Biochem. J. (1989) 257, 859–863 (Printed in Great Britain)

Nitroreductase activity of NADH dehydrogenase of the respiratory redox chain

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1. An NADH-dependent nitroreductase from the inner membrane of ox liver mitochondria copurified with Complex I of the respiratory redox chain (NADH:ubiquinone oxidoreductase, EC 1.6.5.3). 2. The corresponding nitroreductase from ox heart mitochondria co-purified with the NADH–cytochrome *c* reductase of Mahler, Sarkar & Vernon [(1952) J. Biol. Chem. **199**, 585–597] [NADH:(acceptor) oxidoreductase, EC 1.6.99.3], a component of Complex I that contains the FMN. 3. The mitochondrial nitroreductase activity is attributed to the flavoprotein component of Complex I.

INTRODUCTION

There are few biologically occurring nitro compounds [1], yet many organisms can metabolize nitro compounds of biological or non-biological origin by the reduction of the nitro group. Such reactions, which can yield either a hydroxylamine or an amine, are mediated by enzymes termed nitroreductases. These enzymes have been found in bacteria, fungi, protozoa, plants and animals, and their biology have been reviewed by Mitchard [2], Hadley [3] and Smyth [4].

In mammals, separate nitroreductases are associated with the cytosol, the microsomal fraction and the mitochondria. Each of these nitroreductases has been identified with a previously described flavoenzyme, for which nitro compounds can serve as alternative electron acceptors. Thus cytosolic nitroreductase activity has been attributed to aldehyde oxidase (EC 1.2.3.1) [5], xanthine oxidase (EC 1.2.3.2) [6] and DT-diaphorase (EC 1.6.99.2) [7]; microsomal nitroreductase activity has been attributed to NADPH–cytochrome reductase (EC 1.6.2.4) and cytochrome *P*-450, which act in sequence in the reduction of nitro compounds [8]; and mitochondrial nitroreductase activity has been attributed to the matrix enzyme dihydrolipoamide dehydrogenase (EC 1.6.4.3) [9], the outer-membrane enzyme cytochrome b_5 reductase (EC 1.6.2.2) [10] and the inner-membrane enzyme succinate dehydrogenase (EC 1.3.99.1) [11]. (The ability of succinate dehydrogenase to function as a nitroreductase has, however, been questioned [12].)

We have investigated an NADH-dependent nitroreductase associated with the mitochondrial inner membrane. The enzyme, which catalyses the reduction of nitroaromatic compounds to the corresponding hydroxylamines, was first discovered in rat brain [13] and ox brain [14], and subsequent work in our laboratory showed it to be present in every mammalian tissue and species investigated.

Among the oxidoreductases of the mitochondrial inner membrane, only hydroxybutyrate dehydrogenase (EC 1.1.1.30) and the flavoprotein NADH dehydrogenase (ubiquinone) (EC 1.6.5.3) are known to oxidize NADH. Whereas hydroxybutyrate dehydrogenase has not been reported to catalyse the NADH-dependent reduction of alternative electron acceptors, NADH dehydrogenase is known to have a wide specificity for alternative acceptors [15]. Furthermore, NADH dehydrogenase can, under certain conditions, undergo fragmentation to yield a flavoprotein with an altered specificity for electron acceptors; this flavoprotein has been allotted a separate EC number, 1.6.99.3 [16].

In the present paper we consider the question whether the NADH-dependent nitroreductase from the mitochondrial inner membrane can be identified with a previously described enzyme. The most likely candidate for such an identification is NADH dehydrogenase.

MATERIALS AND METHODS

Materials

1,3-Dinitrobenzene and $K_3Fe(CN)_6$ were from BDH Chemicals, Poole, Dorset, U.K.; NADH (disodium salt, grade II, 98%) was from Boehringer Corporation (London), Lewes, East Sussex, U.K.; 2,6-dichloroindophenol was from Dr. Theodor Schuchardt und Co., Hohenbrunn-Waechsterhof, West Germany; cytochrome *c* (from horse heart, type II-A), sodium cholate and sodium deoxycholate were from Sigma Chemical Co., Poole, Dorset, U.K. Calcium phosphate gel was prepared as described by Dixon & Webb [17]. Tissue samples were obtained from a local abattoir not more than 15 min after the animal's death.

Determination of protein concentrations

Protein concentrations were determined by the method of Markwell *et al.* [18], except when published procedures required the adjustment of preparations to certain values of protein concentration according to the biuret assay [19]. The standard protein for both assays was bovine serum albumin.

Assay of enzyme activities

Enzyme assays were carried out with a Gilford 240 spectrophotometer coupled to a Rikadenki model B-104 chart recorder. One unit of enzyme is taken as the

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amount of enzyme that catalyses the transformation of 1 μmol of substrate/min under the conditions specified.

Nitroreductase activity was determined at 25 °C by monitoring the disappearance of NADH at 340 nm. The reaction mixture (3 ml) contained 60.7 mM-sodium pyrophosphate buffer, pH 9.2, 1 mM-1,3-dinitrobenzene and 0.14 mM-NADH. (Dinitrobenzene was added as 100 μl of a methanolic solution.) The reaction was started by the addition of the enzyme (50 μl). The specific activity of the nitroreductase in a mitochondrial preparation was 0.0125 unit/mg of protein (in terms of NADH oxidized).

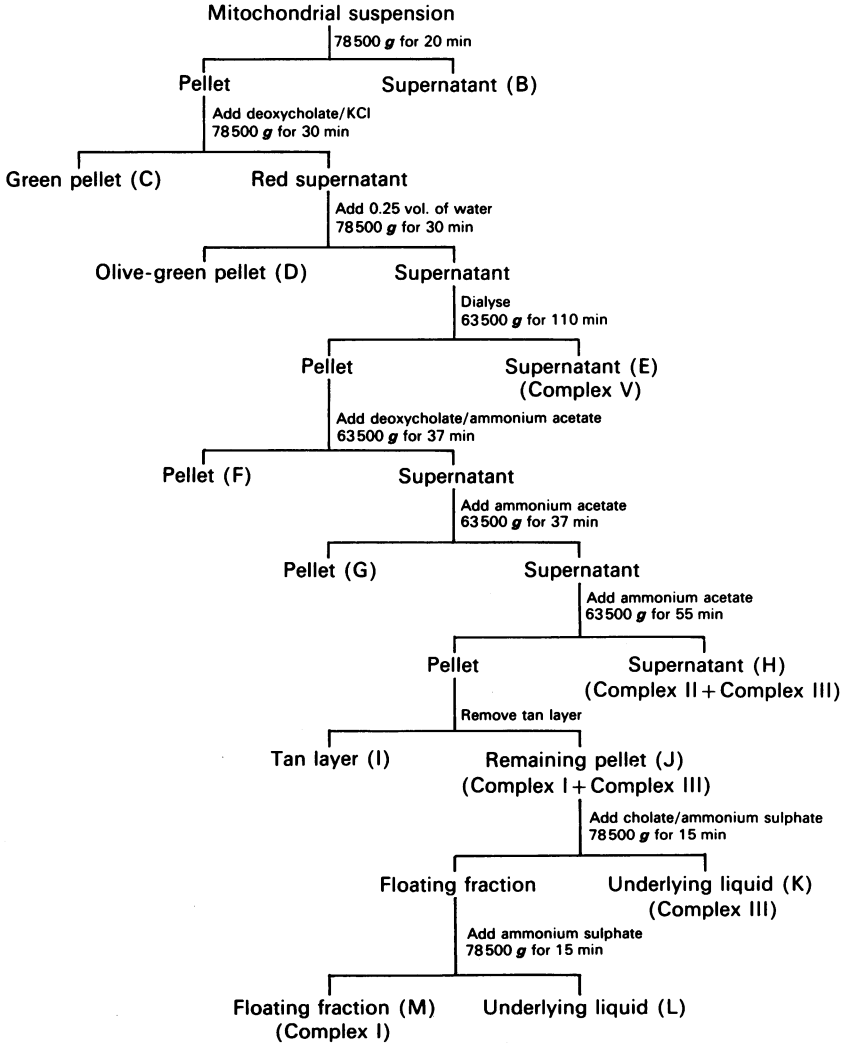
Complex I was assayed at 30 °C by measuring the NADH-dependent reduction of ferricyanide as described by Hatefi [20]. The NADH–cytochrome *c* reductase described by Mahler *et al.* [21] was assayed at 25 °C by measuring the reduction of either cytochrome *c* or 2,6-dichloroindophenol as described by Mahler [22].

Preparation of NADH dehydrogenase

Complex I was prepared from ox liver mitochondria as described by Hatefi & Stiggall [23] and Hatefi [20] (Scheme 1). This procedure was developed specifically for ox heart and had not previously been applied to ox liver. The procedure allows the isolation of the four electron-transfer complexes and the oligomycin-sensitive ATPase from a single batch of mitochondria, and is based on the use of deoxycholate, cholate and KCl for the selective solubilization of the membrane proteins, and of ammonium acetate and ammonium sulphate for the precipitation of the enzyme complexes [24].

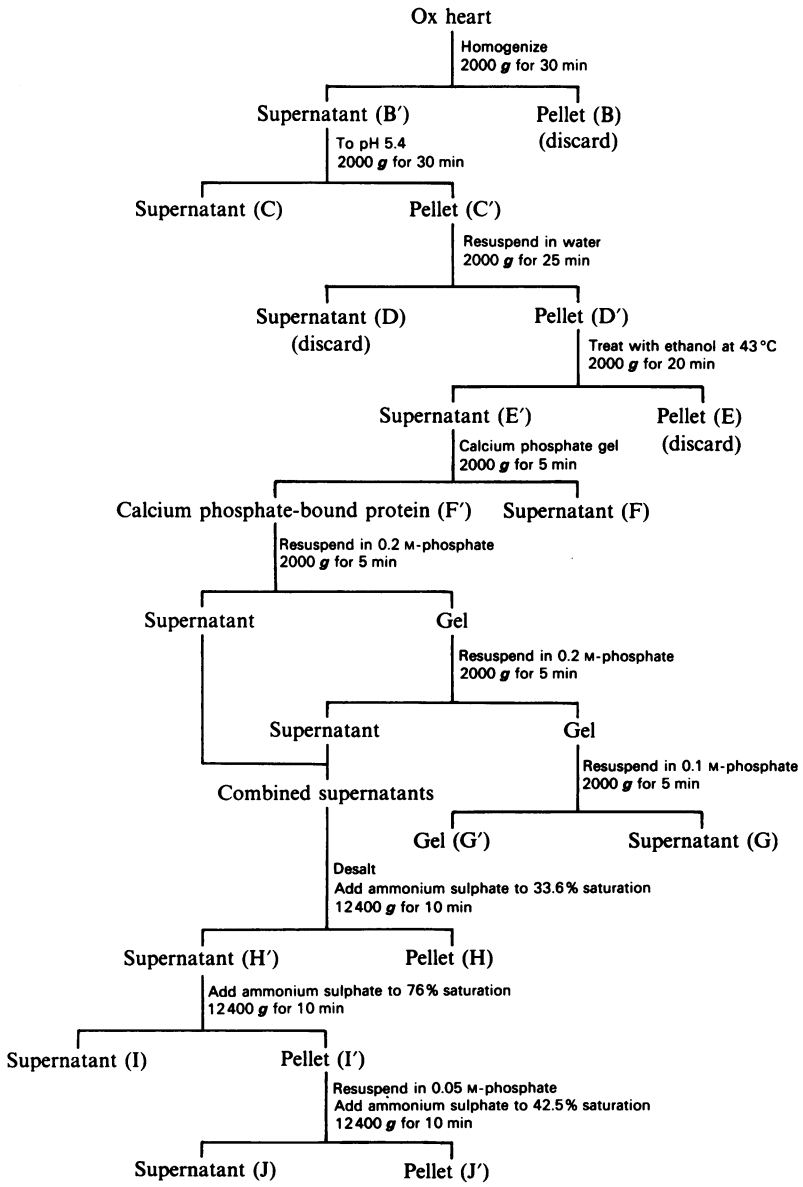
The NADH–cytochrome *c* reductase described by Mahler *et al.* [21] was prepared from ox heart by the method of Biggs *et al.* [25] (Scheme 2), which involves ethanol extraction at pH 5.4 and 44 °C, concentration on calcium phosphate gel and ammonium sulphate fractionation.

All the fractions derived from the preparation of Complex I were assayed for Complex I, and all the fractions derived from the preparation of the NADH–cytochrome *c* reductase described by Mahler *et al.* [21] were assayed for NADH–cytochrome *c* reductase. The fractions from both experiments were also assayed for



Scheme 1. Resolution of Complexes I to V from ox liver mitochondria

The scheme summarizes the procedure described by Hatefi & Stiggall [23] and Hatefi [20].



Scheme 2. Preparation of NADH–cytochrome *c* reductase described by Mahler *et al.* [21] from ox heart

The method of Biggs *et al.* [25] was used. Four more ammonium sulphate fractionation steps are required to give a completely pure preparation. The letters B to J and B' to J' in this scheme are not related to the letters B to M in Scheme 1.

nitroreductase activity and protein. The distributions of Complex I and nitroreductase from the fractionation of the respiratory chain were displayed by the method of de Duve *et al.* [26].

Gel-permeation chromatography

A Sephadex G-100 column (3 cm × 40 cm) was equilibrated with 10 mM-potassium phosphate buffer, pH 7.2, containing 0.1 M-KCl, and calibrated with myoglobin, α-chymotrypsinogen, ovalbumin and bovine serum albumin.

RESULTS

Preparation of Complex I of the respiratory redox chain

Ox liver mitochondria were fractionated by the method of Hatefi [24] as far as the precipitate of Complex I + Complex III (fraction J in Scheme 1). Complex I was enriched 40-fold in fraction J, reported to consist only of Complex I and Complex III, but not more than 5-fold in any other fraction (Fig. 1*a*). The distribution of nitroreductase (Fig. 1*b*) was virtually identical with that of Complex I: the nitroreductase was enriched 48-fold in fraction J, but not more than 8-fold in any other fraction; and the two activities paralleled one another in all fractions except E, where substantial ferricyanide reductase activity was found in the absence of nitroreductase activity.

Preparation of NADH–cytochrome *c* reductase

The NADH–cytochrome *c* reductase described by Mahler *et al.* [21], an iron–sulphur flavoprotein resulting from the fragmentation of Complex I, was partially purified from ox heart mitochondria (see Scheme 2).

Nitroreductase activity was extracted along with the NADH–cytochrome *c* reductase, which was assayed with either cytochrome *c* or 2,6-dichloroindophenol as the acceptor (see Table 1). Furthermore, the specific activity

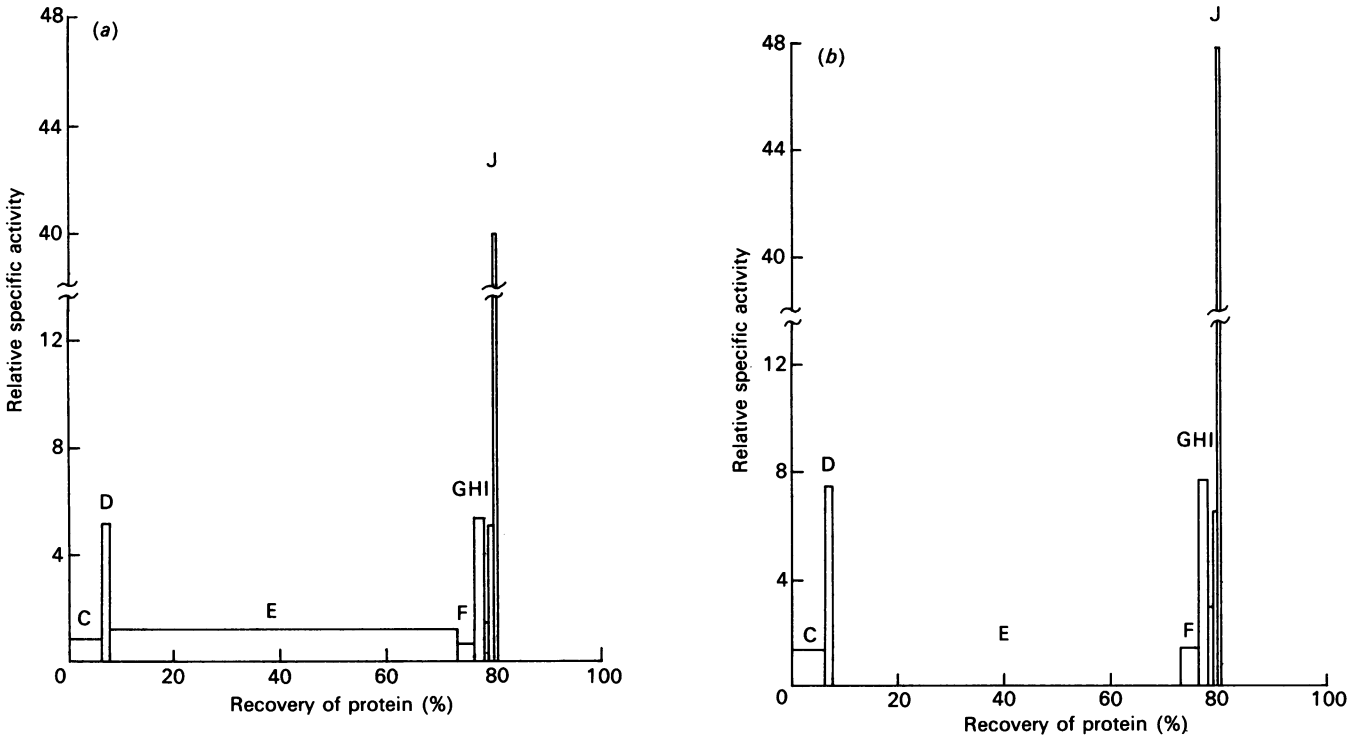


Fig. 1. Distributions of (*a*) Complex I and (*b*) nitroreductase from ox liver mitochondria

Ox liver mitochondria were fractionated as described by Hatefi & Stiggall [23] as far as fraction J (the Complex I + Complex III pellet); the letters C to J correspond to the fractions so denoted in Scheme 1. The specific activity shown is relative to whole mitochondria. Complex I (*a*) was assayed by measuring the NADH-dependent reduction of ferricyanide. Its specific activity in fraction J was 30.4 units/mg of protein (in terms of ferricyanide reduced) and the recovery of activity from the mitochondria was 117%. Nitroreductase (*b*) was assayed by measuring the NADH-dependent reduction of 1,3-dinitrobenzene. Its specific activity in fraction J was 0.54 unit/mg of protein and the recovery of activity from the mitochondria was 58%. Enzyme activity and protein were determined in triplicate and the mean standard deviation in the specific activity is 8%.

Table 1. Relative specific activities of nitroreductase and NADH–cytochrome *c* reductase throughout the purification scheme of Biggs *et al.* [25]

The specific activities are relative to the mitochondrial preparation. The letters refer to the preparations so denoted in Scheme 2. The specific activity of the reductase in fraction J′ was 27.2 units/mg of protein (in terms of cytochrome *c* reduced); for comparison, the specific activity reported by Mahler *et al.* [21] for the same stage of the purification was 18.4 units/mg of protein.

		Relative specific activity						
Acceptor	Fraction...	F	G	I	J	E′	G′	J′
1,3-Dinitrobenzene		1.5	26.5	0.0	6.0	28.0	62.0	119
2,6-Dichloroindophenol		0.0	31.0	0.0	15.0	22.0	52.0	119
Cytochrome *c*		1.25	45.0	0.0	9.5	44.0	62.0	206

of the nitroreductase increased throughout the purification in parallel with the specific activity of the NADH–cytochrome *c* reductase (fractions E′–J′). The activity towards each acceptor also had comparable distributions among the discarded fractions (Table 1): little or no activity was present in fraction F or I, moderate activity in fraction J, and high activity in fraction G. The most highly purified preparation of NADH–cytochrome *c* reductase (J′) was chromatographed on a column of Sephadex G-100 and the eluted fractions were assayed for protein, nitroreductase and NADH-dependent activity towards cytochrome *c* and 2,6-dichloroindophenol. The three activities were co-eluted and their profiles, normalized with respect to area, were indistinguishable (results not shown). The M_r of the reductase was 69200 (a range of 75000 to 80000 was reported by Mahler *et al.* [21] from the $s_{20,w}$ value).

DISCUSSION

Hatefi & Rieske [27] found that Complex I could be purified 12.5-fold from ox heart mitochondria. Fig. 1(*a*) shows that Complex I from ox liver mitochondria, however, can be purified at least 40-fold; this large purification factor is consistent with the comparatively low abundance of Complex I in ox liver [28].

Submitochondrial-distribution studies revealed an NADH-dependent ferricyanide reductase associated with outer-membrane preparations [4] {the enzyme probably corresponds to the NADH–cytochrome b_5 reductase of the outer membrane of rat liver mitochondria (EC 1.6.2.2) [29]}. Therefore the distribution of activity towards ferricyanide (Fig. 1*a*) must reflect the behaviour of at least two enzymes. The only conspicuous difference between the profile for ferricyanide reductase activity and that for nitroreductase activity, however, is the absence of nitroreductase from fraction E, where there is considerable activity towards ferricyanide. The activity in this fraction may be due to the outer-membrane ferricyanide reductase.

Baugh & King [30] prepared the respiratory-chain NADH dehydrogenase by extraction with 0.25 mg of Triton X-100 per mg of protein at pH 8.5. This method is similar to one developed in our laboratory [4] to solubilize the nitroreductase (0.1 mg of Triton X-100 per mg of protein at pH 10.0), so it was suspected that NADH dehydrogenase would be present in our nitroreductase extract. These suspicions were strengthened by the fact that the extract displayed NADH-dependent activity towards ferricyanide and 2,6-dichloroindophenol, compounds known to be reduced by NADH dehydrogenase.

A single nitroreductase is listed in Enzyme Nomenclature (1984) [16]. This is NAD(P)H:4-nitroquinoline *N*-oxide oxidoreductase (EC 1.6.6.10). However, its activity could be attributable to DT-diaphorase [7], in which case it would be represented by the EC number for that enzyme, 1.6.99.2. There may therefore be a case for deleting the EC number of nitroquinoline *N*-oxide reductase.

Nitro compounds are a novel class of acceptor for Complex I, all acceptors previously reported being ferric complexes or quinoids. However, like ubiquinone-1, the physiological acceptor for Complex I, 1,3-dinitrobenzene is reduced at only about 2% of the rate of ferricyanide reduction; ferricyanide therefore remains by far the most rapidly reduced acceptor. We also found that activity towards tetrazolium salts accompanied both Complex I and the NADH–cytochrome *c* reductase described by Mahler *et al.* [21]. Such activity has not been reported to be a property of the mitochondrial NADH dehydrogenase, though it is known to be associated with certain bacterial NADH dehydrogenases [31,32].

Although Complex I accounts for the NADH-dependent nitroreductase activity of the mitochondrial inner membrane, other flavoenzymes of the inner membrane may be capable of functioning as nitroreductases when provided with a suitable electron donor. Such flavoenzymes include choline dehydrogenase (EC 1.1.99.1), glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), succinate dehydrogenase (EC 1.3.99.1), proline dehydrogenase (EC 1.5.99.8) and electron-transfer-flavoprotein dehydrogenase (EC unclassified). Nitroreductase activity is not yet known to be a property of any of these enzymes.

We acknowledge maintenance grants from the Department of Education and Trinity College, Dublin, to G.E.S. We thank Truda McCullagh for typing the manuscript.

Received 9 May 1988/15 August 1988; accepted 25 August 1988

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