Simplified isolation and molecular composition of NADH dehydrogenase of the respiratory chain

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A simplified procedure for the isolation of NADH dehydrogenase from the inner membrane of ox heart mitochondria is presented which permits relatively rapid preparation of the enzyme in a more stable form than that afforded by published methods. The protein thus isolated displays more than eight different subunits in gel electrophoresis under denaturing conditions, three of which are also present in the 'low-molecular-weight form' of the enzyme prepared under more drastic conditions. Complex I contains several subunits, mostly of low molecular weight, not seen in soluble purified NADH dehydrogenase. It is suggested that some of these may be 'binding peptides' necessary in linking NADH dehydrogenase to ubiquinone reduction, analogously to the role of small peptides in linking succinate dehydrogenase to ubiquinone. The dehydrogenase isolated by the rapid method contains equimolar amounts of non-haem iron and labile sulphur, but on further manipulation non-haem iron (but no labile sulphur) is lost, resulting in ratios of S/Fe in excess of unity, as previously reported for preparations isolated by longer procedures.

NADH dehydrogenase (EC 1.6.99.3) from ox heart mitochondria may be isolated in soluble purified form with the aid of phospholipase A at moderate temperature (Cremona & Kearney, 1964), with retention of the iron–sulphur content and most of the catalytic properties of the membrane-bound form as seen in Complex I (Hatefi & Stigall, 1976). It may also be isolated in a low-molecular-weight form by heat/acid-ethanol treatment, proteolysis and other means (Mahler *et al.*, 1952; Singer & Gutman, 1971; Galante & Hatefi, 1979). Such preparations show drastically altered composition and properties, but retain appreciable reactivity with NADH. The purity of available preparations of the former ('high-molecular-weight form') has been uncertain because the properties of the protein do not lend themselves to accurate physical measurements, but was thought not to exceed approx. 70% (Cremona & Kearney, 1964). The fact that the isolation is time-consuming and the product is markedly labile has been a major problem in structural studies of the enzyme. We now present a simplified isolation procedure which yields considerably more stable

preparations. It use has permitted clarification of the non-stoichiometric ratio of Fe/S which has been reported for earlier preparations (Lusty *et al.*, 1965). The present paper also compares the subunit composition of the three forms of the dehydrogenase and points to the probable structural differences between Complex I and the high-molecular-weight form of the soluble enzyme.

Materials and methods

NADH dehydrogenase was isolated from inner membranes of mitochondria (electron-transport particles) by the method of Cremona & Kearney (1964) or by the modifications given below. Complex I was prepared as described by Hatefi & Rieske (1967), and the low-molecular-weight form of NADH dehydrogenase as described by Galante & Hatefi (1979). NADH dehydrogenase activity was assayed as described by Singer (1974); FMN, non-haem iron and acid-labile sulphur were determined by the procedures of Burch (1957), Brumby & Massey (1967) and Lovenberg *et al.* (1963) respectively. Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis was performed either on 14 cm × 10 cm × 1.5 mm slabs in a discontinuous buffer system (Laemmli, 1970) or in cylindrical gels

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(Weber & Osborn, 1969). Protein was determined by the biuret method (Gornall *et al.*, 1949) or by a modified Lowry method (Bensadoun & Weinstein, 1976).

Results and discussion

The revised isolation procedure for NADH dehydrogenase followed the initial steps of the method of Cremona & Kearney (1964). After the first $(\text{NH}_4)_2\text{SO}_4$ precipitation, however, the pellet was resuspended in 25 mM-Tris/phosphate, pH 8.5, to a protein concentration of approx. 10 mg/ml and a total volume of 50–60 ml. This solution was desalted on Sephadex G-25 (medium grade; bed volume 500 ml; equilibrated with 25 mM-Tris/phosphate, pH 8.5), and the eluate was concentrated to a protein concentration of 30–40 mg/ml by ultrafiltration in an Amicon cell with filter PM30.

Turbidity was removed by sedimentation at 14 000 *g* for 10 min and the enzyme was diluted with 0.1 vol. of 1 M-glycine, pH 10. Samples (2 ml) of this were layered on top of 10.8 ml of a 13–50% (w/v) linear gradient of sucrose in 50 mM-glycine, pH 10, in tubes of the SW 41 rotor of the Beckman L3-50 ultracentrifuge. The tubes were centrifuged for 14–16 h at 41 000 rev./min (200 000 *g* at r_{av} , 10.95 cm) at 1°C, and were unloaded from the bottom. The first 4.8 ml was discarded. Then 0.4 ml fractions were collected in test tubes containing 30 μ l of 0.2 M-potassium phosphate, pH 7.4. The tubes were immersed in, and the gradient-collecting device was surrounded by, ice/water to minimize inactivation of the enzyme while at pH 10. Fractions with the highest specific activity were pooled. Sucrose was removed by passing the solution through Sephadex G-25 (medium grade; bed volume 250 ml; equilibrated with 25 mM-Tris/phosphate,

Fig. 1. Protein and activity distribution after gradient centrifugation of NADH dehydrogenase

The enzyme was extracted by phospholipase treatment of 14.5 g of electron-transport particles from ox heart mitochondria (Cremona & Kearney, 1964) and divided into two equal parts. (a) Purification by the Cremona & Kearney (1964) method. After gel filtration and concentration, 2 ml of enzyme (26 mg/ml) was applied to the sucrose gradient and centrifuged as described in the Results and discussion section. Fractions (0.4 ml) were collected and activity at fixed (1.66 mM) ferricyanide concentration and protein were determined in each fraction. Fractions 16–22 were pooled and assayed at V_{max} (ferricyanide). The specific activity was 400 μ mol of NADH/min per mg, the yield of activity 18% as compared with electron-transport particles, and the FMN, non-haem iron and labile sulphur were 0.95, 17.5 and 25.1 nmol/mg respectively. (b) Rapid purification procedure, as described in the text. The concentrated, desalted enzyme (34 mg/ml) was applied to the sucrose gradient, centrifuged, and fractionated as in (a). The specific activity of the pooled fractions was 520 μ mol of NADH/min per mg, the yield of activity 29%, and the FMN, non-haem iron and labile sulphur were 1.21, 22.9 and 24.2 nmol/mg respectively.

pH 8.5), and the resulting eluate was concentrated by ultrafiltration to a protein concentration of 15–20 mg/ml.

Fig. 1 compares the protein and activity profiles of the sucrose gradient in the original and in the modified isolation procedure. Clearly contaminating proteins are present in the enzyme isolated by either method, particularly in the lighter fractions (21 and 22). In contrast with the original method, the impurities in the rapidly isolated enzyme consist partially of iron–sulphur proteins, as judged by the colour of fractions 24–30 of the sucrose gradient (Fig. 1). This explains the higher contents of iron and sulphur in pooled fractions containing most of the NADH dehydrogenase (Table 1). A second density-gradient centrifugation with a shallow sucrose gradient will almost certainly improve the purification, as shown in the original purification scheme by Cremona & Kearney (1964).

The rapid isolation procedure represents a substantial improvement over the original method in that the time required was cut from 5 days to less

than 30 h. Possibly, because of less manipulation, the stability of the purified enzyme is much higher than that prepared by the Cremona & Kearney (1964) procedure. At 0°C activity was lost at the rate of 5–10% per week over a period of 5–6 weeks, after which time precipitation and irreversible denaturation occurred.

Table 1 compares the FMN, iron and labile-sulphur contents of several preparations isolated by the Cremona & Kearney (1964) procedure with those prepared by the short method. Although the FMN content per mg of protein is very similar in the products obtained by the two procedures, the non-haem iron content is usually higher and the labile S lower in the revised method. Most significantly, although the S:Fe ratio of the enzyme prepared by the original method is always 1.4 or higher, as previously reported (Lusty *et al.*, 1965) and also found in preparations isolated by other laborious procedures (Baugh & King, 1972), the ratio in samples prepared by the present method is near unity. The high S:Fe ratio in the earlier preparation cannot be due to the use of an erroneous absorption coefficient, as has been speculated (Hatefi & Stigall, 1976), since the internal standards gave recovery >95% (results not shown) and since a correct S:Fe ratio was obtained with other enzymes, such as ferredoxins. It may have been due, however, to partial loss of Fe–S clusters during isolation, with release of the Fe in soluble form and capture of the S by thiol groups as persulphide. In the presence of reducing agents, which the thiol groups of the enzyme can provide (Gutman *et al.*, 1970), persulphide sulphur may be released and form Methylene Blue in the assay for labile S.

Some support for this interpretation is found in Table 2. When the enzyme isolated by the short procedure was subjected to two additional precipitations with $(\text{NH}_4)_2\text{SO}_4$ and dialyses, as in the original method, a significant part of the Fe was lost, but 93% of the labile S remained protein-bound, resulting in an increase in the S:Fe ratio from 1.06

Table 1. FMN, non-haem iron and acid-labile sulphur contents of NADH dehydrogenase preparations

Data of Expts. 1–5 were taken from Paech *et al.* (1981) and represent preparations by the method of Cremona & Kearney (1964). Data of Expts. 6–8 were obtained from samples of the rapidly isolated enzyme.

Expt. no.	Content (nmol/mg of protein)			Ratio S:Fe
	FMN	Fe	S	
1	1.00	18.8	24.0	1.36
2	1.04	18.6	26.6	1.42
3	0.90	14.6	22.1	1.51
4	0.95	16.8	23.3	1.39
5	1.00	16.7	24.8	1.48
6	0.97	21.7	22.6	1.04
7	0.92	19.9	21.3	1.07
8	0.95	20.8	23.3	1.12

Table 2. Effects of various treatments on the S:Fe ratios of NADH dehydrogenase

Expt. 1, rapidly isolated enzyme. Expt. 2, same after two precipitations with 243 g of $(\text{NH}_4)_2\text{SO}_4$ /litre, each followed by 14 h dialysis against 30 mM-potassium phosphate, pH 7.8. The protein yield was 84%; activity declined by 25%. Expt. 3, same sample after equilibration with 50 mM-potassium phosphate, pH 8.0, and incubation with 5 mM-KCN for 4 h at 25°C under N_2 , followed by dialysis overnight. Specific activity was 55% of that of sample 1. Expt. 4, enzyme isolated by the original Cremona & Kearney (1964) procedure. Expt. 5, as Expt. 4 but after treatment as in Expt. 3. Activity declined to 76% of that in Expt. 4.

Expt. no.	Content (nmol/mg of protein)			Ratio S:Fe	Specific activity [V_{max} for ferricyanide ($\mu\text{mol}/\text{min}$ per mg)]
	FMN	Fe	S		
1	0.96	20.8	22.1	1.06	400
2	0.78	14.8	20.6	1.39	300
3	0.66	11.8	12.2	1.03	220
4	0.88	16.2	23.5	1.45	380
5	0.71	12.8	15.3	1.19	290

to 1.39. Cyanolysis, which is expected to cleave persulphides, caused release of the extra labile S, and changed the S:Fe ratio to 1.03. Cyanide treatment of the Cremona & Kearney (1964) preparation also resulted in a lowering of the S:Fe ratio.

The Fe:FMN ratio in preparations isolated by the Cremona & Kearney (1964) procedure averaged 17.4 (Table 1), in accord with the value reported by these workers and others and in reasonable agreement with the presence of two tetranuclear and four binuclear Fe-S clusters in the enzyme (Paech *et al.*, 1981). In contrast, the Fe:FMN ratio of preparations isolated by the revised method averaged 21.9 (Tables 1 and 2). The excess Fe and labile S

present, over that accounted for by cluster-extrusion studies, in these and other (Baugh & King, 1972) preparations most probably represent impurities.

The determination of the subunit composition of a complex and large protein such as NADH dehydrogenase is always difficult, particularly since the purity of both soluble and particulate preparations cannot be accurately measured. It is not surprising, therefore, that considerable disparity exists in the many reports in the literature on this subject (see Singer *et al.*, 1981, for a review). However, the facts that the enzyme can be isolated in three forms (Complex I, the high-molecular-weight and low-molecular-weight forms) by very different procedures and that the last of these is available in homogeneous form provide a way out of this dilemma, since the constituent peptides of the low-molecular-weight form can serve as reference material for the other two preparations. Thus peptides in Complex I or the high-molecular-weight soluble enzyme at concentrations below those of the reference peptides may be regarded as impurities. Only one previous paper has compared the subunits of the three forms, but the data for the soluble enzyme isolated by the phospholipase method were

Fig. 2. Peptide pattern of Complex I, of the low-molecular-weight NADH dehydrogenase and of the high-molecular-weight soluble NADH dehydrogenase at various stages of purification

Sodium dodecyl sulphate / polyacrylamide-gel electrophoresis patterns of protein samples at different stages of purification of NADH dehydrogenase from electron-transport particles. Tracks (1)–(12) are 11%-acrylamide gels, (13) and (14) are 9% gels. Tracks: (1) electron-transport particles; (2) supernatant after second phospholipase-A digestion; (3) pellet after first $(\text{NH}_4)_2\text{SO}_4$ precipitation; (4) supernatant after protamine sulphate precipitation; (5) pellet after third $(\text{NH}_4)_2\text{SO}_4$ precipitation; (6) eluate from Sephadex G-200 column; (7) pooled fractions after sucrose-density-gradient centrifugation; (8) pooled fractions after sucrose-density-gradient centrifugation of the rapidly isolated enzyme; (9) Complex I; (10) low-molecular-weight NADH dehydrogenase; (11) succinate dehydrogenase; (12) molecular-weight markers [phosphorylase *a* (94 000 Da), bovine serum albumin (68 000 Da), alcohol dehydrogenase (44 000 Da), trypsin (23 000 Da), and cytochrome *c* (12 000 Da)]; (13) same as (8); (14) Complex I. X is the 30 kDa peptide found only in the high-molecular-weight form of NADH dehydrogenase.

Table 3. Peptide composition of different forms of NADH dehydrogenase

Data are from densitometry after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970). Numbers in parentheses represent estimates of mol of each peptide per mol of flavin. No concentrations are given for the peptides of <25 kDa, since the analytical procedure is not reliable in this range with many components of similar molecular weight. The high-molecular-weight form of the enzyme was isolated by the procedure given in this paper.

Complex I	High-molecular-weight form	Low-molecular-weight form
75 kDa (1)	75 kDa (1)	
56 kDa (2)	56 kDa (2)	56 kDa (1)
53 kDa (2)	53 kDa (2)	
40 kDa (2)	40 kDa (2)	
33 kDa (2)	33 kDa (2)	
	30 kDa (2–3)	
27 kDa (1)	27 kDa (1)	27 kDa (1)
23 kDa		
21 kDa		
19 kDa		
16 kDa	16 kDa	
14 kDa	14 kDa	
12 kDa		
11 kDa	11 kDa	11 kDa (1)
9 kDa	9 kDa	
5 kDa		

not documented (Ragan, 1976). Fig. 2 compares the peptide composition of the high-molecular-weight soluble NADH dehydrogenase, obtained by the method of Cremona & Kearney (1964) and by the shortened version described in this paper, with that of Complex I and low-molecular-weight NADH dehydrogenase. Table 3 is a list of these peptides, and the numbers in parentheses are estimates of the number of mol of each peptide present per mol of flavin and are approximate values because of the error inherent in the quantification of staining intensities.

Comparison of the three preparations suggests that the 30kDa peptides in the high-molecular-weight form are impurities, since they are not seen in Complex I. On the other hand, Complex I contains several peptides not seen in the soluble enzyme. Our data (Table 3) and those of others (see Singer *et al.*, 1981) show several peptides in the 9–23 kDa range in Complex I preparations, but not in the soluble enzyme. Although the possibility that some of these are impurities cannot be ruled out, neither can be the alternative that they are genuine constituents of the membrane-bound form and that some of these peptides confer coenzyme Q reductase activity on the enzyme, by analogy with the small binding peptides associated with succinate dehydrogenase in Complex II (Ackrell *et al.*, 1980).

If one were to assume that all or virtually all the peptides regularly found in Complex I are derived from this complex, it would follow that the small number of peptides detected in the soluble enzyme, but not in Complex I (Table 3), is insufficient to account for the 30–35% 'impurity' detected in the ultracentrifuge after sucrose-density-gradient centrifugation by Cremona & Kearney (1964). The possibility arises, therefore, that at least some of this impurity may have represented inactivated and aggregated enzyme, so that the degree of contamination of such preparations with foreign proteins is much less than has been believed.

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References

- Ackrell, B. A. C., Ball, M. B. & Kearney, E. B. (1980) *J. Biol. Chem.* **255**, 2761–2769
- Baugh, R. F. & King, T. E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1165–1173
- Bensadoun, A. & Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250
- Brumby, T. E. & Massey, V. (1967) *Methods Enzymol.* **10**, 463–474
- Burch, H. B. (1957) *Methods Enzymol.* **3**, 960–962
- Cremona, T. & Kearney, E. B. (1964) *J. Biol. Chem.* **239**, 2328–2334
- Galante, Y. & Hatefi, Y. (1979) *Arch. Biochem. Biophys.* **192**, 559–568
- Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–765
- Gutman, M., Mersmann, H., Luthy, J. & Singer, T. P. (1970) *Biochemistry* **9**, 2678–2687
- Hatefi, Y. & Rieske, J. S. (1967) *Methods Enzymol.* **10**, 235–239
- Hatefi, Y. & Stigall, D. L. (1976) *Enzymes*, 3rd Ed. **13**, 175–297
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lovenberg, W., Buchanan, B. B. & Rabinowitz, J. C. (1963) *J. Biol. Chem.* **238**, 3899–3913
- Lusty, C. J., Machinist, J. M. & Singer, T. P. (1965) *J. Biol. Chem.* **240**, 1804–1810
- Mahler, H. R., Sarkar, N. K., Vernon, L. P. & Alberty, R. A. (1952) *J. Biol. Chem.* **199**, 585–597
- Paech, C., Reynolds, J. G., Singer, T. P. & Holm, R. H. (1981) *J. Biol. Chem.* **256**, 3167–3170
- Ragan, C. I. (1976) *Biochem. J.* **154**, 295–305
- Singer, T. P. (1974) *Methods Biochem. Anal.* **22**, 123–175
- Singer, T. P. & Gutman, M. (1971) *Adv. Enzymol.* **34**, 79–153
- Singer, T. P., Ramsay, R. R. & Paech, C. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schatz, G. & Dallner, G., eds.), pp. 155–190, Addison-Wesley, Reading
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412