samples were diluted with 1.0 M urea/50 mM Tris-HCl, pH 7.8, containing 2 mM DTT. ISP-II was not soluble at this pH and was dissolved by addition of 1 M NaOH to a final pH of approximately 10.5. Spectra were run as follows: (1) oxidized minus buffer; (2) $Na_2S_2O_4$-reduced minus buffer; (3) $Na_2S_2O_4$-reduced and sodium mersalyl treated minus buffer; (4) buffer minus buffer (base line). Protein concentrations were the following: ISP-I, 0.63 mg/mL; ISP-(II + III), 0.70 mg/mL; ISP-II, 1.32 mg/mL; ISP-III, 0.66 mg/mL.

Table III: Iron and Flavin Distribution in Principal Fractions Obtained by Chaotropic Resolution of Complex I[a]

| fraction | FMN (nmol/mg of protein) | Fe (ng-atom/mg of protein) | Fe/complex I FMN (mol/mol) |
|---|---|---|---|
| complex I | 0.976 | 22.1 | 22.6 |
| P | 0.040 | 7.2 | 6.6 |
| IP | 0.52 | 48.2 | 9.3 |
| DH | 12.5 | 77.9 | 6.2 |

[a] The numbers in the last column for P and IP refer to original complex I flavin. Values were calculated from Table II as follows. For complex I the values were calculated from line 1 of Table II. For P, the iron content (532 nmol) was corrected for residual DH [(3.0/61.1) × 381 = 19 nmol of Fe] and contaminating complex III (6 nmol of Fe) giving 507 nmol of Fe or 5.2 mol/mol of complex I FMN. Losses of iron in S2 [192 − [(1.9/27.6) × 1435] = 93 nmol of Fe] and in S3 [149 − [(16.5/61.1) × 381] = 46 nmol of Fe] amounted to 139 nmol or 1.4 mol/mol of complex I FMN. For IP, the iron contents of IP and MAS were added (986 nmol of Fe) and corrected for residual DH [[(10 + 2.7)/61.1] × 381 = 79 nmol of Fe], giving a final value of 907 nmol or 9.3 mol/mol of complex I FMN. For DH, measured iron and flavin contents in this fraction were used to calculate the Fe/FMN ratio. Further explanation of the corrections is given in the text.

P-III) L (mol of Fe)$^{-1}$ cm$^{-1}$. Spectra of more pure ISP-I and ISP-III obtained from alkali-solubilized samples were very similar to those of Figure 5.

*Recovery and Distribution of Iron and Flavin during Chaotropic Resolution of Complex I.* The iron to flavin ratio of complex I and similar preparations is sufficiently high and imprecise to make it difficult to estimate the number of possible iron–sulfur centers from the stoichiometry. An attempt to get around this problem is shown in Tables II and III where we followed the distribution of protein, iron, and flavin into the various fractions obtained from chaotropic resolution of complex I.

As shown in Table II, the supernatant from $NaClO_4$ treatment of 100 mg of complex I (S1) contains 27.6 mg of protein, while a second treatment of the residue with $NaClO_4$ solubilizes a further 1.9 mg of protein (S2). The amount of S1 protein trapped in the pellet is given by the difference between the figures calculated from recovered or initial volume (columns a and b, respectively) and comes to 2.1 mg. This is very close to the 1.9 mg recovered in S2 and shows that solubilization is essentially complete after the first $NaClO_4$ treatment. In support of this, the polypeptide composition of S2 is the same as that of S1 (results not shown). Subsequent

calculations of the protein recovered in the ammonium sulfate fractions are based on the column b figures, i.e., total volume, to take account of the trapped material in the pellet fraction, P.

Similar treatment of the figures for iron distribution reveals that S2 contains rather more iron that expected from its protein content. Thus, the iron to protein ratio is 101 nmol/mg compared with 52 nmol/mg in S1. The difference can be attributed to loss of iron from centers belonging to the pellet fraction, P. The amount of such loss (approximately 90 nmol) may also have occurred during the first $NaClO_4$ treatment and, of course, will decrease the iron content of the pellet fraction, P. Of the iron in S1, much of it ends up in the iron–protein fraction (IP) and the dehydrogenase (DH). The iron in the middle ammonium sulfate cut (MAS) seems to be due to residual IP, since the iron to protein ratios are similar (44 and 48 nmol/mg, respectively) and the flavin content is low. The iron to protein ratio of the ammonium sulfate supernatant (S3) is considerably higher than that of any other fraction (201 nmol/mg) presumably because iron which has been lost from iron–sulfur centers ends up in this fraction.

The flavin to protein ratio of S1 and S2 is very similar, and only traces remain in the pellet fraction, P. Thus, unlike protein and iron, virtually all FMN is solubilized by the first $NaClO_4$ treatment. While the bulk of the flavin is found in the DH fraction, appreciable amounts are found in IP, which, as shown in Figure 1, contains the DH polypeptides in small amounts. Considerable amounts of flavin are also found in the S3 fraction. Since the flavin content of the dehydrogenase agrees very well with 1 mol of FMN/mol of enzyme (Galante & Hatefi, 1979), it is unlikely that the isolated enzyme is flavin deficient. Therefore the flavin in the S3 fraction is probably due to unprecipitated dehydrogenase. While the flavin to protein ratio of the S3 fraction (22 nmol/mg) is apparently higher than that of DH (12.5 nmol/mg), the protein concentration was extremely low and, therefore, rather inaccurate. The same is true of the iron concentration in S3 which was at the limit of detection. Using the iron to flavin ratio of the DH fraction, we can calculate that, of the 149 nmol of iron in S3, 103 nmol are due to unprecipitated dehydrogenase. Therefore, only 46 nmol have been lost from iron–sulfur centers during the fractionation. Losses of this magnitude or more from the pellet fraction, P, would have been anticipated during the first $NaClO_4$ treatment.

The results are summarized in Table III as ratios between iron, flavin, and protein. The values for the pellet fraction, P, have been corrected for a contribution from the non-heme iron of contaminating complex III (based on the cytochrome *b* content) and of residual dehydrogenase (based on FMN content). No correction could, however, be made for residual IP, though this is likely to be very small. The iron to flavin ratio is calculated on total complex I flavin to give the number of complex I iron atoms which end up in the pellet fraction, P. The value obtained, 5.2, will be underestimated by the amounts of iron lost from this fraction and recovered in S2 and S3. These two fractions contain 1.4 mol of iron/mol of original flavin, suggesting that the true iron content of P is 6.6 mol/mol of complex I flavin.

The iron content of IP has been calculated in a similar way with a small correction for residual dehydrogenase and for recovery of some IP material in the MAS fraction. The iron to flavin ratio of DH is based on recovered iron and flavin in this fraction since there is no reason to suppose that flavin is labilized or that there is more than one flavoprotein in complex I. The sum of the values in the third column of Table III is 22.1 mol of iron/mol of flavin, which agrees well with that found for complex I (22.6 mol of iron/mol of flavin).

## Discussion

In a previous publication, we reported the purification of two iron–sulfur proteins from the NADH dehydrogenase fragment of complex I (Ragan et al., 1982). In total, therefore, we have isolated five iron–sulfur proteins from this enzyme, some of which may contain more than one iron–sulfur center. With the assumption that our analyses for flavin and iron and our calculated results in Table III are correct, then the iron content of the insoluble residue following $NaClO_4$ resolution suggests the presence in that fraction of at least two further centers, bringing the total to a minimum of seven. This is one more than the number suggested from EPR studies (e.g., Ohnishi, 1979) or from core extrusion (Paech et al., 1981). As already pointed out, some centers may be EPR silent, while the core extrusion experiments were performed not on complex I but on a soluble high molecular weight dehydrogenase whose iron content was lower than that of complex I. The iron to flavin ratio of complex I reported in the present paper (22.6) agrees quite well with values obtained by us from other recent preparations (23.9 and 24.0) and with values in the literature [23.1, Ragan & Racker (1973); 22.1, Fry & Green (1981)]. However, the iron content of the preparations used by Paech et al. (1981) averaged only 17.7 mol/mol of FMN, suggesting that iron might have been lost from certain centers during purification. Obviously, similar core extrusion experiments on complex I would clarify the position.

Iron contents expressed per mole of protein are prone to potentially serious systematic errors in the assay of protein concentration and in the determination of molecular weights by $NaDodSO_4$ gel electrophoresis. For this reason, the values obtained may not be a very reliable guide to the cluster structure. Iron contents expressed per mole of original flavin as in Table III are likely to be more accurate, but careful attention must be paid to the recovery of iron during the fractionation process. The results of Tables II and III show that of the original 22.6 iron atoms per complex I flavin, 9.3 can be accounted for by the iron–protein fraction. In a second experiment, the value obtained was 9.1. If we assume a "molecular weight" for the IP fraction of approximately 220000 based on subunit molecular weights from $NaDodSO_4$ gels, the iron content of 37–48 nmol/mg of protein suggests again the presence of nine iron atoms per "molecule". Most of these can be accounted for in the isolated ISP-I and ISP-(II + III) fractions which together account for seven iron atoms on average (Table I) or eight if the individual numbers are rounded up to four per molecule. Whether the difference between eight and the measured values of around nine iron atoms merely reflects the precision with which these numbers can be obtained is not clear, and we cannot totally discount the presence of yet another iron–sulfur protein in the IP fraction.

As yet we can make no firm conclusions as to the cluster structure of these iron–sulfur proteins. The absorption spectra more closely resemble those of typical binuclear centers in proteins than those of tri- or tetranuclear centers (Hall et al., 1974). This would agree with the iron analyses for ISP-(II + III) and the separated ISP-II and ISP-III. However, the iron content of ISP-I is considerably more than can be accounted for by a single binuclear center. Preliminary EPR studies at 12 K revealed the presence of two species of iron–sulfur center in dithionite-reduced IP, one of which fractionated into ISP-I and the other into ISP-(II + III) (C. I. Ragan, Y. M. Galante, Y. Hatefi, and T. Ohnishi, unpublished re-

sults). Further studies are in progress to determine the EPR characteristics of these isolated iron–sulfur proteins and to determine their cluster structure.

In the dehydrogenase fraction, the summed iron contents of the separated subunit fractions (Ragan et al., 1982) account for 5.5 iron atoms per flavin, in satisfactory agreement with the values obtained with the intact enzyme (Galante & Hatefi, 1979; Table 3). Here again, the cluster structures are uncertain although it is likely that at least one binuclear center is present.

In the $NaClO_4$ pellet fraction, P, the corrected iron content of approximately six iron atoms per original flavin clearly points to the presence of more than one iron–sulfur center. Since the measured iron content is appreciably lower than this, one or more of these centers may be rather labile. Purification of any iron–sulfur protein from this fraction presents a formidable problem in view of the low iron content and extreme insolubility.

Identification of the isolated iron–sulfur proteins with centers detected by EPR in intact complex I is going to be very difficult. Ohnishi et al. (1981) have found that an N-1 type center, perhaps N-1b, is present in the dehydrogenase. Center N-2 is the most likely donor of electrons to ubiquinone and its midpoint potential is phospholipid dependent (Ohnishi et al., 1974). Since subunits of the pellet fraction, P, are the only ones susceptible to photolabeling by an arylazidophospholipid analogue (Earley & Ragan, 1981) or by the hydrophobic probe 5-iodonapth-1-yl azide (Earley & Ragan, 1980), it is likely that the iron–sulfur protein containing center N-2 is to be found in this fraction. Which, if any, of the iron–sulfur proteins described in the present paper contain the other centers N-1a, -3, -4, or -5 remains to be answered.

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