An Analysis of the Polypeptide Composition of Bovine Heart Mitochondrial NADH–Ubiquinone Oxidoreductase by Two-Dimensional Polyacrylamide-Gel Electrophoresis

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Purified preparations of Complex I (NADH–ubiquinone oxidoreductase) from bovine heart mitochondria may be resolved into 26 polypeptides by two-dimensional analysis combining isoelectric focusing and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. Similar analyses of the fragments obtained from chaotropic resolution of the enzyme show that each of these fragments contains a distinct and non-overlapping set of polypeptides. Evidence that the polypeptides seen in the intact enzyme are true constituents comes from analyses of immunoprecipitates obtained by allowing Complex I or solubilized submitochondrial particles to react with antisera directed against the whole enzyme and a subfragment of the enzyme.

The mitochondrial NADH–ubiquinone oxidoreductase complex (Complex I of Hatefi *et al.*, 1962), in common with other respiratory assemblies, apparently consists of several different polypeptides. However, this enzyme is far and away the most complicated, since purified preparations may be resolved into at least 16 components when analysed by conventional gel electrophoresis in the presence of SDS (Ragan, 1976). Analysis by discontinuous gel electrophoresis increased the number of resolvable components to 18 (Crowder & Ragan, 1977). These results are broadly in agreement with the findings of others (Hare & Crane, 1974; Dooijewaard *et al.*, 1978*a,b*), although it is obvious from the literature that the number of polypeptides reported is merely a function of the resolving power of the analytical system used. We have therefore used a technique whose resolving power (approx. 1000 polypeptides) should exceed by a comfortable margin the number of components in Complex I, namely two-dimensional gel electrophoresis (O'Farrell, 1975; Ames & Nikaido, 1976; Cabral & Schatz, 1979). To try and determine whether the polypeptides resolved by this method are indeed Complex I subunits or just contaminants, we have also analysed immunoprecipitates obtained by allowing detergent-solubilized Complex I and submitochondrial particles to react with antisera to Complex I and to a fragment of Complex I, the iron-protein fraction (Smith & Ragan, 1978). In addition,

we have identified several impurities in isolated Complex I by two-dimensional analysis of other mitochondrial enzymes.

Materials and Methods

Biological preparations

Submitochondrial particles were prepared by the method of Racker (1962). Complex I (EC 1.6.99.3) and Complex III were prepared by the methods of Hatefi & Rieske (1967) and Rieske (1967) respectively. F₁-ATPase was partially purified as described by Beechey *et al.* (1975). Chaotropic resolution of Complex I followed the procedure described by Hatefi & Stempel (1969), except that NaClO₄ was used instead of urea (Ragan, 1976). The iron-protein and flavoprotein fractions were purified as described by Hatefi & Stempel (1969). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V from Sigma, Poole, Dorset, U.K.) as standard.

One-dimensional SDS/polyacrylamide-gel electrophoresis

Separation on gels (12 cm × 4 mm internal diam.) containing 12.5% (w/v) acrylamide and 0.34% bisacrylamide was as described by Crowder & Ragan (1977), by the procedure of Weber & Osborn (1969). Alternatively, two discontinuous systems were used. Both employed a 3% (w/v) acrylamide and 0.24% bisacrylamide stacking gel (1 cm long) and a running gel of the above composition. The buffers used were those of Laemmli (1970) or the J4179 system (Jovin

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

et al., 1971) described by Neville & Glossman (1974). Protein bands were stained with either Coomassie Blue (Weber & Osborn, 1969) or, in the later experiments, with Brilliant Blue R, both from Sigma.

Two-dimensional gel electrophoresis

The procedure of Cabral & Schatz (1979) was followed. Protein samples were dialysed extensively against distilled water at 4°C before they were dissociated by addition of urea, Nonidet P-40 (BDH, Poole, Dorset, U.K.), pH3-10 ampholines (LKB Instruments, Selsdon, Surrey, U.K.) and β -mercaptoethanol to final concentrations of 9.5M, 2% (w/v), 2% (w/v) and 0.5% (v/v) respectively. Freshly dissociated samples containing up to 100 μ g of protein in 50 μ l were applied to gels (9cm $\times$ 2mm internal diam.) containing 4.5% (w/v) acrylamide, 0.06% bisacrylamide, 9.5M-urea, 2% (w/v) Nonidet P-40 and 2% (w/v) pH3-10 ampholines. Samples were overlaid with 25 μ l of a solution containing 5M-urea and 2% (w/v) pH3-10 ampholines, and the remaining volume of the gel tubes was filled with 0.4% (v/v) ethylenediamine (basic electrode solution). The acidic electrode solution was 0.2% (v/v) phosphoric acid. Isoelectric focusing was then conducted with the following voltage schedule: 100 V for 10 min, 200 V for 15 min, 300 V for 30 min, 400 V for 11 h and 800 V for 1 h. Slab gels (total dimensions 14cm wide $\times$ 9cm long $\times$ 2mm thick) consisted of a running gel and a 1cm-long stacking gel of the compositions described for one-dimensional separations. The buffers used were those of Laemmli (1970). After equilibration of the isoelectric-focusing gel with buffer containing 50mM-Tris/HCl, pH 6.8, 10% (v/v) glycerol, 3% (w/v) SDS and 3% (v/v) β -mercaptoethanol, the gel was sealed to the top of the slab gel with warm 1% (w/v) indubiose (L'Industrie Biologique Française S.A., Gennevilliers, France). Slots were cut into the solidified indubiose at either end of the isoelectric-focusing gel to accommodate protein samples dissociated with SDS and β -mercaptoethanol. Electrophoresis was performed for 2.5 h at 25 mA. Gels were stained and destained as for one-dimensional gels. To remove a band of stained ampholines at the dye front, the gels were then soaked for 24 h in a solution containing 0.0005% Coomassie Blue (or Brilliant Blue R), 7.5% (v/v) acetic acid and 50% (v/v) ethanol (O'Farrell, 1975). The low concentration of stain present in this mixture largely prevented destaining of protein bands by the high ethanol content. If required, gels could be restained by conventional means after removal of the ampholine band.

Preparation of antisera to Complex I and the iron-protein fraction

Complex I or the iron-protein (5mg of protein diluted to 1 ml with water) was mixed with 1 ml of

Freund's complete adjuvant and was injected into the back of a rabbit at several intradermal sites. After 4 weeks identical samples were injected (intramuscularly) into each hind-limb. The rabbit antibodies were further boosted, at monthly intervals or 2 weeks before bleeding, with 1-2 mg of protein in 1 ml without adjuvant. Blood (10-20 ml) was removed at each bleeding from the ear vein. After allowing it to clot at 21°C for 1 h, serum was clarified by centrifugation on a bench centrifuge for 15 min and finally in the 10ml $\times$ 10ml rotor of an MSE 65 centrifuge for 30 min at 40000 rev./min. Antisera were stored frozen at -20°C.

Preparation of immunoprecipitates from solubilized Complex I

Complex I was diluted to 0.17 mg of protein/ml of 0.25M-sucrose/50mM-Tris/HCl, pH 8.0 at 4°C. Triton X-100 was added to a final concentration of 1% (w/v). Per ml of solution, 50 μ l of anti-(Complex I) serum or 200 μ l of anti-(iron-protein) serum was added. After incubation overnight at 4°C the immunoprecipitate was collected by centrifugation at 20000 rev./min for 20 min. The pellet was washed twice by homogenization and recentrifugation in a solution containing 1% (w/v) Triton X-100 and 0.9% (w/v) NaCl.

Preparation of immunoprecipitates from solubilized submitochondrial particles

Submitochondrial particles were diluted to 1 mg of protein/ml in a solution containing 0.25M-sucrose/50mM-Tris/HCl, pH 8.0, 0.5% (w/v) sodium deoxycholate and 0.5M-KCl. After centrifugation in the 10 $\times$ 10ml rotor of an MSE 65 centrifuge at 25000 rev./min for 40 min, the supernatant was made 1% (w/v) in Triton X-100 and left overnight at 4°C. The solution was then recentrifuged as above. Per mg of initial submitochondrial particle protein, 0.45 ml of anti-(Complex I) serum or 1.5 ml of anti-(iron-protein) serum was added together with Triton X-100 to maintain the final concentration at 1% (w/v). After incubation overnight at 4°C, the immunoprecipitate was collected and washed as described above.

Results

One-dimensional separations by SDS/polyacrylamide-gel electrophoresis

Although two-dimensional methods give the best possible resolution, polypeptides with isoelectric points outside the range covered by the ampholines will be absent from the gels. Consequently, we have tried to resolve as many components as possible by conventional SDS/polyacrylamide-gel electrophoresis, since this method should show every constituent polypeptide, even if they are not com-

Fig. 1. Polyacrylamide-gel electrophoresis of Complex I and NaClO_4 -extracted Complex I. Complex I (100 μg of protein) and the insoluble fraction from treatment of Complex I with NaClO_4 (equivalent to 100 μg of Complex-I protein) were electrophoresed in parallel by either the Weber & Osborn (1969) system (a, b) or the discontinuous system of Neville & Glossman (1974) (c, d). (a) and (c) Complex I; (b) and (d) insoluble fraction. Molecular weights (in thousands) are indicated.

Fig. 2. Polyacrylamide-gel electrophoresis of the iron-protein and flavoprotein fractions from Complex I. (a) The iron-protein fraction (25 μg of protein) and (b) the flavoprotein fraction (15 μg of protein) were electrophoresed as described by Neville & Glossman (1974). Molecular weights (in thousands) are indicated.

pletely resolved. Fig. 1 shows the gel profiles obtained by electrophoresis by the method of Weber & Osborn (1969) (Fig. 1a) or Neville & Glossman (1974) (Fig. 1c). Molecular weights were taken from Crowder & Ragan (1977). The discontinuous system of Laemmli (1970) gave results very similar to those of Fig. 1(c). Figs. 1(b) and 1(d) are of the insoluble fraction obtained from NaClO_4 resolution of Complex I electrophoresed under the conditions of Figs. 1(a) and 1(c) respectively. As previously reported (Ragan, 1976; Crowder & Ragan, 1977), this fraction is almost completely free from certain subunits. From the 'Weber & Osborn' gels, these have mol.wts. of 75 000, 53 000, 30 000 and 27 000. In the discontinuous gels, the missing polypeptides have mol.wts. of

75 000, 53 000, 49 000 (the later two co-electrophoresing in Fig. 1a), 27 000 and 15 500. The 30 000-mol.wt. subunit is only partially depleted, suggesting that the polypeptides of mol.wts. 33 000 and 30 000 seen in Fig. 1(a) co-migrate in the discontinuous system of Fig. 1(c) (Crowder & Ragan, 1977).

As reported by Ragan (1976), the polypeptides of mol.wts. 75 000, 49 000 and 30 000 co-purify into the iron-protein fraction, which also contains some other lower-molecular-weight polypeptides. The latter are more clearly resolved by discontinuous gel electrophoresis (Fig. 2a) and have mol.wts. of 22 000, 18 000 and 15 500. The flavoprotein fraction or type-II dehydrogenase obtained from chaotropic resolution contains polypeptides of mol.wts. 53 000, 27 000 and

15 500 (Fig. 2*b*). Although we have reported that the content of the latter polypeptide is variable (Ragan, 1976), others (Dooijewaard *et al.*, 1978*b*) have proposed that it is a genuine constituent of this fraction. The polypeptides of mol.wts. 75 000, 53 000, 49 000, 27 000 and 15 500 can be almost completely accounted for in these soluble fragments of Complex I. However, the 22 000- and 18 000-mol.wt. components of the iron-protein fraction constitute only a portion of the protein of these molecular weights found in whole Complex I. In view of the intense staining of these two bands on gels of whole Complex I, it is likely that more than one polypeptide of these molecular weights is present in each Complex-I molecule. Thus the incomplete separation of some polypeptides by chaotropes may be more apparent than real.

Immunoprecipitates of Complex I are complicated by the presence of the heavy and light chains of immunoglobulins. These form diffuse and heavily stained bands in the gels and in particular the 53 000- and 49 000-mol.wt. polypeptides are obscured. In Fig. 3, the polypeptide composition of Complex I (Fig. 3*a*) is compared with those of immunoprecipitates of detergent-solubilized Complex I allowed to react with either anti-(Complex I) serum (Fig. 3*b*) or anti-(iron-protein) serum (Fig. 3*c*). The profiles are very similar except for the presence of the heavy and light chains of IgG and the absence of the 42 000-mol.wt. subunit in the immunoprecipitates (Smith & Ragan, 1978).

The specificity of the antiserum for Complex I is shown by the experiment of Fig. 4 in which detergent-solubilized submitochondrial particles were allowed to react with anti-(Complex I) serum. The immunoprecipitate (Fig. 4*b*) contains all the subunits of isolated Complex I with the exception of the 42 000-mol.wt. polypeptide. The interference with the profile by the heavy and light chains of IgG is always more pronounced than in experiments such as those of Fig. 3, indicating that the antibody/antigen ratio of the immunoprecipitates is not constant but depends on conditions. In addition, the precipitate of Fig. 4 is contaminated with minor amounts of several high-molecular-weight polypeptides, which are also found when control serum is used (Fig. 4*c*). These are probably mitochondrial proteins which come out of solution during incubation with the serum, since no such proteins can be seen in Fig. 3(*b*) or 3(*c*). For comparison, the polypeptide profile of the original submitochondrial particles (Fig. 4*a*) is clearly different from that of the immunoprecipitate. Immunoprecipitates obtained by allowing solubilized submitochondrial particles to react with anti-(iron-protein) serum are indistinguishable from those obtained with anti-(Complex I) serum, except that the ratio of immunoglobulin/Complex-I protein is generally higher (results not shown).

Fig. 3. Polyacrylamide-gel electrophoresis of Complex I and immunoprecipitated Complex I

Electrophoresis was conducted by using the Neville & Glossman (1974) system. (a) Complex I (100 µg of protein); (b) immunoprecipitate obtained by allowing 150 µg of Complex I to react with anti-(Complex I) serum; (c) immunoprecipitate obtained by allowing 150 µg of Complex I to react with anti-(iron-protein) serum. The densitometer sensitivity was the same for all three traces. The lower intensity of the stain in (b) and (c) is due to loss of protein incurred during washing of the immunoprecipitates. Molecular weights (in thousands) are indicated. HC, IgG heavy chains; LC, IgG light chains.

To improve the resolution of the low-molecular-weight polypeptides and to try to minimize interference with the Complex I analysis by immunoglobulins, we turned to two-dimensional separation methods.

Two-dimensional separation of Complex-I subunits

Fig. 5(*a*) shows a two-dimensional separation of Complex-I subunits. The pattern obtained is highly

Fig. 4. Polyacrylamide-gel electrophoresis of submitochondrial particles and immunoprecipitated Complex I from submitochondrial particles

Electrophoresis was conducted by using the Neville & Glossman (1974) system. (a) Submitochondrial particles (100 µg of protein); (b) Complex I immunoprecipitated with anti-Complex I serum from 2mg of submitochondrial particles; (c) the same as (b) but using control serum. HC, IgG heavy chains; LC, IgG light chains. Molecular weights (in thousands) are indicated.

reproducible, except at the extreme alkaline end of the focusing dimension, which unfortunately contains a high proportion of low-molecular-weight subunits. Polypeptides at the alkaline extreme do not focus as sharp spots and their position is dependent on the length of time for which focusing is conducted. For example, if the focusing time is extended to say, 16h, several of the more alkaline polypeptides are lost from the focusing gel as a result of cathodic drift, i.e. loss of ampholines to the cathodic electrolyte with consequent flattening of the pH gradient. Lack of reproducibility at the alkaline extreme was noted by O'Farrell (1975). The polypeptides in Fig. 5(a) have been labelled with their molecular weights (in thousands) and a letter to subdivide those of the same molecular weight (Fig. 5b). Of the high-

Fig. 5. Two-dimensional separation of Complex I subunits. Complex I (104 µg of protein) was analysed as described in the Materials and Methods section. (a) Photograph of stained gel; (b) key identifying individual polypeptides.

molecular-weight subunits, 75, 49 and 39 resolve into single spots. Polypeptide 53 has a number of satellite spots of the same molecular weight but more acidic isoelectric point. The gradually decreasing intensity of these spots is characteristic of artificial charge heterogeneity caused by hydrolysis of asparagine and glutamine residues to aspartate and glutamate respectively (O'Farrell, 1975). Polypeptide 42, which is always rather diffuse on one-dimensional gels, resolves into a number of closely spaced species in the focusing dimension. Only one polypeptide of mol.wt. 30000 can be seen (as a pair of closely spaced spots), although results given above showed that two polypeptides of this apparent molecular weight are found in Complex I. Subunits 27 and 25 appear as single spots, and subunit 26 resolves into two components 26A and 26B. Subunit 23.5 does not appear on the two-dimensional gel. Several components of mol.wt. 22000 are resolved, namely 22A, 22B and 22C. On slab gels, the subunit of mol.wt. 20500 is resolved into two components, here labelled 20 and 21. Of these two, only 20 appears on the two-dimensional gel at the extreme alkaline edge. This subunit is frequently lost completely owing to cathodic drift. Two components of mol.wt. 18000

can be seen, subunits 18A and 18B. Nevertheless the stain intensity would not seem to be adequate to account for the material seen on one-dimensional gels, and it is likely that there are other components of this molecular weight that are missing. A single component of mol.wt. 16500 can be seen at the alkaline end of the gel, and several components of mol.wt. 15500 (15.5A, 15.5B, 15.5C and 15.5D). A single weak spot of mol.wt. 8000 appears at the acidic side of the gel. As with subunit 18, the stain intensity suggests that there are other missing components of this molecular weight. The subunit of mol.wt. 5000 does not appear on the two-dimensional gel.

Fig. 6 shows two-dimensional gels of the fractions obtained from NaClO_4 resolution of Complex I. The protein loadings were approximately in the proportions in which these fractions make up Complex I. The iron-protein fraction (Fig. 6a) contains subunits 75, 49, 30, 22A, 18A, 15.5B, 15.5C and 15.5D. In addition there are traces of the flavoprotein subunits (Fig. 6b) and some of subunit 26B, which seems to be split between the insoluble fraction (Fig. 6c) and the iron-protein fraction in this experiment. The flavoprotein fraction contains subunits 53, 27 and 15.5A (the latter not clearly visible in the photograph). The two larger subunits show extensive charge heterogeneity, which we have been unable to prevent. The pattern is unaffected by increasing the β -mercaptoethanol concentration (to prevent possible oxidation of cysteine to cysteic acid) or by varying the sample storage time before isoelectric focusing. Thus the heterogeneity seems to be a consequence of the NaClO_4 -resolution process and may be the basis of the heterogeneity of the iron-sulphur e.p.r. spectrum of this fraction (Orme-Johnson *et al.*, 1974).

Several minor spots on the gels have not been considered as Complex-I subunits, because they appear to be present in relatively small amounts or are not consistently observed. Some have been identified with subunits of other mitochondrial enzymes. Thus the spots labelled α and β in Fig. 5 are the major subunits of the F_1 -ATPase, which are usually visible in one-dimensional separations. Those labelled a (which shows extensive charge heterogeneity), b and c are the two core proteins and iron-sulphur protein respectively of Complex III with reported mol.wts. of 50000, 47000 and 25000 (Gellefors & Nelson, 1975) in good agreement with our molecular-weight calibration. The cytochrome c_1 haem-binding protein of mol.wt. 29000 forms a broad diffuse band in the isoelectric-focusing dimension and is therefore not visible at the loading of the gel in Fig. 5. The other subunits of Complex III do not appear on isoelectric-focusing gels under our conditions. The subunit present in Complex I with a mol.wt. of 87000 (Ragan, 1976) does not appear on isoelectric focusing. The molecular weight is probably

Fig. 6. Two-dimensional separations of Complex I subunits

Conditions were as described in the Materials and Methods section. (a) Iron-protein fraction ($21\mu\text{g}$ of protein); (b) flavoprotein fraction ($10\mu\text{g}$ of protein); (c) NaClO_4 -insoluble fraction ($73\mu\text{g}$ of protein).

underestimated on our rather high-concentration gels and this polypeptide is almost definitely the nicotinamide nucleotide transhydrogenase, which consists of a single polypeptide of mol.wt. 95000 (Höjeberg & Rydström, 1977).

Two-dimensional analysis of immunoprecipitated Complex I produces much more satisfactory results than one-dimensional separation (Fig. 7). Fig. 7(a) shows an analysis of purified rabbit IgG. The heavy

Fig. 7. Two-dimensional separations of IgG and immunoprecipitated Complex I

Conditions were as described in the Materials and Methods section. (a) Rabbit IgG (50 μ g of protein); (b) immunoprecipitate obtained by allowing 150 μ g of Complex I to react with anti-(iron-protein) serum; (c) immunoprecipitate obtained by allowing 2 mg of submitochondrial particle protein to react with anti-(Complex I) serum.

incomplete dissociation of the IgG molecule during equilibration of the isoelectric-focusing gel with SDS-containing buffer. Fig. 7(b) shows an analysis of Complex I immunoprecipitated with anti-(iron-protein) serum as in Fig. 3(c). The 53 000- and 49 000-mol.wt. subunits are clearly resolved from the heavy chains of IgG, and the light chains have more acidic isoelectric points than the Complex-I subunits of comparable molecular weight. This is also true of Complex I immunoprecipitated from submitochondrial particles (Fig. 7c), where the relative intensity of the heavy and light chains is much greater. Several impurities are visible, particularly in the high-molecular-weight region of the gel. However, some subunits found in isolated Complex I are absent from the gel of Fig. 7(c), in particular, subunit 42, as reported by Smith & Ragan (1978). Several others which were present in minor amounts in the gels of isolated Complex I are also missing and these have not been included in the numbering of Fig. 5. Immunoprecipitates obtained by allowing either Complex I (Fig. 7b) or submitochondrial particles (results not shown) to react with anti-(iron-protein) serum contain all the components that are numbered in Fig. 5. The fact that antisera directed against a maximum of eight subunits co-precipitate all the others is strong evidence that the latter are genuine Complex-I subunits.

Two-dimensional analysis of immunoprecipitates that have a high ratio of antibody to antigen protein is complicated by the insolubility of the material in Nonidet P-40 and urea. A considerable proportion of the protein therefore remains at the focusing origin and produces a vertical streak of stained material after electrophoresis (e.g. Fig. 7c). To some extent this accounts for the observation that some polypeptides of Complex I appear rather weakly on two-dimensional gels, but in normal concentrations on one-dimensional gels of immunoprecipitates. To get round this, we treated the immunoprecipitates with SDS before addition of Nonidet P-40 and urea, as described by Ames & Nikaido (1976). The relative intensities of the Complex-I subunits then appeared normal, but unfortunately the SDS treatment appeared to make all the polypeptides more basic, so that those with alkaline isoelectric points were missing from the gel.

Table 1 summarizes our conclusions: 21 subunits are consistently observed in the purified enzyme and in immunoprecipitates analysed by the two-dimensional method; 4 others (33, 23.5, 21 and 5) are seen on one-dimensional gels of the isolated enzyme and immunoprecipitates. Subunit 42 is missing from immunoprecipitates and is discussed below. For reasons given above, there are probably more components of mol.wts. 18 000 and 8000 than have been seen on either one- or two-dimensional gels. The grand total is therefore twenty-six polypeptides,

and light chains show extensive charge and size heterogeneity and consequently are spread over a wide area of the gel. Additional higher-molecular-weight components showing similar charge heterogeneity to the heavy chains are probably a result of

Table 1. *Subunits of Complex I*

10 ⁻³ × Subunit mol.wt.	Localization after NaClO ₄ resolution		
	Iron-protein fraction	Flavoprotein fraction	Insoluble fraction
75	✓		
53		✓	
49	✓		
42*			✓
39			✓
33†§			✓
30	✓		
27		✓	
26A			✓
26B‡			✓
25			✓
23.5§			✓
22A	✓		
22B			✓
22C			✓
21§			✓
20			✓
18A	✓		
18B			✓
16.5			✓
15.5A		✓	
15.5B	✓		
15.5C	✓		
15.5D	✓		
8			✓
5§			✓

* Not found in immunoprecipitates from Triton-solubilized material.

† Molecular weight from 'Weber & Osborn (1969)' gels.

‡ Some found in the iron-protein fraction.

§ Not seen on two-dimensional gels.

|| There may be other components of these molecular weights which do not appear on two-dimensional gels.

which may be increased if it is shown that there are further species of mol.wts. 18000 and 8000.

Discussion

The application of two-dimensional electrophoresis and specific immunoprecipitation leads us to propose that Complex I is a single enzyme of enormous intricacy, comprising some 26 polypeptides. Although the function of any individual polypeptide is unknown, the large number of such constituents allows us to look for statistically significant trends in molecular weight, isoelectric point or other general properties. The two-dimensional separation of Fig. 5 may be regarded as a plot of molecular weight versus isoelectric point. Certainly the distribution of polypeptides does not appear to be random and tests of co-variance of molecular weight with isoelectric point indicate a strong correlation ($P < 0.01$). A further comparison was made between those subunits that are solubilized by chaotropic agents and those that are not, on the basis of isoelectric point. The

former group have a lower mean isoelectric point than the latter ($P < 0.01$, unpaired t test). The correlation of molecular weight with solubility is not so strong ($P < 0.1$). It has already been shown (Ragan, 1976) that the accessibility to external labelling of those subunits solubilized by chaotropic agents is limited. Thus only subunits 75 and 49 were extensively labelled with ¹²⁵I by lactoperoxidase-catalysed iodination of intact Complex I, whereas label was taken up heavily into the NaClO₄-insoluble fraction. In one experiment, only 8% of the total protein-bound radioactivity was solubilized by NaClO₄, but 30% of the total protein was extracted. These considerations lead to the following generalizations about Complex-I structure. There is a central core consisting of high-molecular-weight hydrophilic polypeptides, which bind FMN and one or more iron-sulphur centres. These are surrounded by a number of lower-molecular-weight hydrophobic polypeptides with a high average isoelectric point. It is possible that these latter polypeptides interact with the phospholipid bilayer not only by hydrophobic

interactions with the fatty acid chains but also via electrostatic interactions with the negatively charged phosphate groups, thereby explaining the basic nature of these proteins. The hydrophilic core polypeptides would not need to have any particular isoelectric point. The correlation of molecular weight with isoelectric point or with solubility is puzzling and we can offer no suggestions as to what these trends in subunit size signify.

The subunit composition reported here and previously (Ragan, 1976; Crowder & Ragan, 1977) is in substantial agreement with the analyses of Hare & Crane (1974) and Dooijewaard *et al.* (1978*a,b*). The latter authors reported that the subunit of Complex I of approx. mol.wt. 40000 is not a constituent of the type-I NADH dehydrogenase. We have also found that this subunit (subunit 42) is not co-ordinately immunoprecipitated with the other subunits from solubilized mitochondria, but the possibility remains that this component is required for ubiquinone reductase activity, since neither the type-I NADH dehydrogenase nor Triton-treated mitochondria retain activity towards this acceptor. Dooijewaard *et al.* (1978*b*) found that the flavoprotein fraction occasionally contains a low-molecular-weight polypeptide (15.5A in our terminology), whose presence was correlated with a higher specific content of iron and acid-labile sulphide. Their conclusion that the iron-protein fraction contains most of the polypeptides of Complex I is a reflection of the inferior resolving power of their analytical techniques. As shown in the present paper, the iron-protein fraction has a quite different subunit composition from either Complex I or the flavoprotein fraction. The degree to which the iron-protein fraction contains subunits normally found in the insoluble residue from chaotropic resolution depends on the residual detergent present in the Complex I. Thus, at high enough cholate concentrations, most of the protein of Complex I is apparently 'solubilized' and fractionates into the iron-protein fraction (C. I. Ragan, unpublished observations). Only the subunits designated in the present paper are found in the iron-protein fraction prepared from dialysed (i.e. detergent-free) Complex I. In our hands, and in agreement with Hatefi & Stempel (1969), $(\text{NH}_4)_2\text{SO}_4$ fractionation of the material solubilized by chaotropic agents produces only two well-separated fractions, the iron-protein and flavoprotein. The fractions obtained by Dooijewaard *et al.* (1978*b*) at somewhat different $(\text{NH}_4)_2\text{SO}_4$ concentrations appear to be mixtures of these two fractions further contaminated with polypeptides of the NaClO_4 -insoluble fraction.

We would like to propose a simple and logical reason for the mitochondrial NADH dehydrogenase consisting of 26 different polypeptides but we can think of none. The function of the enzyme is to oxidize NADH by ubiquinone and couple this

process to translocation of protons across the membrane. There seems to be no reason why this should not be achieved by two or three polypeptides, and, indeed, several ion pumps do seem to have a relatively simple polypeptide composition. We are left with the impression that we are missing something.

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References

- Ames, G. & Nikaido, K. (1976) *Biochemistry* **15**, 616–623
- Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D. & Munn, E. A. (1975) *Biochem. J.* **148**, 533–537
- Cabral, F. & Schatz, G. (1979) *Methods Enzymol.* in the press
- Crowder, S. E. & Ragan, C. I. (1977) *Biochem. J.* **165**, 295–301
- Dooijewaard, G., De Bruin, G. J. M., Van Dijk, P. J. & Slater, E. C. (1978*a*) *Biochim. Biophys. Acta* **501**, 458–469
- Dooijewaard, G., Slater, E. C., Van Dijk, P. J. & De Bruin, G. J. M. (1978*b*) *Biochim. Biophys. Acta* **503**, 405–424
- Gellefors, P. & Nelson, B. S. (1975) *Eur. J. Biochem.* **52**, 433–440
- Hare, J. F. & Crane, F. K. (1974) *J. Subcell. Biochem.* **3**, 1–25
- Hatefi, Y. & Rieske, J. S. (1967) *Methods Enzymol.* **10**, 235–239
- Hatefi, Y. & Stempel, K. E. (1969) *J. Biol. Chem.* **244**, 2350–2357
- Hatefi, Y., Haavik, A. G. & Griffiths, D. E. (1962) *J. Biol. Chem.* **237**, 1676–1680
- Höjeberg, B. & Rydström, J. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1183–1190
- Jovin, T. K., Dante, M. L. & Chramback, A. (1971) *Multiphasic Buffer Systems Output*, Federal Scientific and Technical Information, U.S. Department of Commerce, PB 196085-196091, Springfield
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Neville, D. M. & Glossman, H. (1974) *Methods Enzymol.* **32**, 92–102
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Orme-Johnson, N. R., Hansen, R. E. & Beinert, H. (1974) *J. Biol. Chem.* **249**, 1922–1927
- Racker, E. (1962) *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1657–1663
- Ragan, C. I. (1976) *Biochem. J.* **154**, 295–305
- Rieske, J. S. (1967) *Methods Enzymol.* **10**, 239–245
- Smith, S. & Ragan, C. I. (1978) *Biochem. Soc. Trans.* **6**, 1349–1352
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412