IMMUNOLOGICAL ASSAYS OF THE NADH DEHYDROGENASE CONTENT OF BOVINE HEART MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Stuart SMITH, Ian R. COTTINGHAM and C. Ian RAGAN
Department of Biochemistry, University of Southampton, Southampton, Hants S09 3TU, England

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1. Introduction

The NADH dehydrogenase content of mitochondria and submitochondrial particles is more difficult to measure than the content of other electron-transport complexes. The absence of unambiguously identifiable chromophores has, until recently, necessitated the use of more indirect measurements based on turnover numbers. For example, a turnover number of 8×10^5. min^{-1}. mol FMN^{-1} in the NADH–$K_3Fe(CN)_6$ oxidoreductase assay was determined [1] for the soluble type 1 NADH dehydrogenase. From this value and the specific activity of NADH–$K_3Fe(CN)_6$ oxidoreductase in submitochondrial particles, a value for the NADH dehydrogenase content of the latter was calculated (0.03 nmol/mg protein). That this value (as nmol dehydrogenase FMN/mg protein) was considerably less than the total acid-extractable FMN content of these particles showed that the use of total FMN content was an unreliable way of estimating NADH dehydrogenase content.

However, the turnover numbers of purified NADH dehydrogenase preparations, all containing similar concentrations of FMN, vary considerably with the purification procedure between 4×10^5 for complex I and 1.5×10^6 for the preparation of [2]. In the absence of clear evidence that the lower range represents inactivation, dehydrogenase contents based on NADH–$K_3Fe(CN)_6$ oxidoreductase activities should be treated with reservation. NADH–ubiquinone oxidoreductase activity is probably also unreliable in this respect since the kinetics of this reaction are very dependent on the phospholipid environment [3].

Here we describe two assays of NADH dehydrogenase content using antibodies to purified complex I. From these values we calculate the true turnover number of the enzyme in the membrane and show that previous estimates of the NADH dehydrogenase content of submitochondrial particles require revision.

2. Materials and methods

Submitochondrial particles (SMP) and complex I from bovine heart were prepared by the methods in [4,5], respectively. Antibodies to complex I were raised in rabbits as in [6] and their ability to immunoprecipitate NADH dehydrogenase from detergent-solubilised SMP was tested as in [7]. Fluorimetric analysis of non-covalently bound FMN and FAD was carried out as in [8]. FMN and FAD standards were purified from commercial FMN and FAD by DEAE-cellulose chromatography [9]. Fluorescence was measured in a Perkin Elmer MPF-44A fluorescence spectrophotometer with excitation at 450 nm and emission at 520 nm. Rocket electrophoresis was carried out as in [10] except that protein samples were solubilised in Triton X-100 (1%, w/v) and Na-deoxycholate (2.5 mg/mg protein) instead of Triton and SDS. NADH–$K_3Fe(CN)_6$ oxidoreductase activity was measured at 30°C exactly as in [11] for the so-called '30°C enzyme'. Protein was measured as in [12] using bovine serum albumin (Sigma fraction V) as a standard.

3. Results and discussion

In the experiment of table 1, non-covalently bound flavin was released from protein by boiling [8] and its fluorescence at neutral pH compared with an FMN standard. Thus the value of 0.157 nmol/mg protein for SMP contains contributions from both FMN and

Table 1
Estimation of NADH dehydrogenase FMN by fluorescence

Sample and treatment	Fluorescence (arbitrary units)	Flavin content (nmol/mg protein)
Complex I (23.3 μg protein)	88	0.80
Complex I + antiserum (total)	132	
Complex I + antiserum (sup.)	50	
Immunoprecipitate (by diff.)	82	0.75
SMP-1 (267 μg protein)	158	(0.157)
SMP + antiserum (total)	206	
SMP + antiserum (sup.)	140	
Immunoprecipitate (by diff.)	66	0.066

Complex I (58.2 μg protein) was incubated in 2.5 ml final vol. of 0.67 M sucrose/50 mM Tris–HCl (pH 8.0) for 2 h at 0°C. Where indicated, 2 ml anti-(complex I) serum and 0.14 ml 20% (w/v) Triton X-100 replaced part of the sucrose–Tris buffer. Flavin was released by heating to 100°C for 3 min in the dark. Another sample containing antiserum and Triton was centrifuged at 50 000 × *g* for 15 min, and the supernatant was heated as above. Portions of SMP (668 μg protein) were treated identically. The heated samples were cooled, centrifuged at 100 000 × *g* for 15 min and filtered to remove traces of insoluble material. The fluorescence of 1 ml portions diluted with 1 ml sucrose–Tris buffer was compared with that of FMN (0–100 pmol) in 2 ml same buffer

FAD. The fluorescence of the sample from SMP which had been treated with Triton X-100 and sufficient antiserum to completely agglutinate the NADH dehydrogenase present was higher than that of SMP alone. This increase was due to traces of fluorescent material in both the Triton and the antiserum. After removal of the immunoprecipitate by centrifugation, the fluorescence released from the supernatant by boiling was decreased. This decrease in fluorescence represents the loss of the FMN-containing NADH dehydrogenase and therefore can be directly converted into nmol FMN without consideration of the FAD originally present. Thus, the NADH dehydrogenase content of the SMP was found to be 0.066 nmol enzyme-bound FMN/mg protein. The total non-covalently bound FMN and FAD contents of these SMP were 0.133 and 0.246 nmol/mg protein, respectively. A second SMP preparation (SMP-2) was found to have an NADH dehydrogenase content of 0.075 nmol/mg protein.

The validity of this experimental approach is shown by the results of table 1 obtained with complex I. The immunoprecipitatable flavin was almost exactly the same as the total flavin, as would be expected. Thus, the fluorescent impurities introduced with the Triton and antiserum did not interfere with the assay. The only other source of error would be if the antiserum was not specific for complex I. We have analyzed immunoprecipitates by one and two dimensional gel electrophoresis [6] to establish their purity. However, the assay of table 1 requires only that the antiserum should not precipitate any other flavoproteins.

A direct estimate of the NADH dehydrogenase content as a % of mitochondrial or SMP protein was obtained by rocket immunoelectrophoresis (fig.1). Rocket area rather than height gave the more linear relationship with antigen concentration. The complex I content of heavy layer mitochondria was found to be 4.8% based on protein (0.036 nmol/mg) while SMP-1 and SMP-2 contained 9.3% (0.070 nmol/mg) and 8.5% complex I (0.064 nmol/mg). The values for SMP are in good agreement with those obtained from fluorescence measurements. On the whole, the reproducibility of the rocket assay with crude membrane preparations is not as good as the fluorescence assay. Factors contributing to this include non-specific precipitation of membrane proteins leading to distortion of the rocket, and fragmentation of NADH dehydrogenase during electrophoresis.

Table 2 summarizes specific activities and turnover numbers based on the dehydrogenase content. The addition of Triton X-100 and antiserum to SMP did not alter the V_{max} of NADH–$K_3Fe(CN)_6$ oxidoreduc-

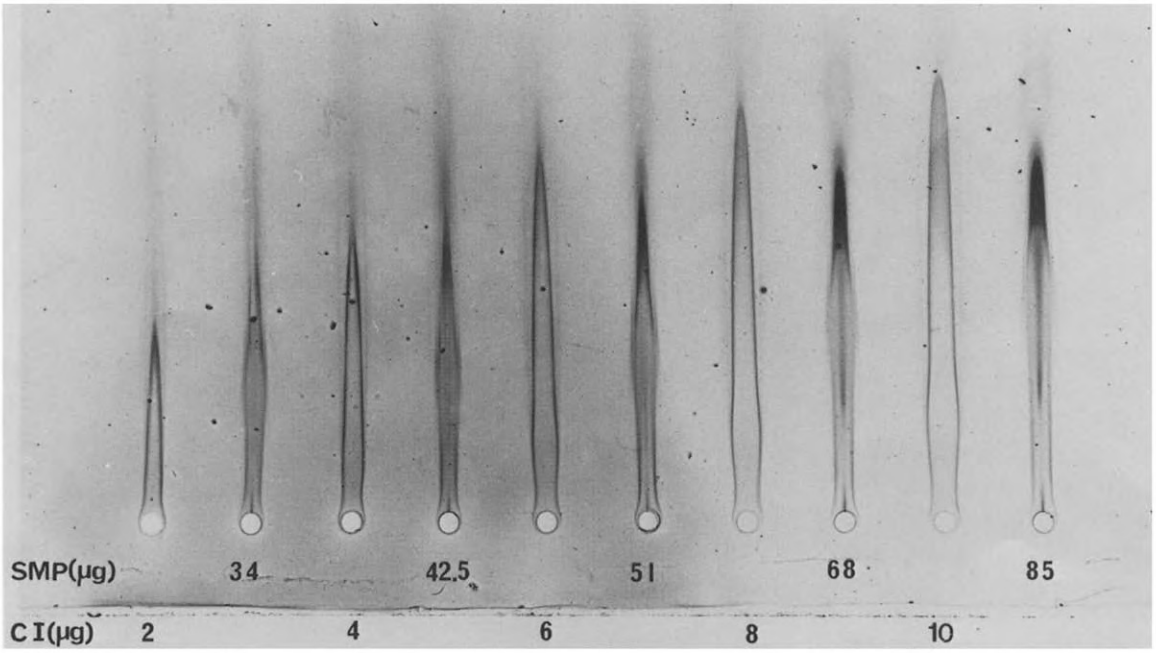

Fig.1. Rocket immunoelectrophoresis of complex I and submitochondrial particles. Immunoelectrophoresis was carried out in 1% (w/v) agarose gels containing 30 mM Tris–glycine (pH 8.6) 1% (w/v) Triton X-100 and 43 μl anti-(complex I) serum/ml gel. Complex I was diluted to 2 mg protein/ml Tris–glycine buffer containing 1% (w/v) Triton X-100 and 0.4 mg Na deoxycholate/mg protein. SMP-1 were diluted to 10 mg/ml with the same buffer and dissolved with Triton X-100 and deoxycholate as above. The amount of protein (in μg) applied to each well is shown. Electrophoresis was at 2.5 V/cm for 24 h at 4°C.

tase activity. Some activity was not precipitated by antiserum [7], and could be distinguished from residual NADH dehydrogenase by its very much lower K_m. Thus, under the assay conditions used, this activity did not vary with $K_3Fe(CN)_6$ concentration and amounted to 0.75 and 0.82 μmol NADH . min^{-1}. mg SMP-1 and SMP-2^{-1} $protein^{-1}$, respectively. Correction of the total activities for this residual activity caused a small increase in both the K_m for $K_3Fe(CN)_6$ and the V_{max} as indicated in table 2. Turnover numbers based on the corrected activities were 4.6×10^5/min for SMP-1 and 4.5×10^5/min for SMP-2. Since all complex I activity is precipitated by antiserum [13], no correction to the V_{max} was required and a very

Table 2
Turnover numbers of NADH dehydrogenase

Prep.	NADH dehydrogenase FMN (nmol/mg protein)	NADH–$K_3Fe(CN)_6$ oxidoreductase				
		Before correction		After correction		
		V_{max} (μmol NADH/ min/mg protein	K_m (mM)	V_{max} (μmol NADH/ min/mg protein)	K_m (mM)	Turnover no. (per min)
SMP-1	0.066	30	2.8	31	3.3	4.6×10^5
SMP-2	0.075	33	2.7	34	2.8	4.5×10^5
Complex I	0.75	330	2.2	–	–	4.4×10^5

NADH–$K_3Fe(CN)_6$ oxidoreductase activities were measured at different $K_3Fe(CN)_6$ concentrations [11] to determine V_{max} and K_m for $K_3Fe(CN)_6$. SMP were pretreated with Triton X-100 and antiserum as in table 1. Activities were measured before and after centrifugation so that correction could be made for the activity catalyzed by enzyme(s) other than NADH dehydrogenase

similar turnover number of 4.4×10^5/min was obtained. Thus, there is a strict parallel between enzyme-bound FMN and NADH–$K_3Fe(CN)_6$ oxidoreductase activity during purification of complex I.

This is not true, however, of other NADH dehydrogenase preparations such as those in: [1] (turnover no. 8×10^5/min); [14] (turnover no. 1.5×10^6/min); [15] (turnover no. 7.4×10^5/min); [2] (turnover no. 1.5×10^6/min). Clearly these preparations are activated during purification. The use of high turnover numbers to calculate the NADH dehydrogenase content of SMP obviously produces values which are too low. In view of this it will be necessary to reassess the conclusion that membrane-bound NADH dehydrogenase binds 2 molecules inhibitor piericidin/molecule enzyme [16]. Since the piericidin titre was reported to be 0.05–0.06 nmol/mg SMP protein [16,17] it is possible that the stoichiometry is only 1:1. The halving of the piericidin titre by mersalyl [16] will then require an alternative explanation perhaps based on a multimeric NADH dehydrogenase (e.g. [18]).

Further corroboration of these findings has been provided ([18], Albracht et al. personal communication) by determining the relative stoichiometry of the iron–sulphur centres of NADH dehydrogenase in SMP. The molar ratio of centre 2 to cytochrome c_1 in SMP was found to be 0.28:1. Assuming a typical cytochrome c_1 content of 0.25 nmol/mg protein this leads to an estimated centre 2 (or dehydrogenase) content of 0.070 nmol/mg protein in good agreement with the values we have obtained.

Lastly, we wish to point out that the preparation of complex I used for FMN and activity measurements in these studies was rather impure, and more realistic figures for the dehydrogenase content of SMP and mitochondria would be ~6% and 3%, respectively. In view of this, the yield of complex I from mitochondria (1.1–1.8% [5]) represents a more respectable recovery than was suspected and further suggests that NADH–ubiquinone oxidoreductase activity [5] is lost to some extent during purification.

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