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CHARACTERIZATION OF TYPE-I NADH DEHYDROGENASE

POLYDISPERSITY, MOLECULAR WEIGHT AND POLYPEPTIDE COMPOSITION

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Summary

1. Complex I preparations are polydisperse at 5°C, consisting of equal amounts of two components, one with sedimentation coefficient ($s^0_{20,w}$) of 42 S and specific volume ($\bar{v}$) of 0.83 cm^3/g and the other with $s^0_{20,w}$ of 1.6 S and $\bar{v}$ of $\geqslant 0.97$ cm^3/g. The protein moieties of the two components are similar in polypeptide composition, enzymic activity, and probably in molecular weight. Both are Type-I NADH dehydrogenases.

2. Based on the analogy of the behaviour of amphiphiles it is suggested that the protein of the 42 S component is captured by detergents in a small spherical micelle and that of the 1.6 S in a large lamellar structure. In agreement with this view it is possible to convert the 42 S component into the 1.6 S component by increasing the concentration of detergent or by raising the temperature.

3. The above observations imply that the various Type-I NADH dehydrogenase preparations, as isolated by different investigators, are essentially the same proteins. A scheme is presented that accounts for the supposed differences in solubilization procedure.

4. The molecular weight of the heavy component in the Complex I preparations used in this paper (0.9 nmol FMN/mg protein) is $2.8 \cdot 10^6$, calculated from sedimentation velocity measurements. This implies that Type-I NADH dehydrogenase contains two FMN molecules per molecule, if allowance is made for bound detergents and phospholipids.

5. Conversion of the 42 S into the 1.6 S component results in some purification of the latter (to 1.2 nmol FMN/mg protein). Studies by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate show that polypeptides of

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mass 11—16, 18, 22, 28, 32, 40, 56 and 77 kdaltons remain associated with the NADH dehydrogenase in a ratio of 18, 8, 2, 1, 1, 1, 2 and 1, respectively, to the FMN content.

6. Treatment of Complex I with 0.5% (w/v) Triton X-100 results in a sigmoidal NADH-binding curve, as observed earlier after removal of Q-10 by pentane extraction, and loss of rotenone sensitivity of the NADH-Q oxidoreductase activity. It is concluded that the exchange of phospholipids for detergents weakens the binding of Q-10 to Complex I.

Introduction

The many preparations of NADH dehydrogenase (NADH:(acceptor) oxidoreductase, EC 1.6.99.3) that have been isolated from mitochondria or submitochondrial particles [1—8] can be divided essentially into two classes [9], high molecular weight (Type I) and low molecular weight (Type II). A comparison of the steady-state kinetics of the two types [8,10] led to the conclusion that Type-II NADH dehydrogenase may be considered to be a component of the Type-I enzyme, and that no gross changes in tertiary structure of the subunits occur in this component when it is separated from other subunits present in the Type-I dehydrogenase.

In addition to the above classification some investigators [11,12] discriminate between the particulate preparation, NADH : Q oxidoreductase or Complex I, as isolated by Hatefi et al. [2], and the water-soluble preparations as isolated for instance by Ringler et al. [3]. Although both types can be considered to have a high molecular weight, usually only the latter is denoted as Type-I. It differs from the particulate preparation in that it lacks phospholipids and endogenous ubiquinone.

This paper deals with the characterization of the high molecular weight, Type-I, NADH dehydrogenase. It is shown that, as a consequence of the properties of the detergents used for solubilization of the protein, the dehydrogenase is isolated in two different states of dispersion, with essentially the same subunit composition. It is concluded that the preparations isolated by Hatefi et al. [2], Ringler et al. [3], Kaniuga [5], Huang and Pharo [6], Baugh and King [7], and Ragan and Racker [13] are essentially the same proteins.

Results

Complex I preparations containing 0.9 nmol FMN/mg protein were found to be heterogeneous by sedimentation velocity measurements at 5°C. Fig. 1A shows at least three components that sediment within 1 h at 55 000 rev./min and Fig. 1B shows a very slow component. Plots of sedimentation coefficient ($s_{20,w}$) against protein concentration (nine different concentrations over a range of 2—20 mg/ml) are linear with a negative slope (see Fig. 2). Extrapolation to zero concentration gives $s^0_{20,w}$ values of 42 S and 1.6 S for the main fast component and the slow component, respectively.

The large difference in sedimentation coefficients allowed the separation of the fast and slow components on a preparative scale. After 1 h centrifugation at

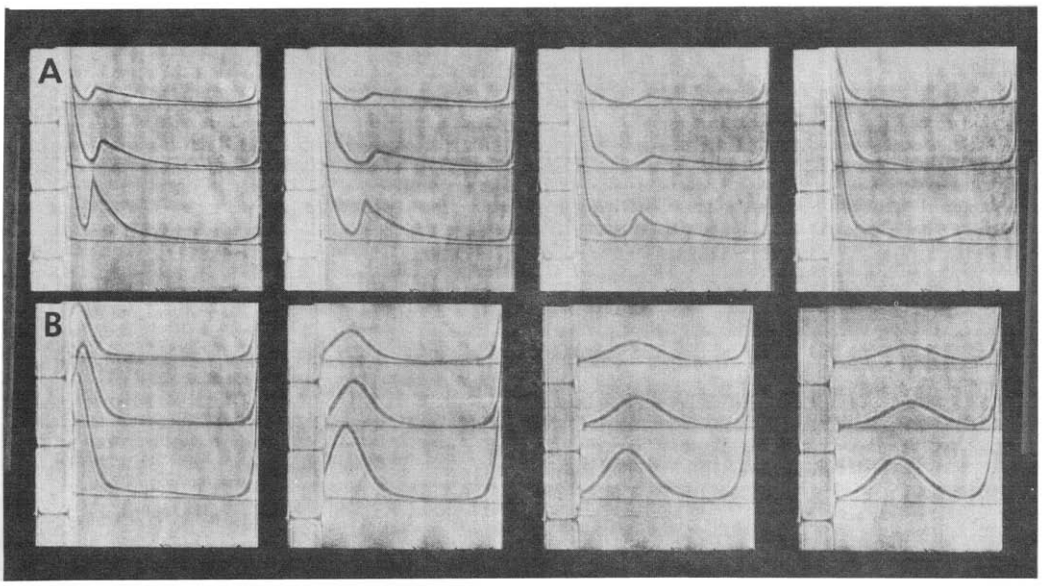


Fig. 1. Sedimentation pattern of Complex I. Complex I (40 mg/ml) was dialysed overnight at 0°C against 100 volumes of 50 mM Tris · HCl buffer (pH 8.0)/1 mM EDTA/1 mM dithiothreitol/100 mM KCl. It was then diluted with the dialysate and placed in three double-sector cells of the ultracentrifuge, two of them equipped with wedge windows. The double-sector cells contained 20, 13, 10 mg/ml protein (lower, middle and upper pattern, respectively) on one side and dialysate buffer on the other. Centrifugation was conducted at 5°C and 55 000 rev./min. The Schlieren photographs shown (left to right) were taken at a phase plate angle of 30° after 3, 6, 9 and 21 min (A) and after 45, 125, 245 and 325 min (B), respectively.

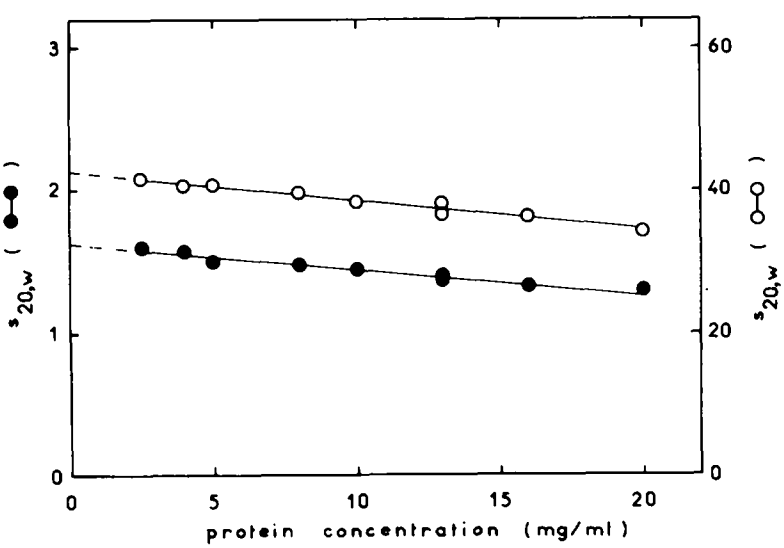


Fig. 2. The effect of protein concentration on the sedimentation coefficient of the slow and the main fast components of Complex I. Complex I at the protein concentrations indicated was centrifuged under the conditions given in Fig. 1.

170 000 × g, a dark-brown jelly-like precipitate and a yellow supernatant were obtained, each containing about half of the total protein. The precipitate could be solubilized only by strong detergents such as dodecyl sulphate and was enzymically inactive.

The supernatant was homogeneous in sedimentation equilibrium experiments, linear plots of log c versus r^2 being obtained. From the slope of these plots a molecular weight of 18 800 was calculated, in which no allowance was made for the buoyancy effect of the solvent (see below).

Isopyknic centrifugation of Complex I in a preformed sucrose gradient showed two bands, one with an estimated specific volume of at least 0.97 cm^3/g and one with 0.83 cm^3/g.

The molecular weights estimated from the above data are compiled in Table I.

The polypeptide composition of the 42 S (precipitate) and 1.6 S (supernatant) components was studied by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. The results for preparations 1 and 2, given in Table II, show that except for the 22-, 30- and 32-kdalton chains there is little difference between the heavy and light components, which suggests that the heterogeneity of Complex I preparations is due mainly to different states of dispersion rather than to the presence of contaminating proteins. The optical and EPR spectra (not shown), the rotenone sensitivity of the NADH-Q oxidoreductase activity and kinetic data (see Table III) of the starting material, Complex I (1.6 S + 42 S), and the supernatant (1.6 S component, Preparation 2 in Tables II and III) were similar, from which it follows that, before sedimentation, the insoluble precipitate (42 S component) was as active as the supernatant.

The different intensities of the 22-, 30- and 32-kdalton polypeptide bands in Preparations 1 and 2 in Table II suggest that some at least of these chains do not belong to Type-I NADH dehydrogenase. Similar differences in the partition of other polypeptides between the two states of dispersion were found after treatment of Complex I in different ways, such as incubation for 2 h at 30°C in the presence of 1 mM EDTA, 1 mM dithiothreitol and residual cholate, or incubation with 0.5% (w/v) Triton X-100. All these treatments resulted in some purification of the 1.6 S component (see Fig. 3), the FMN content increasing from 0.9 in Preparation 2 (FMN : Fe : S = 1 : 28 : 25) to 1.1—1.2 mol per mg protein in Preparation 4 (FMN : Fe : S = 1 : 24 : 21), and the 24-, 30- and 90-

TABLE I

PARTIAL SPECIFIC VOLUME, SEDIMENTATION COEFFICIENT AND MOLECULAR WEIGHT OF COMPLEX I COMPONENTS

All measurements were made at 5°C.

	Component 1	Component 2
Partial specific volume, $\bar{v}$ (cm^3/g)	0.83	⩾0.97
Sedimentation coefficient *, $s^0_{20,w}$ (S)	42	1.6
Molecular weight (M_r) calculated from $s^0_{20,w}$ **	$2.8 \cdot 10^6$	$\geqslant 0.53 \cdot 10^6$
M_r ($1-\bar{v} \cdot \rho$) calculated from sedimentation equilibrium	—	$18.8 \cdot 10^3$
M_r		$\geqslant 0.93 \cdot 10^6$

* No correction was made for the difference in $\bar{v}$ between 5 and 20°C.

** A spherical molecule is assumed.

TABLE II

POLYPEPTIDE COMPOSITION OF TYPE-I NADH DEHYDROGENASE, DETERMINED BY POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE PRESENCE OF DODECYL SULPHATE

Preparation 1, untreated Complex I, 42 S component (1.21 mg protein/nmol FMN); preparation 2, untreated Complex I, 1.6 S component (1.12 mg protein/nmol FMN); preparation 3, Complex I (10 mg/ml) incubated 2 h at 30°C in 50 mM Tris · HCl buffer (pH 8.0)/1 mM EDTA/1 mM dithiothreitol, 1.6 S component (0.88 mg protein/nmol FMN); preparation 4, Complex I (10 mg/ml) incubated 5 min at 30°C with 0.5% (w/v) Triton X 100, 1.6 S component (0.81 mg protein/nmol FMN); preparation 5, same as preparation 4, except incubation was for 2 h (0.81 mg protein/nmol FMN).

Polypeptide (kdaltons)	mol polypeptide per mol FMN				
	Preparation				
	1	2	3	4	5
11–16	16.9	17.6	18.1	18.8	16.8
18	7.63	7.55	7.24	9.0	8.38
22	2.50	3.90	5.45	4.26	5.94
24	0.98	0.76	—	—	—
28	0.93	0.97	1.20	1.18	1.00
30	1.62	0.90	—	—	—
32	5.92	3.42	1.35	1.10	1.03
40	1.72	1.65	0.57	0.89	0.81
52	1.04	1.06	0.22	0.66	0.65
56	2.06	2.10	2.29	2.06	2.12
73	0.90	0.85	0.66	0.46	0.48
77	1.00	1.24	0.98	1.03	1.00
90	0.96	0.90	—	—	—

kdalton chains almost completely remaining in the precipitate. Moreover, 70–80% of the Complex I protein was solubilized by these methods. The results in Table II taken as a whole suggest that the polypeptides of mass 11–16, 18, 22, 28, 32, 40, 56 and 77 kdaltons belong to the dehydrogenase and, according to the relative staining intensity, are present in a ratio to FMN of 18, 8, 2, 1, 1, 1, 2 and 1, respectively.

The steady-state kinetics of the NADH-ferricyanide activity of the supernatants (see Table III) show that under all the above conditions a Type-I NADH dehydrogenase, characterized by double substrate inhibition [8], is retained. However, incubation for 2 h at 30°C with or without Triton X-100 (Preparations 3 and 5) lowers the calculated rate constants. Furthermore, Triton X-100

TABLE III

KINETIC PARAMETERS OF TYPE-I NADH DEHYDROGENASE AFTER DIFFERENT TREATMENTS

The kinetic parameters were determined from the steady-state kinetics of the NADH-ferricyanide activity at pH 7.5 as described in refs. 8 and 10. The preparations are described in the legend to Table II.

Preparation	k_4 (s^{-1})	k_3 ($M^{-1} \cdot s^{-1}$)	K_m^{NADH} (μM)	K_i^{NADH} (μM)
Complex I	$9.3 \cdot 10^3$	$4.3 \cdot 10^6$	100	40
Preparation 2	$8.3 \cdot 10^3$	$4.2 \cdot 10^6$	100	40
Preparation 3	$9.5 \cdot 10^2$	$8.3 \cdot 10^5$	100	40
Preparation 4	$11.8 \cdot 10^3$	$5.5 \cdot 10^6$	sigmoidal	sigmoidal
Preparation 5	$9.5 \cdot 10^2$	$8.3 \cdot 10^5$	sigmoidal	sigmoidal

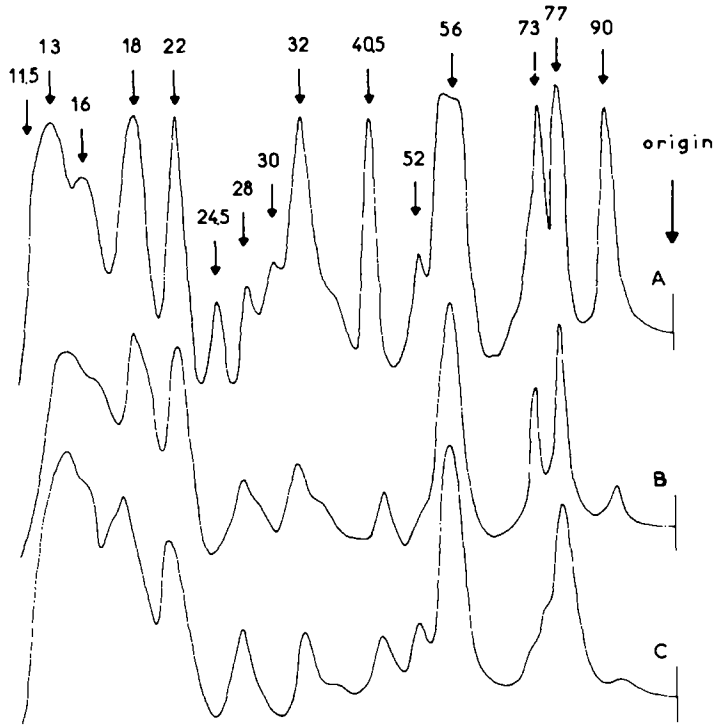


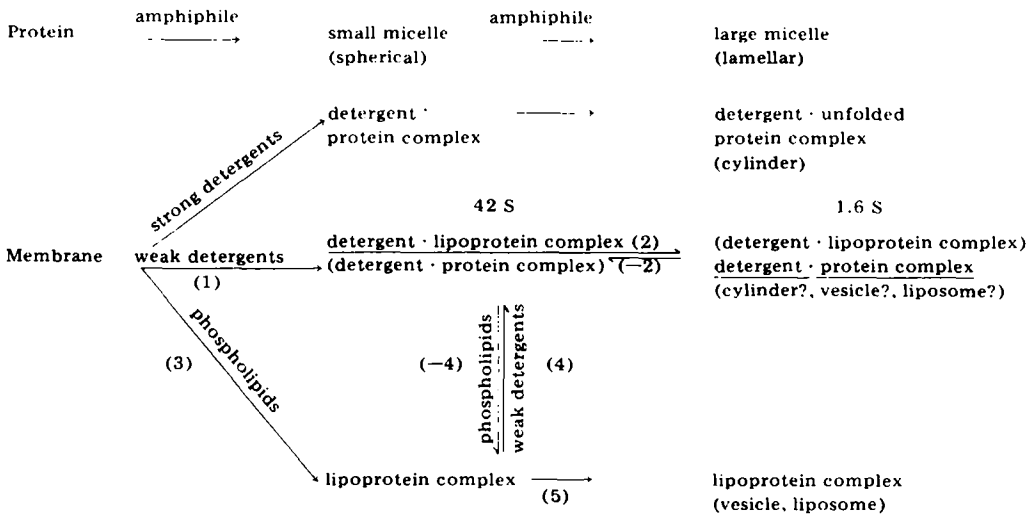
Fig. 3. Polypeptide composition of the soluble 1.6 S component of Complex I obtained by high speed centrifugation of Complex I after different treatments. Electrophoresis in the presence of dodecyl sulphate was performed on 10% acrylamide gels. Molecular weights (10^{-3}) are indicated. A, Complex I, untreated (preparation 2 in Table II); B, Complex I, incubated for 2 h at 30°C in the presence of 1 mM EDTA/1 mM dithiotreitol (Preparation 3 in Table II); C, Complex I, incubated for 5 min at 30°C with 0.5% (w/v) Triton X-100 (Preparation 4 in Table II).

causes a sigmoidal binding of NADH, and a loss of rotenone sensitivity of the NADH-Q oxidoreductase activity.

Discussion

Polydispersity

The presence of two states of dispersion in Complex I preparations is discussed on the basis of Scheme I. Residual detergent (cholate in the case of Complex I) present in the enzyme preparation and responsible for its solubility will, like all amphiphiles, form at moderate concentrations small spherical micelles and at higher concentrations large lamellar micelles [14]. In Scheme I, horizontal direction, it is indicated that a protein may be captured in both forms after dispersion. For instance, the strong detergent dodecyl sulphate is able to form two complexes with any protein, one with 0.4 g dodecyl sulphate per g protein and one with 1.4 g per g [15]. This large amount of detergent bound to the protein will cause the specific volume of the detergent · lipoprotein complex to differ substantially from the average value of 0.734 cm^3 per g given for proteins [16—18], not only because of the high specific volume of the detergents themselves (0.80—0.95 cm^3/g), but also because of the asymmetry and abnormal hydration of the detergent · lipoprotein complex.



SCHEME I

DISPERSION OF TYPE-I NADH DEHYDROGENASE BY AMPHIPHILES

Ringler et al. [3]: phospholipase, Triton X-100,	pathways 3, 4 and 2;
Hatefi et al. [2]: cholate	pathways 1 and 2;
Bangh and King [7]: Triton X-100	pathways 1 and 2;
Kaniuga [5]: Triton X-100	pathways 1 and 2;
Huang and Phavo [6]: Lubrol	pathways 1 and 2;
Ragan and Racker [13]: cholate, phospholipids	pathways 2, 4 and 5.

In Scheme I, vertical direction, the effect of varying the type of amphiphile is considered. The small micelles are all spherical, but the shape of the large micelles depends on the amphiphile used. For structural reasons vesicles or liposomes are obtained with diacylphospholipids and cylinders with monoacyl fatty acids, such as the strong detergent dodecyl sulphate [14]. As a consequence, dodecyl sulphate causes the protein to unfold. The mild detergents take an intermediate position and the precise structure of their protein complexes is not known. The values of $\bar{v}$ and $s^0_{20,w}$ of the slow-sedimenting Complex I component (see Table I) approximate those given for a vesicle [19,20].

The results of the treatments of Complex I listed in Table II illustrate three principles, that apply independently and simultaneously. (1). The transition from small to large micelles is favoured by increasing the concentration of the amphiphile or the temperature [14]. Accordingly, incubation with residual cholate at 30°C, addition of the detergent Triton X-100, or both, increases the solubility of Complex I up to 80% by forming more of the 1.6 S component (pathway 2 in Scheme I). (2). Hydrophobic membrane proteins associate readily with other hydrophobic proteins present in the preparation. That such interactions are strong is illustrated by the fact that an insoluble jelly-like precipitate is formed after sedimentation of the 42 S component. Repeated treatment with mild detergents leads to a purification [21], since the hydrophobic protein-protein interactions are replaced by protein-detergent interactions. The FMN content of the 1.6 S component of Complex I increases from 0.9 nmol/mg protein in Preparation 2 to 1.2 nmol/mg protein in Prepara-

tion 4, and one or two associated Fe-S polypeptides are lost (3). Repeated treatment of lipoproteins with detergents results in the exchange of phospholipids by detergents [17,22], and the release of the apolar Q-10 which is captured in mixed phospholipid-detergent micelles. At the same time the NADH-binding curve changes from hyperbolic to sigmoidal (see Table III), as found earlier [8] after removal of Q-10 from Complex I by pentane extraction, and the rotenone sensitivity of the NADH-Q oxidoreductase activity is lost.

The isolation procedures of Ringler et al. [3], Kaniuga [5,23], Baugh and King [7], Huang and Pharo [6], and Ragan and Racker [13] can now be explained on the basis of the above principles as is summarized in Scheme I.

In Ringler et al.'s procedure incubation with phospholipase catalyses the conversion of diacylphospholipids in the membrane to lysophospholipids (monoacyl), which results in progressive disruption of the ordered bilayer structure of the membrane by forming the more stable globular lysophospholipid-protein micelles (pathway 3). Since the monoacyl structure of lysophospholipids prevents pathway 5 (see above), Ringler had to use Triton X-100 in further purification steps to obtain the soluble 1.6 S form (pathways 4 and 2).

Ragan and Racker [13] obtained by cholate treatment of Complex I a Type-I NADH dehydrogenase preparation that had lost 50% of the phospholipids. This is consistent with the third principle mentioned above. Moreover, they demonstrated the reversibility of the processes described in 1 and 3, above. Thus, by removing cholate by dialysis and addition of phospholipids they changed the type of amphiphile and were able to convert the 1.6 S form of Type-I NADH dehydrogenase via pathway 2, 4 and 5 into the vesicular form, which is favoured by phospholipids.

The heterogeneity of high molecular weight NADH dehydrogenase preparations was demonstrated already in 1963 by Cremona and Kearney [24]. They isolated on a discontinuous sucrose gradient two NADH dehydrogenase-containing fractions with a different specific volume. The heavy component was considered to be a polymer or an impurity and discarded. The soluble light component was used in the later studies by this group [25].

The demonstration in this paper of two components in Complex I preparations, differing only in state of dispersion, confirms these earlier findings. In this respect, the soluble preparations of Ringler et al. [3] and Baugh and King [7] correspond to the exclusively dispersed state, whereas the particulate preparation of Hatefi et al. [2] is a mixture of the two states. The lack of rotenone-sensitive NADH-Q oxidoreductase in the preparations of Ringler et al. [3] and Baugh and King [7] can be explained by replacement of phospholipids by Triton and extraction of Q-10.

Molecular weight

Since the Complex I preparations used contain about $1.1 \cdot 10^6$ g protein per mol FMN and the 42 S component has a molecular weight of $2.8 \cdot 10^6$, it is probable that the latter contains two FMN molecules per molecule, bound detergents and phospholipids being responsible for increasing the molecular weight to $2.8 \cdot 10^6$. Since all small micelles are spherical (see above discussion

and Scheme I) the assumption made in the calculation of the molecular weight that this is the case seems to be justified.

Unfortunately the data of the 1.6 S component do not allow firm conclusions, mainly because of our inability to determine the specific volume of the protein sufficiently accurately. An error in the latter of only 1% would change the molecular weight by 200%. However, since the linear relation with negative slope between $s_{20,w}$ values and protein concentration (see Fig. 2) excludes monomer-dimer equilibria [26], the molecular weights of the 1.6 S and 42 S components are probably the same. This conclusion is confirmed by the similarity in the steady-state kinetics of Complex I and its soluble supernatant (see Table III).

It should be noted that earlier observations in our laboratory are consistent with the picture of two FMN molecules per molecule of dehydrogenase. Studies of the steady-state kinetics of Complex I [8] showed that at least two interacting binding sites for the substrate NADH are present per molecule. Moreover, Albracht et al. [27] have recently reported that only one EPR Centre 1 is present per two FMN molecules.

Polypeptide composition

The acrylamide gel electrophoresis profiles, presented in this paper (see Fig. 3), are similar to those published by others [11,28,29], although the assignment of the molecular weights differs slightly from that by Ragan [11,28]. Such differences are not unusual when this technique is used in different laboratories. Our 90-, 77-, 56-, 40-, 32-, 28-, 22-, 18- and 13-kdalton bands correspond to the 87, 75, 53, 39, 33, 29, 25, 22 and 15.5 kdaltons, respectively, reported by Ragan.

We were unable to achieve the high resolution as reported especially by Ragan [11] on 12.5% acrylamide gels for the polypeptides with molecular weight of 25 000 and lower. In all our calibrations the marker protein cytochrome *c* only slightly separated from the dye front, so that 8- and 5-kdalton chains could not be resolved. Although we did observe peaks and shoulders of 11.5, 13, 16, 18 and 22 kdaltons in this range, we were only able to resolve the 11—16, 18 and 22 kdalton for reliable calculations of the relative intensities (see Table II). This is probably why the 11—16 kdalton polypeptides appear so abundant.

Since the kinetic data (Table III) show that after the treatments described in Table II the quaternary structure of Type-I NADH dehydrogenase is preserved, no essential polypeptide is lost. This enables us, then, to decide in a more rigorous way which polypeptide chains really belong to Type-I enzyme and which do not. The loss of the 24-, 30- and 90-kdalton chains is unambiguous, and that of the 52- and 73-kdalton likely. The 30-, 52- and 73-kdalton chains were also not present in the purer Complex I preparation used by Ragan, and the 90-kdalton chain was not observed [28] in the soluble preparation of Baugh and King [7]. The importance of the 22-kdalton chain is doubtful since this is more soluble than Type-I enzyme itself after all treatments described in Table II. However, at least two polypeptides of this molecular weight, per molecule of FMN, remain with the 42 S component. Although a substantial part of the 32-kdalton chain disappears upon purifica-

tion, one polypeptide per FMN remains. The results for the 40-kdalton chain are ambiguous. Ragan reported two peaks with slightly different molecular weight (39 000 and 42 000) in a ratio to FMN of 1 : 1 : 1. Our results suggest that at least one of them is not essential.

Conclusion

The ideas brought forward in this paper make plausible the concept of one high molecular weight NADH dehydrogenase, the Type-I enzyme, as proposed originally by Slater [9]. Minor differences in polypeptide, phospholipid and detergent composition between different preparations may occur, depending on the history of the enzyme during isolation (see Scheme I).

Experimental

Heart-muscle particles were prepared essentially as described by Keilin and Hartree [30]. Complex I was isolated from these particles according to the procedure of Hatefi et al. [2].

Protein was determined by the biuret method after trichloroacetic acid precipitation as described by Cleland and Slater [31]. The enzyme concentration is expressed on the basis of FMN concentration, determined fluorimetrically as acid-extractable flavin, as described in a previous paper [8].

The NADH-ferricyanide activity was measured at 25°C as described earlier [8].

NADH dehydrogenase was subjected to polyacrylamide gel electrophoresis in the presence of dodecyl sulphate, essentially as described by Weber and Osborn [32]. The protein was denatured in 1% dodecyl sulphate, 1% 2-mercaptoethanol and alkylated with monoiodoacetamide according to the method of Crestfield et al. [33]. Samples containing 25—100 μg protein were subjected to electrophoresis at 3 mA on 10% polyacrylamide gels containing 0.1% dodecyl sulphate. Electrophoresis was carried out vertically in gel columns immersed in buffer at room temperature. The gels were stained with Coomassie Blue and scanned at 600 nm with a scanning attachment for a Gilford spectrophotometer.

The relative areas of the peaks were estimated with a Curve Resolver, Dupont 310. By equating the total area under the peaks with the amount of protein applied on the gel the contributions of the constituent polypeptides could be calculated. From these values, the molecular weights of the polypeptides and the FMN content of the preparation, the polypeptide composition in mol per FMN was obtained. Results were reproducable within 5%.

Molecular weights were estimated by parallel electrophoresis with the marker proteins: bovine serum albumin (68 000), catalase (60 000), ovalbumin (43 000), alcohol dehydrogenase (41 000), chymotrypsinogen (25 700), haemoglobin (15 500) and cytochrome *c* (11 700). The mobility was linearly related to the logarithm of the molecular weight of the marker proteins.

Ultracentrifugation was performed in a MSE Analytical Ultracentrifuge, equipped with a Schlieren optical system and a scanning device for absorption measurements. Sedimentation coefficients were determined from sedimentation velocity experiments described in the legend to Fig. 1. The values obtained

were corrected to the viscosity of water at 20°C [34].

Viscometry at 5.0°C was carried out with a rotating cylinder viscometer designed by Zimm and Crothers [35].

Sedimentation equilibrium measurements for determination of the molecular weight were made by the 3 mm column method of Van Holde and Baldwin [36], using the absorption optical system at 280 nm. The density of solvents and protein solutions was determined by the method of Linderstrøm-Lang and Lanz [37].

Isopyknic centrifugation of NADH dehydrogenase in preformed sucrose gradients was carried out essentially as described by Lusty et al. [25].

All materials were obtained from commercial sources and were used without further purification.

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