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CHAOTROPIC RESOLUTION OF HIGH MOLECULAR WEIGHT (TYPE I) NADH DEHYDROGENASE, AND REASSOCIATION OF FLAVIN-RICH (TYPE II) AND FLAVIN-POOR SUBUNITS

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Summary

1. Type-I NADH dehydrogenase (Complex I) was solubilized and dissociated into subunits by $NaClO_4$. NADH slows the dissociation. On subsequent stepwise addition of $(NH_4)_2SO_4$ the dissociation is partly reversed, as is to be expected from the opposing effects of ClO_4^- and SO_4^{2-}, which are on the salting-in and salting-out sides, respectively, of the lyotropic series.

2. In consequence, the aggregates of subunits that are separated by $(NH_4)_2SO_4$ fractionation consist of randomly associated subunits as well as fragments of Type I enzyme. The fraction precipitating at 27% satd. $(NH_4)_2SO_4$ is flavin-poor, that remaining soluble at 55% satd. $(NH_4)_2SO_4$ flavin-rich and those separating between 27 and 55% satd. $(NH_4)_2SO_4$ intermediate in composition.

3. The fraction remaining soluble at 55% satd. $(NH_4)_2SO_4$ contains the purified low-molecular-weight iron-sulphur flavoprotein (Type-II dehydrogenase). It is a dimer consisting of one molecule of FMN, one 28-kilodalton and one 56-kilodalton subunit per protomer. Work of others indicates that it contains 4 Fe and 4 acid-labile S atoms per molecule of FMN. Sometimes the fraction remaining soluble at 55% satd. $(NH_4)_2SO_4$ contained an additional small subunit (12 kilodaltons) and four additional Fe and acid-labile S atoms per protomer. The sedimentation coefficients ($s^0_{20,w}$) of the two preparations were 5.3 and 6.6 S, respectively, with calculated frictional ratios of 1.5 and 1.24, respectively.

4. The intermediate fractions are mixtures of the various subunits present in Complex I. Specifically a fraction separating at 55% satd. $(NH_4)_2SO_4$ was found to be a mixture of two fragments, the pure iron-sulphur flavoprotein and a 26-S fragment that contained per protomer four subunits of 12 kilodaltons, one each of 28, 32, 56 and 77 kilodaltons, one molecule of FMN and 20 Fe and

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acid-labile S atoms. It was probably tetrameric or even larger.

5. The oxidoreductase activity of the intermediate fractions is dependent on the protein concentration, the activity with ferricyanide increasing and that with ferricytochrome *c* decreasing with increasing protein concentration. This is interpreted as an increased association of subunits present in the intermediate fractions. Similar results are obtained when flavin-rich and flavin-poor fractions are mixed. The association is co-operative. NADH favours the association of the subunits.

6. Association of the subunits is accompanied by a 10-fold increase in k_2 (rate constant for intramolecular electron flow), a 10-fold decrease of the accessibility of ferricyanide to the reduced enzyme and a 10^4-fold decrease of the accessibility of ferricytochrome *c*. The K_s (NADH) is also decreased. Although the changes are in the direction to be expected from a conversion of Type II enzyme to Type I, the value of k_2 is still much less than in the latter enzyme.

Introduction

In a previous paper [1] it was shown that the various types of high-molecular-weight (Type I) NADH dehydrogenase, isolated by different investigators, are essentially the same. The main difference is in the proportion of molecules that are captured by detergents in small micelles (42 S) and in large lamellar structures (1.6 S).

Various treatments (phospholipase digestion at 37°C, or treatment with acid/ethanol, urea, thiourea or proteolytic enzymes) convert the Type I enzyme to a low-molecular-weight form (Type II), with a striking change in kinetics and acceptor specificity [2—8]. It has been shown, however, that the steady-state kinetics of the NADH dehydrogenase activity catalysed by both types of preparation can be described by a single mechanism, in which an ordered and a ping-pong mechanism operate simultaneously [9—11]. The rate constants for association of NADH with the oxidized enzyme (k_1), dissociation of NADH from the resulting complex (k_{-1}) and dissociation of NAD^+ (k_4) are the same in both types of preparation. The main differences lie in the rate constant k_2 for electron flow from enzyme-bound NADH to the electron-accepting group in the enzyme (presumably flavin), which is at least four orders of magnitude lower in Type II, and in k_3, the rate constant for oxidation of reduced enzyme by acceptor, which is two orders (with ferricyanide) or more (with ferricytochrome *c*) higher. The relative inaccessibility of the acceptors to the Type-I enzyme is reflected by double substrate inhibition, suggesting that the electron-donating and -accepting groups in the enzyme can be reached by the substrates only by the same cleft.

In this paper, the physical basis for the difference in properties between Type I and Type II dehydrogenase is examined. Type I enzyme has been partially resolved by treatment with the chaotropic reagent $NaClO_4$, followed by $(NH_4)_2SO_4$ fractionation, a technique introduced by Hatefi and Stempel [12,13]. The flavin-poor, flavin-rich and intermediate fractions, thus obtained, were examined by ultracentrifugation and gel electrophoresis. Reassociation of

flavin-poor with flavin-rich subunits by mixing the separated fractions, or by increasing the concentration of intermediate fractions, results in a conversion of the kinetics from that characteristic for Type II enzyme towards that characteristic for Type I enzyme.

Results

Resolution of Complex I and isolation of fragments

Complex I was incubated at 30°C with 0.5 M $NaClO_4$ in the presence of 1 mM EDTA, 1 mM dithiothreitol and under nitrogen in order to prevent destruction of the iron-sulphur centres. In agreement with the results of others [14] on the transformation of Type I into Type II enzyme, the NADH-ferricyanide oxidoreductase activity decreases and the NADH-ferricytochrome *c* oxidoreductase activity increases (see Figs. 1A and 1B). When the enzyme is kept in the reduced state by the presence of 2 mM NADH in the incubation mixture, the transformation is slowed down.

After 15-min incubation, the insoluble material was removed by centrifugation, and the soluble fragments, containing iron-sulphur proteins and flavoproteins, were isolated from the supernatant by $(NH_4)_2SO_4$ fractionation. The fraction precipitating at 27% satd. $(NH_4)_2SO_4$ could not be solubilized and was dispersed in 0.67 M sucrose, 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol. Increasing the concentration of $(NH_4)_2SO_4$ in the supernatant to 40% satn. resulted in the separation of a phase on top of the solution,

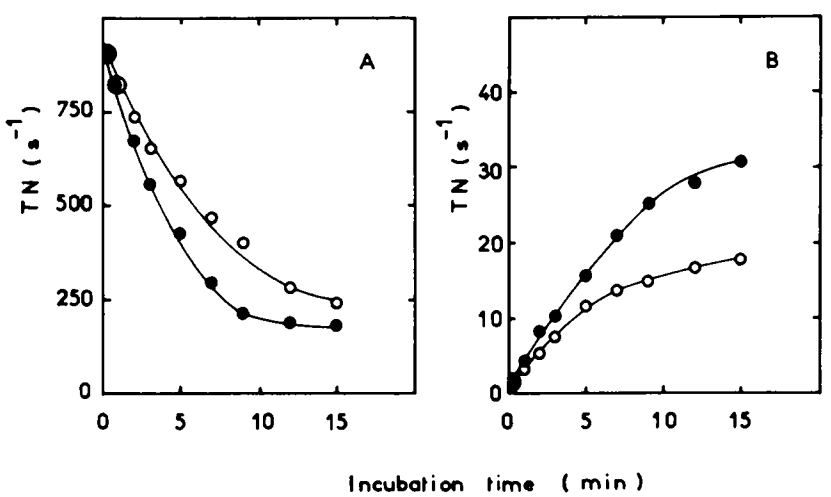

Fig. 1. Effect of treatment of Type I NADH dehydrogenase with perchlorate. Complex I (10 mg/ml) was treated with 0.5 M $NaClO_4$ at 30°C in 50 mM Tris-HCl buffer (pH 8.0), 0.66 M sucrose and 1 mM dithiothreitol in the presence (○———○) or absence (●———●) of 2 mM NADH. At zero time a concentrated solution of $NaClO_4$ was added and samples of the reaction mixture were taken at various time intervals, diluted 20-fold in ice-cold Tris/sucrose buffer and assayed for activity. The NADH-ferricyanide (A) or NADH-ferricytochrome *c* reductase activity (B) of the NADH dehydrogenase is plotted as function of the incubation time. The activity assays were carried out by adding 5 nM enzyme to a reaction mixture containing 1 mM EDTA, 100 μM NADH, 20 mM sodium phosphate (pH 7.5) (A) or 20 mM glycylglycine buffer (pH 8.5) (B), and 1 mM ferricyanide (A) or 15 μM cytochrome *c* (B). The activity is expressed as turnover number (TN) in mol NADH oxidized per mol NADH dehydrogenase (FMN basis) per s.

the precise amount of $(NH_4)_2SO_4$ required varying slightly from experiment to experiment. The fraction remaining soluble at 55% satn. was concentrated and desalted by ultrafiltration, washing with 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol. This fraction contained about 30% of the total flavin and 3% of the total protein. Two types of preparation were obtained in this way, probably dependent upon the extent of washing. These are referred to as Prepns. 1 and 2.

Sedimentation studies on isolated fragments

Two classes of fragments are obtained in this way (see Table I), one with a low specific volume, like the 42-S component of Complex I, the other with a high specific volume like the 1.6-S component (cf. ref. 1). The proteins with the higher specific volume ($\bar{v} \geqslant 0.97$ cm^3/g) start floating at the lower $(NH_4)_2SO_4$ saturation.

Sedimentation-velocity experiments (Fig. 2) show that the fragments investigated are homogeneous, except for the fraction separating at 55% satn. that clearly consists of two components with $s_{20,w}$ values of 4.8 and 26 S (see Fig. 2C). The fragments not separating at 55% satn. represent the purest Type II NADH dehydrogenase preparations, so far as their flavin content is concerned. However, their $s^0_{20,w}$ values vary, both 5.3-S and 6.6-S preparations being obtained (Prepns. 1 and 2, respectively, in Table I). The molecular weight of the latter, determined by sedimentation-equilibrium experiments (see Fig. 3, trace 2), is 223 000, which is double the minimum molecular weight calculated from the FMN content. The molecular weight of the 5.3-S fragment, calculated

TABLE I

PHYSICAL PARAMETERS OF FRAGMENTS OF TYPE-I NADH DEHYDROGENASE

Complex I (10 mg/ml) was incubated under N_2 for 15 min at 30°C with 0.5 M $NaClO_4$ in 0.05 M Tris-HCl buffer containing 0.67 M sucrose, 1 mM EDTA and 1 mM dithiothreitol, final pH 8.0, and fractionated with $(NH_4)_2SO_4$ as described in the text. Physical measurements were made at 5°C.

	Fraction			
	Separated at		Soluble at 55% satd. $(NH_4)_2SO_4$	
	41% satd. $(NH_4)_2SO_4$	55% satd. $(NH_4)_2SO_4$	Prepn. 1	Prepn. 2
Partial specific volume, $\bar{v}$ (cm^3/g)	⩾0.97	0.83	0.83	0.83
Sedimentation coefficient * $s^0_{20,w}$ (S)	1.3	4.8 *** 26	5.3	6.6
Molecular weight ($M_r \times 10^{-3}$), calculated from $s^0_{20,w}$ **	⩾400	112 1370	129	180
$M_r \times 10^{-3}$, calculated from sedimentation equilibrium	—	—	52	223
Minimum $M_r \times 10^{-3}$, calculated from FMN content (kg protein/mol FMN)	1000	179	105	115

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

** A spherical molecule is assumed.

*** Mixture of two components, $s_{20,w}$ values.

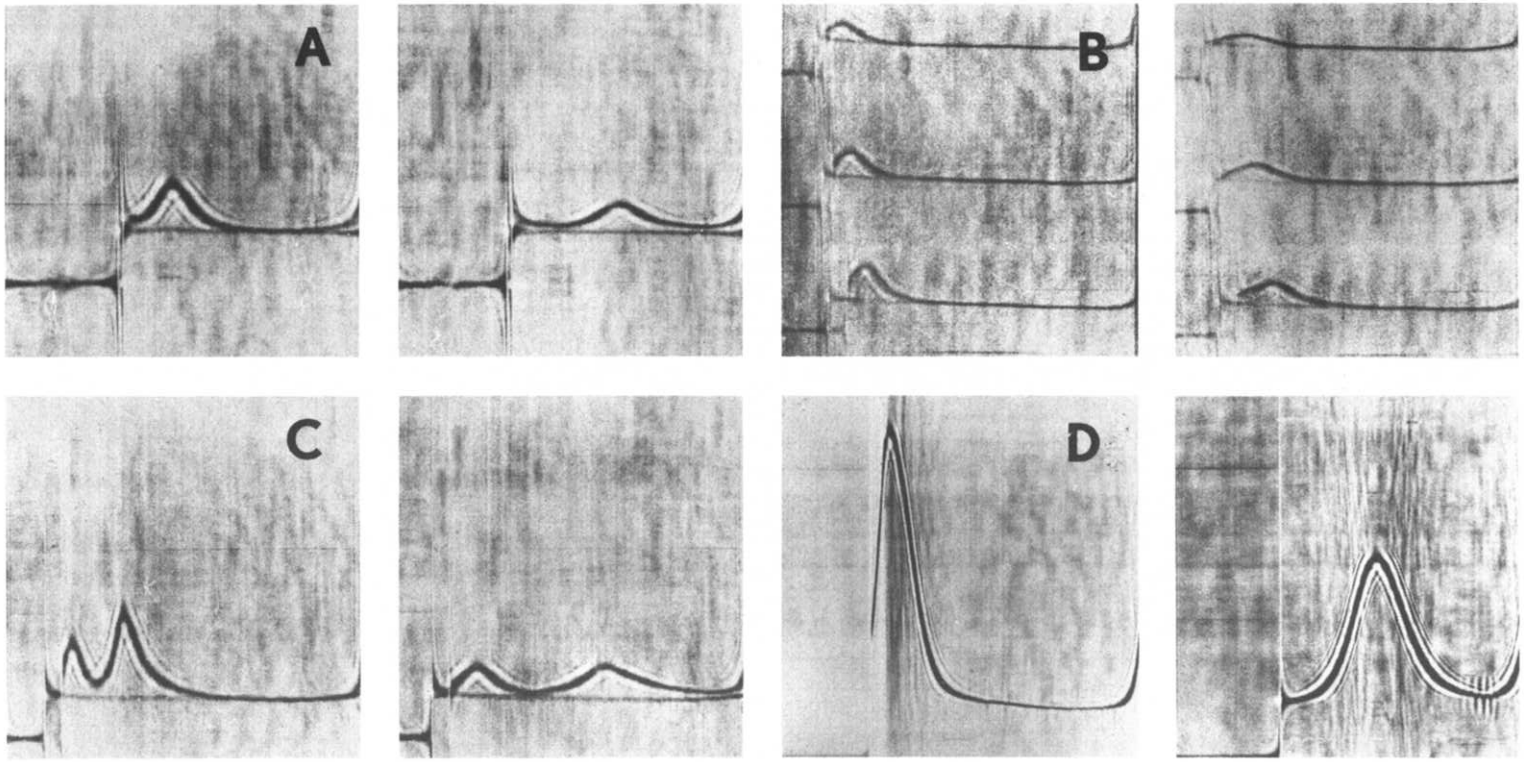

Fig. 2. Sedimentation pattern of fragments of Type I NADH dehydrogenase. (A) Fraction soluble at 55% satd. $(NH_4)_2SO_4$, Prepn. 1. (B) Fraction soluble at 55% satd. $(NH_4)_2SO_4$, Prepn. 2. (C) Fraction separated at 55% satd. $(NH_4)_2SO_4$. (D) Fraction separated at 41% satd. $(NH_4)_2SO_4$. The preparations were dialysed overnight at 0°C against 100 vols. of 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol and 100 mM KCl. They were then diluted with the dialysate and placed in double-sector cells of the ultracentrifuge (A, B and C) or a single-sector cell (D). The cells contained 3 mg/ml protein in A, 2.2, 1.65 and 1.1 mg/ml in B (lower, middle and upper pattern, respectively), 2.2 mg/ml in C and 10 mg/ml in D. The double-sector cells were filled with dialysate buffer on the other side. Centrifugation was conducted at 5°C (A, C and D) or 20°C (B) at 55 000 rev./min (A, B, C) or 60 000 rev./min (D). The photographs shown (left to right) were taken at a phase plate angle of 30°C after 15, 60 min (A), 6, 15 min (B), 6, 20 min (C) and 60, 240 min (D), respectively.

from the $s^0_{20,w}$ value, is only 129 000 (however, see Discussion). The molecular weight could not be determined by sedimentation-equilibrium measurements, since a straight line was not obtained in the plot of ln c vs. r^2 (see Fig. 3, trace 1), suggesting that a mixture of components with different molecular weight was present. This is supported by the experiment given in Fig. 4, which shows that the weight-average molecular weights, calculated from the straight part of the plots of ln c vs. r^2, vary with the measuring wavelength (Fig. 4, trace 6), since the component with lower molecular weight absorbs relatively more at 270 and 285 nm, compared with 280 nm, than the component of higher molecular weight (cf. traces 3 and 4 with traces 1 and 2 in Fig. 4).

Composition of isolated fragments

The polypeptide composition of the fragments was investigated by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. The profiles are shown in Fig. 5, traces A—F, and the concentrations of the different polypeptides in Table II, together with the FMN, iron and acid-labile sulphur content.

The insoluble material (0% $(NH_4)_2SO_4$) clearly represents denatured Type I enzyme, that has lost much of its FMN, Fe and acid-labile sulphur.

The fraction precipitated at 27% satd. $(NH_4)_2SO_4$ is the iron-sulphur protein fraction of Hatefi and Stempel [12,13], consisting primarily of the smaller

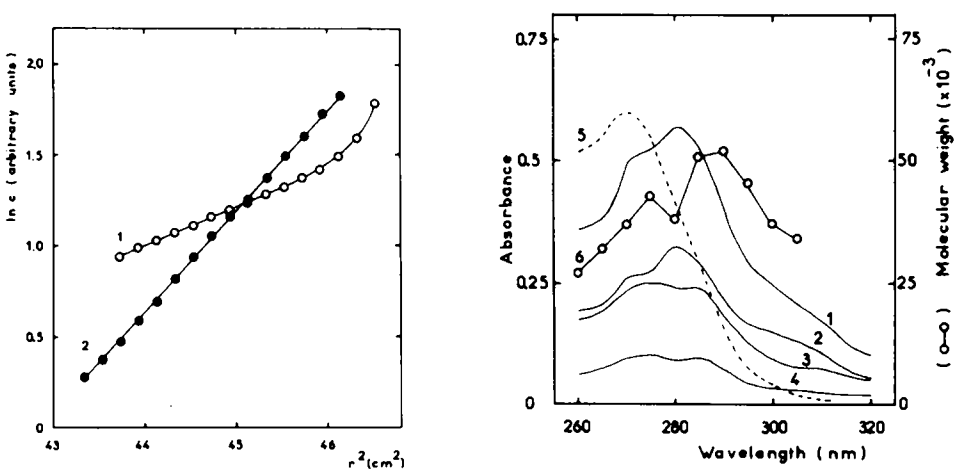

Fig. 3. Plots of ln c vs. r^2 of sedimentation-equilibrium experiments of two preparations (trace 1, Prepn. 1; trace 2, Prepn. 2) of Type II NADH dehydrogenase (soluble at 55% $(NH_4)_2SO_4$ satn.). The enzyme was dialysed as described in Fig. 2 and then centrifuged at 8000 rev./min at 5°C until equilibrium had been established. The absorption at 290 nm was taken as an estimate for the concentration (c). The weight-average molecular weights, calculated from the straight portions of the plots, are shown in Table I.

Fig. 4. Absorption-difference spectra of Type II NADH dehydrogenase (soluble in 55% satd. $(NH_4)_2SO_4$, Prepn. 1) taken at different distances r from the middle of the rotor during the sedimentation-equilibrium experiment described in Fig. 3. The difference spectra were calculated at r 6.821 cm minus 6.613 cm (trace 1); 6.821 cm minus 6.777 cm (trace 2), 6.777 cm minus 6.613 cm (trace 3) and 6.673 cm minus 6.613 cm (trace 4). Trace 5, the spectrum of pure FMN, obtained under the same conditions. o———o, the weight average molecular weights, calculated from the straight portions of the plots of ln c vs. r^2 as a function of the measuring wavelength.

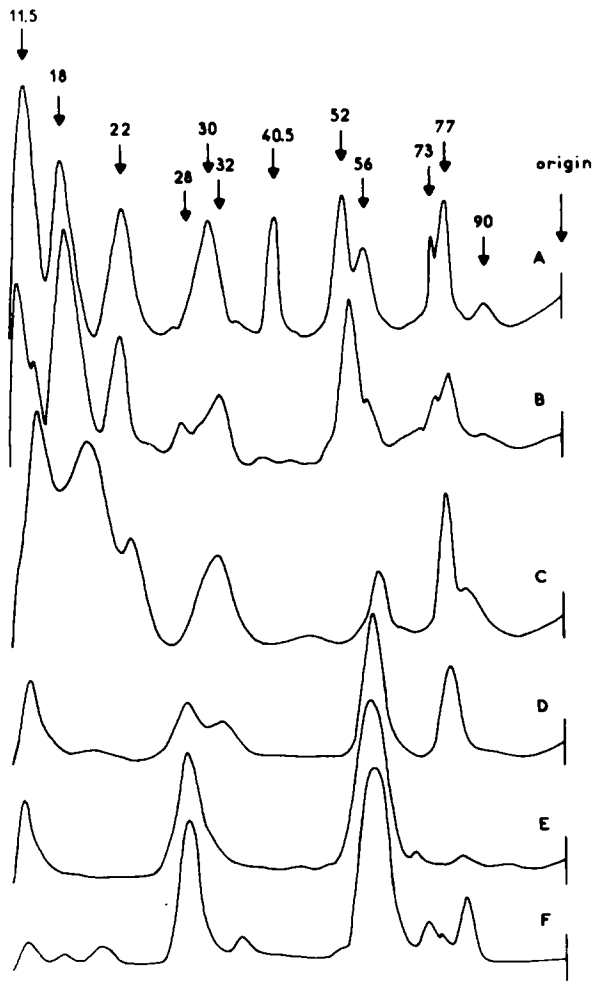

Fig. 5. Polypeptide composition of fragments of Type I NADH dehydrogenase. Electrophoresis in the presence of dodecyl sulphate was performed on 10% acrylamide gels. Molecular weights are indicated. (A—D) Fractions separated at 0, 27, 41 and 55% satd. $(NH_4)_2SO_4$, respectively. (E and F) Fraction soluble at 55% satd. $(NH_4)_2SO_4$ (Prepns. 2 and 1, respectively).

polypeptides. It is, however, very impure (see Discussion) and will be referred to as the flavin-poor fraction.

The fraction not separating at 55% satd. $(NH_4)_2SO_4$ contains an equal number of 28- and 56-kilodalton chains. One preparation (the one shown in Fig. 3 to be homogeneous) contained in addition a smaller polypeptide of 12 kilodaltons (see Fig. 5, trace E). Both preparations contain FMN and iron-sulphur centres. The difference in iron-sulphur content between the two preparations suggests that the smaller polypeptide present in Prepn. 2 is a [4Fe-4S] iron-sulphur protein.

The fractions separating between 27 and 55% satn. are intermediate in flavin content. These will be referred to as the intermediate fractions.

TABLE II

POLYPEPTIDE COMPOSITION, FLAVIN, IRON, AND ACID-LABILE SULPHUR CONTENT OF FRAGMENTS OF TYPE-I NADH DEHYDROGENASE

	Complex I [a]	Fraction					
		Separated at [d]				Soluble at 55% satd. $(NH_4)_2SO_4$	
		0%	27%	41%	55%	Prepn. 2	Prepn. 1
Polypeptide composition (mol%)							
11–16 [b]	49	44	34	37	43	38	—
18	22	22	35	43	—	—	—
22	11	14	11	7	—	—	—
28	3		3	9	16	32	50
32	3	8	5		11	—	—
40	3	4	—	—	—	—	—
52	3	4	6	—	—	—	—
56	6	3	2	2	20	30	50
77	3	4	3	3	9	—	—
mol Fe/mol FMN	24	67	81	21	12	9	4 [c]
mol S/mol FMN	21	34	76	—	12	8	4 [c]
nmol FMN/mg protein	1.2	0.1	0.3	1.0	5.6	8.7	9.5
nmol Fe/mg protein	29	7	24	21	67	78	38

[a] See ref. 1.
[b] Kilodaltons.
[c] From ref. 13.
[d] % satd. $(NH_4)_2SO_4$.

Association of flavin-rich and flavin-poor subunits

Fig. 6A shows that the NADH-ferricyanide oxidoreductase activity of the pure flavoprotein (Prepn. 1) is increased about 3-fold by addition of the flavin-poor fraction precipitated by 27% satd. $(NH_4)_2SO_4$. The intersection point in the titration suggests that 14 nM of the flavoprotein interacts with 33 μg/ml of the flavin-poor subunits for optimal activity. Taking into account the 0.3 nmol FMN/mg protein in the latter, this mixture contains 0.7 nmol FMN/mg protein, somewhat lower than in the starting material, Complex I. If the concentration of the flavin-poor subunits added can be equated with the concentration of free ligand (see Discussion), the data can be converted to the saturation curve (Fig. 6B) and to the Hill plot (Fig. 6C), which shows positive cooperative binding ($h = 2.0$). Under the conditions of the experiments in Fig. 6 the NADH-ferricytochrome *c* oxidoreductase activity is only slightly affected (not shown).

In Fig. 7 it is seen that the turnover rate of an intermediate fraction is dependent on the enzyme concentration in the reaction cuvette. The sigmoidal 5-fold increase in ferricyanide oxidoreductase activity and 20-fold decrease in cytochrome *c* oxidoreductase activity, when the enzyme concentration is increased, suggest that at high protein concentration the flavoprotein becomes associated with other subunits to give a preparation more like the Type I enzyme. The enzyme concentration giving half-maximal turnover rate (K_e) is decreased by pretreatment with NADH before the assay (Fig. 7), or by increasing the concentration of NADH in the assay mixture (Fig. 8). The lower the

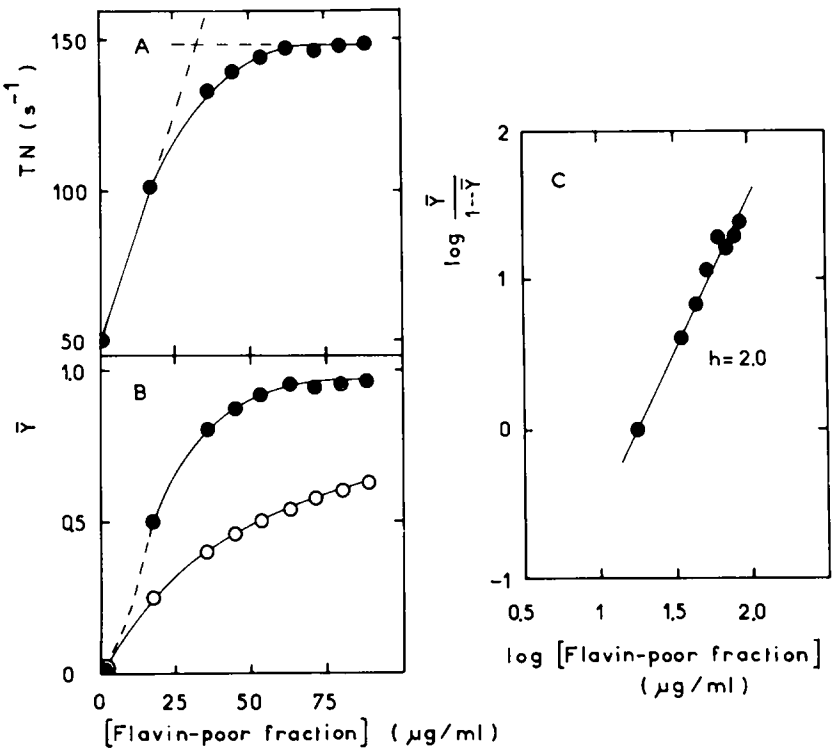

Fig. 6. (A) Stimulation of the NADH-ferricyanide oxidoreductase activity of flavoprotein by addition of the flavin-poor fraction precipitating at 27% satd. $(NH_4)_2SO_4$. Various amounts (25—250 μl) of the flavin-poor fraction, suspended to 1 mg protein/ml in 0.67 M sucrose, 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothreitol were added to 25 μl flavoprotein fraction in 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (Prepn. 1 in Table I) containing 3.7 μg protein and 35 pmol FMN. After 1 min at 25°C, the mixture was diluted to 2.5 ml with 20 mM sodium phosphate buffer (pH 7.5), 1 mM EDTA and the activity measured with 100 μM NADH and 1 mM $K_3Fe(CN)_6$. The activities are expressed as turnover number, calculated on the basis of total FMN content, taking into account the FMN (0.3 nmol/mg protein) present in the flavin-poor fraction. (B) Data of A converted to saturation curve (●——●). The fraction of flavoprotein saturated with subunits ($\bar{Y}$) was calculated from the equation:

$$\bar{Y} = \frac{\text{turnover number} - 50\ s^{-1}}{(152 - 50)\ s^{-1}}$$

$50\ s^{-1}$ is the turnover number of the flavoprotein alone (see A) and $152\ s^{-1}$ the turnover number of the flavin in the flavin-poor fraction. The latter turnover number was constant between 10 and 100 μg/ml of this fraction. The curve, ○——○, gives the theoretical saturation curve, calculated from the equation:

$$\bar{Y} = \frac{[\text{flavin-poor fraction}]\,(\text{nM})}{[\text{flavin-poor fraction}] + 14\ (\text{nM})}$$

for a mixture of 14 nM of the flavoprotein and indicated amounts of the flavin-poor fraction, if there were no activation. (C) Data of B, converted to a Hill plot.

FMN content of the intermediate fractions (i.e. the higher the concentration of the FMN-free subunits), the lower is the flavoprotein concentration, K_e, at which association occurs (see Table III) and the greater is the effect on the enzyme activity (cf. Fig. 7 with Fig. 8).

It should be noted (Fig. 7A) that, at low flavoprotein concentrations, the

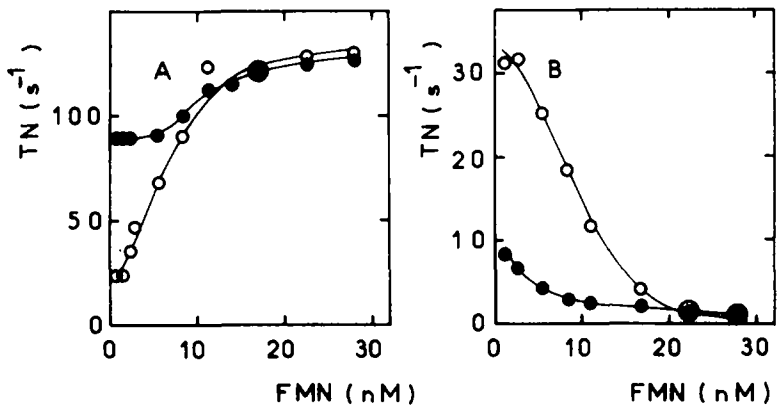

Fig. 7. NADH-ferricyanide (A) and NADH-ferricytochrome *c* (B) oxidoreductase activity as function of the enzyme concentration (FMN basis) in the reaction cuvette of: ○———○, intermediate fraction; ●———●, intermediate fraction, preincubation for 30 s with 100 μM NADH at 25°C in the assay medium before the reaction was started by addition of acceptor. The intermediate fraction (0.5 nmol FMN/mg protein) was separated at 36% satd. $(NH_4)_2SO_4$. Further conditions as described in Fig. 1.

NADH-ferricyanide oxidoreductase activity of the intermediate fraction is less than that of Type II enzyme (see Fig. 9), suggesting the presence of an inhibitor in the former preparation that is revealed only at low enzyme concentrations. This is confirmed in the experiment in Fig. 9, in which the effect of adding a constant amount of intermediate fraction to various amounts of Type

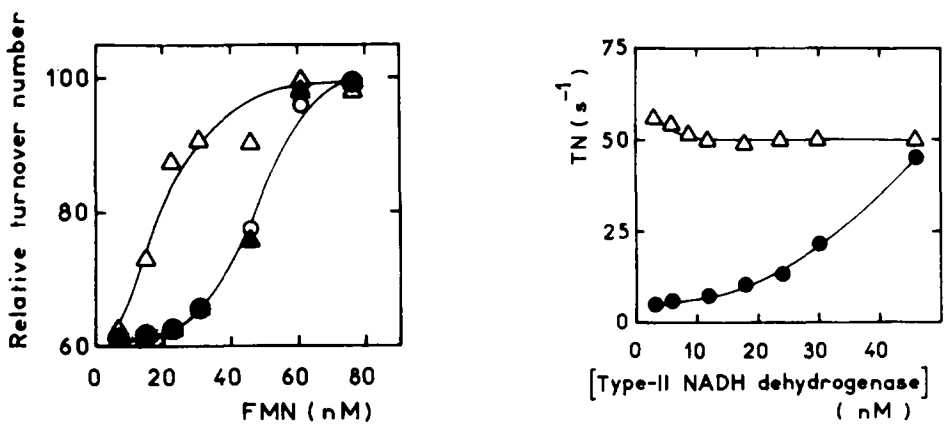

Fig. 8. The NADH-ferricyanide activity of an intermediate fraction containing 6.2 nmol FMN/mg protein as function of the enzyme concentration (FMN basis) in the reaction cuvette, measured at different NADH concentrations. The intermediate fraction was separated at 54% satd. $(NH_4)_2SO_4$. The activity assays were carried out in 1 mM EDTA, 20 mM sodium phosphate (pH 8.0) with 1 mM ferricyanide and 400 μM (△———△), 100 μM (▲———▲) or 33 μM (○———○) NADH. The relative turnover number is expressed as percentage of the maximum value obtained at each NADH concentration. Further conditions as described in Fig. 1.

Fig. 9. Inhibitory effect of an intermediate fraction, containing 0.5 nmol FMN/mg protein (separated at 36% satd. $(NH_4)_2SO_4$) on the NADH-ferricyanide oxidoreductase activity of the flavoprotein (Prepn. 1). The conditions were as described in Fig. 6. △———△, no intermediate fraction; ●———●, 2.8 nM (FMN) intermediate fraction.

TABLE III

THE RELATION BETWEEN FMN CONTENT OF INTERMEDIATE FRACTIONS AND THE FLAVOPROTEIN CONCENTRATION FOR HALF-MAXIMAL TURNOVER RATE (K_e), AS MEASURED BY THE NADH-FERRICYANIDE ACTIVITY ASSAY

The measurements were made as in Fig. 7.

FMN content (nmol FMN/mg protein)	K_e (nM)
0.5	5
4.6	17
5.1	30
5.5	40
6.2	50

II enzyme is shown. Low concentrations of this intermediate fraction (containing 2.8 nM FMN) are able nearly completely to inhibit the NADH-ferricyanide oxidoreductase activity of up to 20 nM of Type II enzyme.

Steady-state kinetics of isolated fragments

In order to obtain more information on the association processes described, a kinetic analysis of the NADH-ferricyanide and -ferricytochrome *c* oxidoreductase activities of the different fragments was made. A sample of the chaotropic-resolved Complex I, that was desalted immediately after the 15-min incubation, was also included. The results are assembled in Table IV. The rate constants refer to the unitarian mechanism described in a previous paper [11]. The turnover numbers of some of the fragments, measured under standard conditions, i.e. fixed concentrations of NADH (100 μM) and ferricyanide (1 mM) or ferricytochrome *c* (15 μM), are listed in Table V.

The data in Table IV show that under conditions favouring association, the k_2 increases by one order of magnitude compared with the value for the Type II dehydrogenase and the accessibility of the acceptors (k_3) decreases by one (ferricyanide) or four (ferricytochrome *c*) orders. The K_s (NADH) also decreases. However, it should be emphasized that the value of k_2 ($8.5 \cdot 10^6$ s^{-1}), found in intact Complex I, is not approached in the reconstituted systems.

Discussion

Isolation of fragments of Complex I

The dissociation of Complex I by the so-called chaotropic agent $NaClO_4$ and the fractionation with $(NH_4)_2SO_4$ of the perchlorate extract are understandable in terms of the lyotropic or Hofmeister series, perchlorate being a salting-in anion and sulphate a salting-out anion. Thus perchlorate will tend to break the ordered structure of water (thereby increasing the solubility of the protein) [14,15] and to weaken the interactions both between the subunits of the protein (thereby loosening the quaternary structure) and within the subunits (thereby loosening the tertiary structure) [16]. Sulphate will tend to have the opposite effects (e.g. see ref. 17). The effects are additive.

Ideally Complex I would be split by perchlorate into separate soluble subunits without any irreversible damage to the latter. Indeed, a comparison of the

TABLE IV

STEADY-STATE KINETICS OF FRAGMENTS OF TYPE-I NADH DEHYDROGENASE

Measurements were made (pH 8.0) and the kinetic data analyzed from plots as described in refs. 10 and 11. The rate constants refer to the unitarian reaction mechanism in ref. 11: $k_{-2} = 8.5 \cdot 10^5\ s^{-1}$ and $k_4 = 10^4\ s^{-1}$.

Preparation	kg protein/ mol FMN	Enzyme * (nM)	K_S (NADH) (μM)	k_2 (s^{-1})	k_3 (ferricyanide) ($M^{-1} \cdot s^{-1}$)	k_3 (cytochrome c) ($M^{-1} \cdot s^{-1}$)
Complex I	1100		25	$8.5 \cdot 10^6$	$1.8 \cdot 10^6$	—
Complex I, resolved with 0.5 M $NaClO_4$ and then desalted	1100		20	$9.9 \cdot 10^3$	$2.5 \cdot 10^7$	$7 \cdot 10^6$
Flavin-poor fraction (precipitated at 27% satd. $(NH_4)_2SO_4$)	3333		40	$1.2 \cdot 10^4$	$2.5 \cdot 10^7$	$7 \cdot 10^6$
Intermediate fraction (separated at 36% satd. $(NH_4)_2SO_4$)		5.6	40	$1.1 \cdot 10^4$	$7.7 \cdot 10^6$	$7 \cdot 10^6$
	2000	28	10	$1.4 \cdot 10^4$	$7.7 \cdot 10^6$	$1 \cdot 10^5$
Intermediate fraction (separated at 54% satd. $(NH_4)_2SO_4$)		19	100—10 **	$1.2 \cdot 10^3$	$6.7 \cdot 10^7$	$1 \cdot 10^9$— $2.5 \cdot 10^5$ **
	161	60	10	$1.2 \cdot 10^3$	$6.7 \cdot 10^7$	$2.5 \cdot 10^5$
Flavoprotein (soluble at 55% satd. $(NH_4)_2SO_4$, Prepn. 1)	105		100—25 **	$1.1 \cdot 10^3$	$1 \cdot 10^8$	$3.3 \cdot 10^9$

* Enzyme concentration in reaction cuvette, expressed on the basis of FMN. The turnover rate is independent of the enzyme concentration, unless specified.

** Depending on NADH concentration.

TABLE V

TURNOVER NUMBERS OF FRAGMENTS OF TYPE-I NADH DEHYDROGENASE, MEASURED UNDER STANDARD CONDITIONS

100 μM NADH, 1 mM ferricyanide in 20 mM phosphate buffer (pH 7.5) or 15 μM ferricytochrome *c* in 20 mM glycylglycine buffer (pH 8.5).

Preparation	kg protein/ mol FMN	NADH-ferricyanide activity (s^{-1})	NADH-ferricytochrome *c* activity (s^{-1})
Complex I, resolved with 0.5 M $NaClO_4$ *	1100	170	32
Complex I, resolved and then desalted	1100	170	33
Flavin-poor fraction (precipitated at 27% satd. $(NH_4)_2SO_4$)	3333	170	35
Intermediate fraction (separated at 36% satd. $(NH_4)_2SO_4$)	2000	25—125 **	32—1.7 **
Flavoprotein (soluble at 55% satd. $(NH_4)_2SO_4$, Prepn. 1)	105	50	32

* Treated with $NaClO_4$ for 15 min; see Figs. 1A and B.

** Turnover rate is dependent on enzyme concentration.

steady-state kinetics of Type I and Type II NADH dehydrogenases [11] shows that the only common rate constant that is altered is that for the intramolecular electron transfer in the NADH · flavoprotein complex (k_2). Although we cannot exclude the possibility that some units involved in partial reactions specific for Type I dehydrogenase are irreversibly modified, the simplest explanation is that association with other polypeptides is necessary to stabilize a conformation of the flavoprotein polypeptide that favours intramolecular electron transfer. However, the kinetic analysis in this paper (Table IV) shows that, in agreement with Singer's "Humpty-Dumpty principle" [8], this conformation is not restored when the polypeptides are reassociated by adding $(NH_4)_2SO_4$. Apparently, new associations, either fragments of Type I enzyme with a limited number of polypeptides or aggregates of randomly associated subunits, are formed and salted-out. Many of the flavin-poor subunits become associated in this way and are precipitated at low $(NH_4)_2SO_4$ concentrations together with some FMN-containing fragments so that the pure flavoprotein subunit, remaining soluble even at 55% satn., is left after fractionation.

The amphiphiles (lipids and cholate) present in Complex I play an important role in the sedimentation behaviour of the species present in the perchlorate/sulphate solutions. In a previous paper [1] it has been shown that the proportion of molecules of Complex I that are captured by amphiphiles in small micelles (42 S) and in large lamellar structures (1.6 S) depends on the ratio of protein species to amphiphiles. Since this ratio increases substantially upon chaotropic resolution, one would expect only small micelles to be present after the resolution.

The calculations given in Table VI support this conclusion, since no floating separated phase, that would be formed by large micelles at 27% satd. $(NH_4)_2SO_4$, was observed. After the removal of much protein by precipitation with 27% satd. $(NH_4)_2SO_4$, however, the ratio of amphiphiles to protein in the supernatant is increased. The fragments formed at 40% satd. $(NH_4)_2SO_4$ comprise both large micelles, that form a floating separated phase, and small micelles that hardly float or sediment at this concentration of $(NH_4)_2SO_4$, but would

TABLE VI

APPARENT MOLECULAR WEIGHT OF A PROTEIN, CAPTURED IN SMALL OR LARGE MICELLES, AS A FUNCTION OF THE DENSITY (ρ) OF THE SOLUTION

The calculation is made for a protein of true molecular weight 10^6. M_r is the apparent molecular weight, taking into account the density of the suspending medium. The specific volume of the small and large micelles is assumed to be 0.83 and 0.97 cm^3/g, respectively.

Fraction (% satd. $(NH_4)_2SO_4$)	ρ (g/cm^3)	M_r ($\times 10^{-3}$)	
		Large micelle	Small micelle
27	1.18	−145	+20
40	1.20	−164	+5
55	1.22	−184	−12.5

float at 55% satn. Thus the separated floating phase obtained at 55% satn. is inhomogeneous, containing both the small micelles formed at 40% satn. and fragments formed at 55% satn. Type II dehydrogenase, with a molecular weight of about 200 000, but an effective molecular weight with respect to the $(NH_4)_2SO_4$ solution of only minus 2500, cannot be separated at this concentration of $(NH_4)_2SO_4$. It may be concentrated by ultrafiltration.

Characterization of low-molecular-weight NADH dehydrogenase

The purest preparation of NADH dehydrogenase obtained in this work (Prepn. 1 in Tables I and II) contained equal amounts of 28- and 56-kilodalton subunits. This is in agreement with the findings of Ragan [18,19].

The minimum molecular weight, calculated from the sedimentation coefficient, is 129 000, assuming a spherical molecule. If the molecule contains one protomer (with a molecular weight of 84 000), it would contain 35% amphiphiles. The protein weight per mol FMN (105 000 g) is not neccessarily inconsistent with this conclusion, since it is possible that some FMN, which is not covalently bound, is dissociated during purification and lost in the filtrate during ultrafiltration. Although this preparation is homogeneous in sedimentation-velocity measurements, its instability made it impossible to determine its molecular weight by sedimentation equilibrium (see Figs. 3 and 4).

A second preparation (Prepn. 2 in Tables I and II), containing in addition a smaller Fe-S-containing peptide (12 kilodaltons), was, however, homogeneous in both sedimentation-velocity and sedimentation-equilibrium measurements, without any evidence of dissociation. The molecular weight of this preparation, calculated from sedimentation equilibrium, is 223 000. Since the protomer molecular weight of this preparation is 96 000, it is probable that it is a dimer containing two of each of the three subunits. The protein weight per mol FMN (115 000 g) indicates that it contains also two molecules FMN. The molecular weight calculated from the sedimentation coefficient is 180 000, assuming a spherical molecule, from which a frictional ratio of 223/180 = 1.24 can be calculated. The difference between the molecular weight (223 000) and double the protomer weight (192 000) suggests that the molecule contains about 15% amphiphiles. The presence of these amphiphiles would account for the relatively high specific volume.

It seems likely, then, that Prepn. 1 is also a dimer containing two molecules of each of the 56- and 28-kilodalton subunits and two molecules FMN per molecule. If it also contains 15% amphiphiles (the specific volumes of the two preparations are the same), the molecular weight would be 193 000 and the frictional ratio 193/129 = 1.5.

These results suggest that binding of the iron-sulphur protein (12 kilodalton polypeptide) to iron-sulphur flavoprotein (Prepn. 1) stabilizes the dimeric structure and makes the molecule more spherical. In disagreement to Ragan [18], we consider this small subunit to be an integral part of the NADH dehydrogenase molecule.

The conclusion that Type-II NADH dehydrogenase, like Type I (see ref. 1), is a dimer is consistent with the conclusion drawn earlier from the steady-state kinetics [11] that two interacting sites for NADH binding are present in the molecule.

Characterization of high-molecular-weight fragments

The heavier fragments separating from the solution at lower concentrations of $(NH_4)_2SO_4$ contain most of the polypeptides present in Complex I but relatively little of the flavoprotein. The fraction precipitating at 27% satd. $(NH_4)_2SO_4$ could be designated the iron-sulphur protein fraction [12,13], insofar as it has the highest Fe : FMN ratio, but in fact it contains less Fe per g protein than the purified preparations of Type II NADH dehydrogenase or even intact Complex I. Indeed, four classes of iron-sulphur centres may be clearly distinguished: (i) Those present in the fraction precipitating at 27% satd. $(NH_4)_2SO_4$; (ii) The iron-sulphur centre that is an integral part of the iron-sulphur flavoprotein itself (Prepn. 1 of Tables I and II). (iii) The 12 kilodalton subunit closely associated with the Type-II NADH dehydrogenase, present in Prepn. 2 of Tables I and II (see above). (iv) Additional iron-sulphur centres present in the fraction separating at 55% satd. $(NH_4)_2SO_4$. Further work is required to correlate these classes with the five different types of centre identified in Complex I by EPR spectrometry [20]. Orme-Johson et al. [21] have shown that the flavin-poor fraction has an EPR spectrum similar to iron-sulphur centre of Complex I.

The intermediate fractions, separating between 27 and 41% satd. $(NH_4)_2SO_4$, contain all the subunits of Complex I except the 40 kilodalton. At low concentrations (see Table IV) these fractions behave kinetically like Type II dehydrogenase, at higher concentrations (100 nM or higher) the kinetic behaviour approaches that of Type I dehydrogenase. Thus, in sedimentation studies, where the flavoprotein concentration is at least 10 μM, these subunits would be expected to be associated in the form of fragments of Type I enzyme. The fraction separating at 55% satd. $(NH_4)_2SO_4$ demonstrates this specifically, being a mixture of two molecules, with sedimentation coefficients ($s_{20,w}$) of 4.8 and 26 S, respectively. The former corresponds to Type II dehydrogenase (Prepn. 1 $s^0_{20,w} = 5.3$). It contains polypeptides of 12, 28, 32, 56 and 77 kilodaltons and Fe atoms in a ratio to FMN of about 2 : 1 : 0.5 : 1 : 0.5 : 12, respectively. Since the 4.8-S component (identical to Type II enzyme) contains 28- and 56-kilodalton polypeptides and Fe atoms in a ratio to FMN of 1 : 1 : 4, respectively, it may be calculated that the 26-S component contains 4 of the 12-kilodalton, 1 each of the 28-, 32-, 56- and 77-kilodalton subunits, 20 atoms Fe and

one molecule FMN per protomer. Since the protomer weight is about 250 000 and the molecular weight calculated from the sedimentation velocity is more than 10^6, it is likely that the 26-S component is tetrameric or perhaps larger. The four 12-kilodalton subunits need not be identical, since the electrophoresis technique used did not resolve molecules smaller than cytochrome *c*.

Designating the subunit structure previously proposed for Complex I protomer [1] by $AB_2CDEF_2G_8H_{18}$, the association-dissociation of subunits in the 55% satd. $(NH_4)_2SO_4$ fraction may be described by the equilibria

	$(ABDEH_4)_n \rightleftharpoons$	$(BEH)_2 \rightleftharpoons$	$(BE)_2$
$s^0_{20,w}$	26	6.6	5.3
Fe : FMN	20	8	4

4 Fe atoms are present in the B or E subunits and 4 on each H subunit(s). Most of the 24 iron atoms per molecule of FMN found in Complex I are in the 26-S component. An additional [4 iron-4 sulphur] (or two [2 iron-2 sulphur]) centre(s) is (are) probably present in the fraction precipitating at 27% satd. $(NH_4)_2SO_4$, but this is so impure that it is impossible to assign a subunit to this fraction. It is relatively enriched in the 18- and 52-kilodalton subunits. However, previous work [1] has shown that the 52-kilodalton subunit is an impurity, and this may also be the case with most of the 18-kilodalton subunits. Since none of the 40-kilodalton subunits (C) was solubilized by perchlorate, it is probable that it also does not belong to Complex I, and the same can probably be said for one of the two 56-kilodalton subunits. Ragan [18] has shown that the two 56-kilodalton subunits are not identical. We shall now then refine the subunit structure of the protomer of Complex I to $ABDEF_2G_8H_{18}$, in the expectation that many of the small subunits (FGH) will turn out to be impurities, and those belonging to the enzyme to be heterogeneous. It would, however, be at least premature to assume that Complex I has the same protomer subunit structure as the 26-S particle, since the steady-state kinetics are not the same (see Table IV), and moreover one or two Fe-S centres are probably missing.

Association of flavin-rich and flavin-poor subunits

The similarities and differences in the kinetics of Types I and II dehydrogenases have been summarized in the Introduction.

In this paper the effect of association of subunits, separated from the Type I enzyme by $NaClO_4$ treatment, on the steady-state kinetics has been studied. This association was brought about in three ways: (i) by adding varying amounts of the flavin-poor fraction precipitated at 27% satd. $(NH_4)_2SO_4$ to a constant amount of the flavoprotein subunits (Fig. 6); (ii) by adding varying amounts of the flavoprotein subunit to a constant amount of the 36% satd. $(NH_4)_2SO_4$ fraction (Fig. 9); (iii) by varying the concentration of an intermediate fraction (Figs. 7 and 8). In all cases, association of the flavoprotein subunits with other subunits led to a change in the steady-state kinetics in the direction expected of a transition from a Type II to a Type-I enzyme. NADH favours the transition and presumably the association.

However, the transition was far from complete and the association of sub-

units was much less than in Complex I. Table IV shows that the maximum reconstitution demonstrated in this paper could be obtained simply by removal of perchlorate from the resolved Complex I preparation. In fact, the similar turnover numbers of the resolved Complex I, before and after desalting, shown in Table V suggest that the 2000-fold dilution in the reaction cuvette for measurement of the activity is as effective as desalting. This implies, then, that the decrease of the NADH-ferricyanide oxidoreductase activity and the increase of the NADH-ferricytochrome *c* oxidoreductase activity in Figs. 1A and 1B, respectively, reflect the irreversible changes occurring on resolution. The reversible changes are illustrated in Tables IV and V by comparing the kinetic parameters of the pure flavoprotein with those of the other fragments. Thus the value for k_2 in the flavin-poor fraction is 10 times greater than with the flavoprotein fraction, but still nearly three orders smaller than in Complex I. The value of k_3, reflecting the accessibility of ferricyanide, is one quarter that in the flavoprotein fraction but is still more than 10 times greater than in Complex I. The increased k_2 but decreased k_3 results in a 3-fold increase in the NADH-ferricyanide oxidoreductase activity measured under standard conditions (Table V). That the NADH-ferricytochrome *c* oxidoreductase activities of the flavin-poor fraction and the flavoprotein are the same (Table V) is purely a coincidence, since the 10-fold increase in k_2 is compensated by a 500-fold decrease in k_3 (see Table IV).

Since the turnover number of the flavoprotein in the flavin-poor fraction is independent of the enzyme (FMN) concentration, the flavoprotein remains associated with flavin-poor subunits when diluted for measurement of the enzyme activity. This points to an excess of flavin-poor subunits in this fraction. The increase in the NADH-ferricyanide oxidoreductase activity, measured under standard conditions, on addition of the flavin-poor fraction to the flavoprotein fraction, indicates that the subunits in excess are capable of associating with the dimeric flavoprotein. The Hill plot (Fig. 6C) shows that the association is positively cooperative.

As described above, the fraction precipitating at 55% satd. $(NH_4)_2SO_4$ contains three subunits (A, D, H) in addition to those present in the pure iron-sulphur flavoprotein fraction (B and E). When diluted to 19 nM FMN, in the presence of 33 or 100 μM NADH, the steady-state kinetics are essentially the same as with the pure flavoprotein (Table IV). Increasing the concentration of NADH to 400 μM, however, leads to association (see Fig. 8), resulting in a lowering of the K_s (NADH) 10-fold and of the accessibility of ferricytochrome *c* by nearly 4 orders of magnitude. With a 3-fold greater concentration of protein in Fig. 8, the same degree of association is already reached with 33 μM NADH. This implies that binding of one or more of the subunits involved here (A, D, H) to the dimeric iron-sulphur flavoprotein promotes a conformation that binds NADH tightly. Since in neither case was there any effect on k_2 or k_3 (ferricyanide), it follows that other subunits (F and G are left) must have been responsible for the increased ferricyanide oxidoreductase activity on adding the flavin-poor fraction to the flavoprotein, described above.

In a previous paper [11] we have shown that the binding of NADH to the pure iron-sulphur flavoprotein is positively cooperative, K_s (NADH) changing from 100 to 25 μM at high NADH concentrations. However, in that case the

association processes described here did not play a role, since the turnover number was independent of the enzyme concentration. Moreover, varying NADH or protein concentration had no effect on k_3 (ferricytochrome *c*).

The association of the fraction separated at 36% satd. $(NH_4)_2SO_4$, shown in Fig. 7, can be explained along similar lines. The increased activity with ferricyanide with 28 nM enzyme (Fig. 7A) is mainly due to a decreased K_s (NADH), since k_3 (ferricyanide) is not affected by increasing the enzyme concentration from 5.6 to 28 nM (see Table IV). The greatly decreased activity with ferricytochrome *c* (Fig. 7B) is due to the declining accessibility to ferricytochrome *c* overshadowing the decrease in K_s (NADH). The A, D and H subunits are probably involved in these effects. However, the different concentrations of protein that have half-maximal effect in Figs. 7A and 7B (5 and 9 nM, respectively) suggest that other subunits (perhaps F or G) with a different effect on the NADH-ferricyanide and the NADH-cytochrome *c* oxidoreductase activities also play a rôle.

The low value of k_3 (ferricyanide) found with the 36% satd. $(NH_4)_2SO_4$ fraction, even lower than with the 27% satd. $(NH_4)_2SO_4$ fraction, is worthy of attention. This decreased accessibility channels the kinetics towards the ping-pong mechanism, but does not restore the double substrate inhibition, characteristic of Type I dehydrogenase (not shown). Indeed, this preparation seems to contain an excess of the subunit that is responsible for blocking access to ferricyanide, since it strongly inhibits the NADH-ferricyanide oxidoreductase activity of the iron-sulphur flavoprotein (Fig. 9).

The above considerations show that the association of flavin-poor subunits with the iron-sulphur flavoprotein is complex and takes place in different stages. However, with respect to function two classes of subunits may be distinguished: (i) the subunits (A, D and maybe H) that promote the tight binding of NADH to the flavoprotein (K_s (NADH) is decreased); (ii) the subunits (F and maybe G) that influence the redox potential of the electron-accepting groups in the flavoprotein (k_2 is increased). Binding of both classes (as well as another subunit in the 36% satd. $(NH_4)_2SO_4$ fraction) leads to structural changes in the flavoprotein that make it more inaccessible to ferricytochrome *c* and ferricyanide, but this is not itself of physiological importance. Since many iron-sulphur proteins are involved in the association processes one might speculate about their function (see also ref. 11) in regulating the redox potential of the flavoprotein subunit by inter-subunit interactions. For example, the 12-kilodalton iron-sulphur protein (H) was shown above to change the conformation of the flavoprotein, making it more stable and spherical. Further work, however, is required to ascribe specific functions to the different polypeptides.

Experimental

Heart-muscle particles were prepared essentially as described by Keilin and Hartree [22]. Complex I was isolated from these particles according to the procedure of Hatefi et al. [23]. Complex I was resolved essentially according to the method of Hatefi and Stempel [12,13], using 0.5 M $NaClO_4$ [14] instead of 2.5 M urea. The resolution was carried out in a medium containing 0.67 M sucrose, 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1 mM dithiothrei-

tol. All solutions were de-aerated before use and the enzyme solution was kept under N_2 throughout the isolation procedure. The resolved Complex I was fractionated by $(NH_4)_2SO_4$ as described in Results. The fractions, thus obtained, were desalted by ultrafiltration, stored at the temperature of liquid N_2 and thawed just before use.

Protein was determined by the biuret method after trichloroacetic acid precipitation as described by Cleland and Slater [24]. The enzyme concentration is expressed on the basis of FMN concentration, determined fluorimetrically as acid-extractable flavin, as described in a previous paper [10]. Non-heme iron was determined by the procedure of Lovenberg et al. [25], and acid-labile sulphide by the procedure of Fogo and Popowsky [26].

The NADH-ferricyanide and NADH-ferricytochrome *c* oxidoreductase activities were measured at 25°C as described earlier [10,11].

Complex I and its subunits were subjected to polyacrylamide gel electrophoresis in the presence of dodecyl sulphate, essentially according to the method of Weber and Osborn [27]. Molecular weights and polypeptide composition were calculated as described earlier [1].

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 as described earlier [1]. The values obtained were corrected to the viscosity of water at 20°C [28].

Viscometry at 5.0°C was carried out with a rotating-cylinder viscometer designed by Zimm and Crothers [29].

Sedimentation-equilibrium measurements for determination of the molecular weight were made by the 3-mm column method of Van Holde and Baldwin [30], 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 [31].

Isopycnic centrifugation of fragments of NADH dehydrogenase in preformed sucrose gradients was carried out essentially as described by Lusty et al. [32].

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

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References

1 Dooijewaard, G., De Bruin, G.J.M., Van Dijk, P.J. and Slater, E.C. (1978) Biochim. Biophys. Acta 501, 458–469

2 Mahler, H.R., Sarkar, N.K., Vernon, L.P. and Alberty, R.A (1952) J. Biol. Chem. 199, 585–597

3 Kaniuga, Z. (1963) Biochim. Biophys. Acta 73, 550–564

4 Slater, E.C. (1966) in Flavins and Flavoproteins (Slater, E.C., ed.), BBA Library, Vol. 8, pp. 487–488, Elsevier, Amsterdam

5 King, T.E., Howard, R.L., Kettman, Jr., J., Hegdekar, B.M., Kuboyama, M., Nickel, K.S. and Possehl, E.A. (1966) in Flavins and Flavoproteins (Slater, E.C., ed.), BBA Library, Vol. 8, pp. 441—481, Elsevier, Amsterdam
6 Kumar, S.A., Appaji Rao, N., Felton, S.P. and Huennekens, F.M. (1968) Arch. Biochem. Biophys. 125, 436—448
7 Singer, T.P. and Gutman, M. (1970) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 375—391, Springer-Verlag, Berlin
8 Singer, T.P. and Gutman, M. (1971) Adv. Enzymol. 34, 79—153
9 Slater, E.C. (1975) in Electron-Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 3—14, North-Holland, Amsterdam
10 Dooijewaard, G. and Slater, E.C. (1976) Biochim. Biophys. Acta 440, 1—15
11 Dooijewaard, G. and Slater, E.C. (1976) Biochim. Biophys. Acta 440, 16—35
12 Hatefi, Y. and Stempel, K.E. (1967) Biochem. Biophys. Res. Commun. 26, 301—308
13 Hatefi, Y. and Stempel, K.E. (1969) J. Biol. Chem. 244, 2350—2357
14 Davis, K.A. and Hatefi, Y. (1969) Biochemistry 8, 3355—3361
15 Hatefi, Y. and Hanstein, W.G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1129—1136
16 Von Hippel, P.H. and Schleich, T. (1969) in Structure and Stability of Biological Macromolecules (Timasheff, S.N. and Fasman, G.D., eds.), Vol. 2, pp. 417—574, Marcel Dekker, Inc., New York
17 Davis, K.A. and Hatefi, Y. (1972) Arch. Biochem. Biophys. 149, 505—512
18 Ragan, C.I. (1976) Biochem. J. 154, 295—305
19 Ragan, C.I. (1976) Biochim. Biophys. Acta 456, 249—290
20 Albracht, S.P.J., Dooijewaard, G., Leeuwerik, F.J. and van Swol, B. (1977) Biochim. Biophys. Acta 459, 300—317
21 Orme-Johson, N.R., Hansen, R.E. and Beinert, H. (1974) J. Biol. Chem. 249, 1922—1927
22 Keilin, D. and Hartree, E.F. (1947) Biochem. J. 41, 500—502
23 Hatefi, Y., Haavik, A.G. and Griffiths, D.E. (1962) J. Biol. Chem. 237, 1676—1680
24 Cleland, K.W. and Slater, E.C. (1953) Biochem. J. 53, 547—556
25 Lovenberg, W., Buchanan, B.B. and Rabinowitz, J.C. (1963) J. Biol. Chem. 238, 3899—3913
26 Fogo, J.K. and Popowsky, M. (1949) Anal. Chem. 21, 732—734
27 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
28 Svedberg, T. and Pedersen, K.O. (1940) The Ultracentrifuge, Oxford University Press, Oxford
29 Zimm, B.H. and Crothers, D.M. (1962) Proc. Natl. Acad. Sci. U.S. 48, 905—911
30 Van Holde, K.E. and Baldwin, R.L. (1958) J. Phys. Chem. 62, 734—743
31 Linderstrøm-Lang, K. and Lanz, Jr., H. (1938) C.R. Trav. Lab. Carlberg, Sér. Chim. 21, 315—338
32 Lusty, C.J., Machinist, J.M. and Singer, T.P. (1965) J. Biol. Chem. 240, 1804—1810