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The organo-specific external NADH dehydrogenase of mammal heart mitochondria has an artefactual origin

Laurent Fraisse [a], Emmanuel Rey [b] and Michel Rigoulet [b]

[a] *Groupement de Recherche de Lacq, Artix (France) and* [b] *Institut de Biochimie Cellulaire CNRS, Bordeaux (France)*

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The existence of an organo-specific (heart) external NADH dehydrogenase located on the outer face of the inner mitochondrial membrane has been recently proposed. We have studied the respiration on external NADH in rat and beef heart mitochondrial fractions: (i) by using different mitochondrial isolation procedures on the rat, we observed that the higher the criteria of quality toward classical substrate respiration of mitochondrial fractions, the lower the external NADH-linked respiration; (ii) by using an especially loosely fitting glass-Teflon homogenizer, we obtained rat heart mitochondrial fractions practically free from external NADH linked respiration and with the highest respiratory control ratio on glutamate plus malate respiration. In rat and beef heart mitochondrial fractions containing an external NADH respiration: (i) ethoxyformic anhydride used previously to distinguish internal and external NADH oxidation was shown not to be specific; (ii) external NADH-linked respiration (although associated to the normally functioning respiratory chain as was shown by the effects of classic respiratory inhibitors) did not lead to ADP phosphorylation while glutamate plus malate did; (iii) respiratory activity on glutamate plus malate and external NADH was totally additive and the oxidation corresponded to two separate cytochrome oxidase pools, indicating a total functional separation between the two respiratory systems; (iv) NAD^+ addition stimulated states 3 and 4 glutamate plus malate respiration to the same extent, indicating the presence of anappreciable number of internal dehydrogenases accessible to external cofactors. These results show that external NADH-linked dehydrogenase activity, which is usually detectable in mammal heart mitochondrial fractions, is of artefactual origin.

Introduction

Long ago, it was possible to affirm that the NADH consuming enzyme of the respiratory chain in isolated liver mitochondria was inaccessible to cytoplasmic NADH [1]. This inaccessibility has been extended and it is now accepted that all animal mitochondria contain an internal NADH pool which is spatially and functionally separated from cytoplasmic NADH, and that the inner membrane is impermeable to NADH and NAD^+ [2–4]. This is confirmed by the observation that very different $NADH/NAD^+$ ratios are maintained in vivo in matricial and cytoplasmic compartments of the cell [5].

In contrast to plants [6–8] or yeast [9,10], which are known to contain particular enzymes for cytoplasmic NADH oxidation, the large majority of animal mitochondria are not able to oxidize cytoplasmic NADH and the absence of external NADH-linked respiration today remains a common and simple integrity criterion for isolated animal mitochondria.

However, in contrast to other organs, the ability of muscle mitochondria (heart and skeletal) to oxidize external NADH has often been reported in the literature: for example, in the monkey and rat heart [11] and in human skeletal muscle [12]. However, isolation experiments were conducted on rat and pigeon heart mitochondria which exhibited very low added NADH-linked respiration [13]. In spite of the impossibility to isolate muscle (skeletal or heart) mitochondrial fractions free from external NADH-linked respiration, this latter activity has generally been considered as an artefact of the isolation procedure.

However, the existence of an authentic cytoplasmic NADH dehydrogenase activity located on the outer face of the inner mitochondrial membrane in pigeon [14,15] and rat heart [16] has been demonstrated.

Rasmussen [14] associated external NADH-linked respiration to an authentic activity of intact pigeon heart mitochondria. This activity was equal to two-

Correspondence to: M. Rigoulet, Institut de Biochimie Cellulaire CNRS, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France.

Abbreviations: EFA, ethoxyformic anhydride; IC_{50}, inhibitor concentration which gives 50% inhibition of the respiratory rate for 1 mg mitochondrial protein; RCR, respiratory control ratio.

thirds of maximal site I-linked substrate respiration, which was reproducible and sensitive to classical respiratory chain inhibitors. This external NADH-linked respiration was not coupled to ADP phosphorylation. Rasmussen proposed the existence of an alternative respiratory chain associated with cytoplasmic NADH respiration. Moreover, Rasmussen and Rasmussen [15] confirmed the presence in pigeon heart mitochondria of an external NADH oxidase located on the outer face of the inner mitochondrial membrane, separated from the classic respiratory chain up to the cytochrome *b* level. They found a single cytochrome oxidase pool for external NADH and internal substrate respiration.

Nohl [16] described an organo-specific external NADH dehydrogenase in rat heart mitochondria. In contrast to Rasmussen and Rasmussen [15], this activity was linked to the classic respiratory chain at the site I level and controlled by ADP, with an ADP/O of 2.8, seeming to indicate that external NADH linked respiration was associated to the three classic phosporylation sites. The demonstration of the existence of this enzyme was based essentially on the selectivity of the inhibitor ethoxyformic anhydride (EFA).

It is, however, well known that the preparation of muscle mitochondria is difficult, essentially because tissue homogenization involves the use of proteinase and/or a strong mechanical tissue disruption. Such processes could generate some artefacts of preparation [17]: a modification of inner mitochondrial membrane integrity could lead to oxidation of external NADH. On the other hand, some isolation procedures may be responsible for the disappearance of an authentic NADH dehydrogenase activity. Moreover, we cannot exclude the eventuality of a natural heterogeneity of the heart mitochondrial population, and therefore a different composition of the isolated mitochondrial fractions depending on the procedure used.

In this work we first show in rat heart mitochondria that by using different isolation procedures, we obtained mitochondrial fractions with extremely variable external NADH-linked dehydrogenase activity. Mitochondrial fractions of the best quality (regarding classic substrate respiration) require a quasi-absence of external NADH linked respiration. We show that this latter activity is confined to a subpopulation of damaged mitochondria. The arguments proposed in favour of the existence of an authentic external NADH dehydrogenase are critically discussed.

Materials and Methods

Mitochondrial preparations

Rat heart mitochondria. Rat heart mitochondria were isolated from male wistar rats (200–300 g) according to three procedures in order to test their influence on external NADH-linked respiration. These procedures were different in (i) the use, or not, of proteinase during tissue homogenization, (ii) the use of two different glass homogenizers equipped with Teflon pestle: a commercial homogenizer (clearance between the pestle and the glass of 0.15–0.17 mm) and a broad homogenizer (clearance of 1.5–1.6 mm) made in our laboratory. A single isolation medium was used: 0.3 M sucrose, 20 mM H_3PO_4, 2 mM EDTA and equilibrated to pH 7.4 by Tris addition. All operations were conducted at 4°C. After extraction, two rat hearts were incised and washed four times to remove maximal blood. Hearts were then minced and washed again to obtain a clear and colorless supernatant.

Procedure 1: the mince was disrupted using the commercial homogenizer in 30 ml isolation medium without any addition of proteinase. Two energetic 'up and down' strokes were sufficient to obtain disrupted tissue suspension. The homogenate was centrifuged at $800 \times g$ for 10 min. The supernatant was collected and centrifuged at $8000 \times g$ for 10 min. The mitochondrial pellet was gently resuspended and washed by further centrifugations under the conditions mentioned above. The last pellet was resuspended in a minimal volume and was the stock mitochondrial suspension (40–80 mg protein/ml).

Procedures 2 and 3 were taken from those previously published [18] with slight modifications:

Procedure 2: the mince was incubated with 1.5 mg bacterial proteinase, 10 min at 4°C in 30 ml isolation buffer and homogenized with the same homogenizer used for protocol 1. The suspension was centrifuged 10 min at $8000 \times g$ to remove proteinase localized in the supernatant. The whole pellet was resuspended and centrifuged 10 min at $800 \times g$. The supernatant was further centrifuged 10 min at $3000 \times g$. This pellet was further treated as in procedure 1.

Procedure 3: the broad homogenizer was used here. The mince was immediately homogenized in the presence of bacterial proteinase (1.5 mg in 30 ml medium). After 4 min incubation the very gentle homogenization was repeated until a smooth homogenate was obtained. At this point, procedure 2 was followed. The mitochondrial integrity was verified by measuring the adenylate kinase activity of the mitochondrial fraction.

The protein concentration was estimated by the biuret method using bovine serum albumin as standard.

Bovine heart mitochondria. Mitochondria were isolated from slaughterhouse material according to slight modifications of a mechanical method [19]. The removed hearts were placed in a cold medium containing 0.25 M sucrose, 20 mM KCl, 2 mM EDTA, 10 mM Tris and equilibrated to pH 7.4 with HCl, during transportation (15 min) from the slaughterhouse to the laboratory. Other experimental differences were the following: the homogenate was obtained in a buffer

containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris equilibrated to pH 7.4 with HCl. Mitochondria were washed twice and suspended finally at a concentration of 35–45 mg protein/ml in the same medium described for homogenate obtention. The protein concentration was estimated with a Bio-Rad Kit assay (Bio-Rad, Munich, Germany) using bovine serum albumin as standard.

Assays

Respiratory assays. The rate of oxygen consumption was measured polarographically at 30 °C using a Clark type electrode (Hansatech, King's Lynn, UK) connected to a computer giving an on-line display of rate values. Mitochondria (bovine or rat heart) were suspended in the following medium: 60 mM sucrose, 60 mM KCl, 10 mM H_3PO_4, and equilibrated to pH 7.4 by Tris addition. When added, substrates were 3 mM glutamate, 3 mM malate, 3 mM succinate,1 mM NAD^+ and 2 mM NADH.

For the measurement of respiratory rate additivity, rat and beef heart mitochondria were preincubated for 3 min in a respiratory buffer until oxygen consumption was close to zero, to avoid the influence of the endogenous substrate. State 3 respiration was determined in the presence of 1 mM ADP.

ATP/O assays. ATP/O ratio stoichiometries were determined from the average of ADP phosphorylation vs. respiratory rate at 30 °C under the following conditions: mitochondria (0.8 mg/ml) were suspended in the respiratory buffer (see above) containing 10 mM glucose, 1 mM $MgCl_2$, 1 mM ADP, hexokinase in a non-limiting concentration for maximal state 3 respiratory rate and when added,1 mM NADH or 3 mM glutamate and 3 mM malate. Phosphorylation rate determination: glucose 6-phosphate was measured in aliquots of the incubation medium extracted according to [20] by NADPH formation at 340 nm in the presence of glucose-6-phosphate dehydrogenase. Oligomycin (0.025 mg/ml) was used to estimate the involvement of adenylate kinase activity.

Cytochrome aa_3 measurements. Time-course reductions of cytochrome aa_3 were recorded using a DW 2000 SLM/Aminco spectrometer in the dual wavelength mode (605–752 nm). Recording signals were treated with a SLM/Aminco software installed on an IBM PS2 computer. At 605 nm cytochrome *a* contributes for 80% and cytochrome a_3 for 20% of the total absorbance [21]. The concentration was estimated to be 0.65 nmol/mg protein for each cytochrome *a* form in beef heart mitochondria [21].

Bovine heart mitochondria were incubated from 2 to 40 min in 60 mM sucrose, 60 mM KCl, 15 mM Tris equilibrated to pH 7.4 with HCl, at 20 °C under air and very gentle magnetic stirring. Final mitochondrial protein concentration was 0.3 mg/ml. For spectrometric measurements the sample cuvette was thermostated at 28 °C and contained a 3 ml aliquot of the incubation medium described above and, when added, 5 mM glutamate, 5 mM malate, 1 mM potassium cyanide, 2 mM NADH and a few crystals of sodium dithionite. In the presence of respiratory substrates, cyanide induces a total reduction of cytochrome aa_3 (with a slight modification of the cytochrome a_3 spectrum in the reduced form) in aerobiosis [21]. When necessary, parallel oxygraphy measurements under the same conditions had been conducted to determine the time at which anaerobiosis was reached.

Chemicals

NADH and NAD^+ were from Boehringer Mannheim, Sucrose from Merck. All other chemicals were of the highest purity obtained from Sigma. Bacterial proteinase (Nagarse type XXVII), Hexokinase (EC

TABLE I

Characteristics of added NADH and glutamate plus malate respirations in different heart mitochondrial fractions, taken from the literature and (a) prepared in our laboratory

Ref.	Animal	Added NADH linked respiration		Ratio	Use of proteinase	Procedure (Expt. No.)
		V added NADH [b]	RCR	*V* added NADH/*V* glutamate plus malate state 3		
13	rat	33	1	0.18	+	
	pigeon	40	1	0.24	+	
14	pigeon	200	1	0.67	+	
16	rat	200 [c]	4.25	–	+	
(a)	rat	27±17	1	0.15±0.09	+	3 (7)
(a)	rat	82±35	1	0.52±0.14	+	2 (4)
(a)	rat	133±37	1	1.42±0.33	–	1 (4)
(a)	rat	52	1	0.33	–	1 * (1)
(a)	beef	463±40	1	1.34±0.13	–	(5)

[b] Respiratory rates are expressed in natoms O consumed/min per mg mitochondrial protein. Values are expressed as means±S.E.M.

[c] Determined in the presence of ADP. Isolation procedures are described in Materials and Methods.

* This mitochondrial fraction was isolated according to procedure 1, except that the broad homogenizer was used.

2.7.1.1) and Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were from Boehringer Mannheim.

Results and Discussion

External NADH-induced respiration depends on isolation procedure

In order to study the respiration induced by external NADH in the heart mitochondrial fraction, we first determined the dependence of this activity on isolation procedures (for more details see Materials and Methods). This dependence was studied on rat heart mitochondria for which repetitive isolation experiments are easier to perform. Table I presents some characteristics of heart mitochondrial fractions obtained as described above and compared with results from the literature. For rat heart mitochondria the respiratory rate with external NADH as substrate seemed to be drastically dependent on the isolation procedure used. When the broad homogenizer was used (procedure 1* or 3), mitochondrial characteristics were similar to those published by Tyler and Gonze [13] with a very low ratio between external NADH respiration and glutamate plus malate respiration. When the tightly-fitting homogenizer was employed (procedures 1 and 2), respiratory characteristics were more in keeping with the results presented by Rasmussen and Rasmussen [15] and Nohl [16]. The presence of external NADH dehydrogenase activity was not due to the use of proteinase during tissue homogenization, since procedure 1* gave mitochondrial fractions having a low external NADH respiration capacity. The specifications of the homogenizer (clearance between pestle and vessel) which are rarely given in the literature are decisive for the presence or not of this activity. Commercial homogenizers have a clearance which does not exceed 0.25 mm. Smith [22] recommended increasing this clearance up to 0.4–0.5 mm to isolate heart mitochondria. Our results show that it is possible to increase this clearance to 1.5 mm. The mitochondrial pellet obtained by procedure 3 contained mitochondria which were well coupled and in which external NADH-linked respiration was negligible.

The respiratory control ratio (RCR) with glutamate plus malate as respiratory substrates can be considered as a good criterion of heart mitochondrial integrity. Fig. 1A shows that regardless of the isolation procedure (1, 2 or 3), when the RCR on glutamate plus malate increased, external NADH-linked respiration decreased drastically and the ADP stimulated respiratory flux with glutamate plus malate as substrates increased. The three procedures gave stastistically three different qualities of mitochondrial fractions (Fig. 1B). We observed a strong decrease in the ratio between these two activities (V NADH/V glutamate), which dropped by a factor 10 (from procedure 1 to procedure 3) while the RCR on glutamate plus malate largely increased.

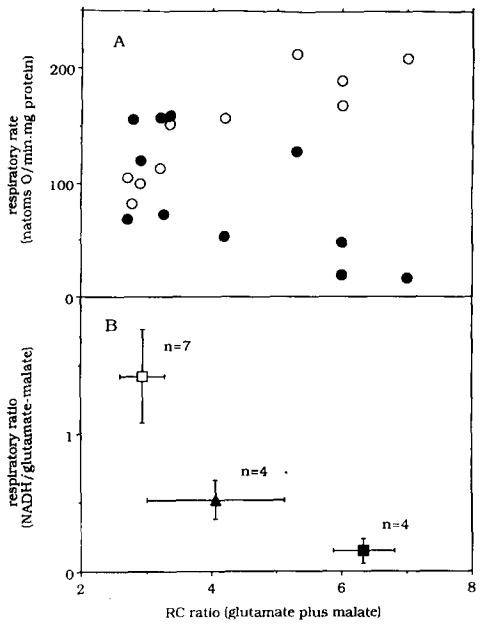

Fig. 1. (A) Relationships between either external NADH or glutamate plus malate respiration and the respiratory control ratio (RC ratio) with glutamate plus malate as substrates of rat heart mitochondrial fractions isolated according to protocol 1, 2 or 3. External NADH-linked respiration: filled symbols; glutamate/malate state 3 respiration: open symbols. (B) Relationship between the ratio of the two respirations (V external NADH/V glutamate-malate state 3) and the respiratory control ratio with glutamate plus malate as substrates of rat heart mitochondrial fractions isolated according to protocol 1, 2 or 3. Each point represents mean ± S.E. according to: □ procedure 1, ▲ procedure 2, ■ procedure 3. Isolation procedures and respiratory assays were conducted as described in Materials and Methods.

These experiments may explain the heterogeneity of the results published previously in this field. Indeed with different procedures, we were able to isolate rat heart mitochondrial fractions with very different properties. The mitochondrial fractions of the best quality were obtained according to procedure 3, and this was correlated with a very low respiration on external NADH. These results seem to indicate that intact mitochondria do not contain external NADH dehydrogenase.

External NADH is oxidized by the classical site I NADH ubiquinone oxidoreductase, located on damaged mitochondria

Effect of respiratory inhibitors. Ethoxyformic anhydride (EFA) was previously described to inhibit selectively intramitochondrial NADH respiration without any effect on either external NADH-linked respiration or external NADH-linked cytochrome *b* reduction in intact rat heart mitochondria and under normal respiration conditions [16]. EFA is known to inhibit NADH-ubiquinone oxidoreductase by an essential his-

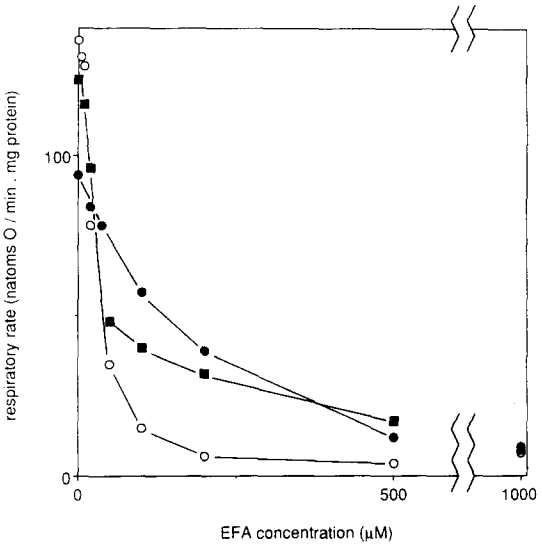

Fig. 2. Dose dependent inhibition by EFA of glutamate plus malate, succinate and added NADH respirations of rat heart mitochondrial fractions isolated according to procedure 1, ○: glutamate plus malate respiration, ●: succinate respiration, ■ Added NADH respiration. Experiments were conducted as described in Materials and Methods, in the presence of 1 mg/ml protein. Respiration was performed in the presence of 500 μM ADP.

tidine modification [23]. This powerful reagent for protein modification is considered to be relatively specific for histidine only under appropriate conditions [24]. Inhibition experiments presented by Vik and Hatefi [24] were performed at 0 °C and with isolated NADH-ubiquinone oxidoreductase. EFA was also described to be able to inhibit complex III [25,26]. Moreover, this compound is very labile at room temperature and at neutral pH. Under our experimental conditions and on rat heart mitochondrial fractions isolated according to procedure 1, EFA inhibited external NADH-linked respiration in a dose-dependent manner (Fig. 2). Such an inhibition was also observed for succinate and glutamate plus malate respirations, which were more sensitive to EFA inhibition than external NADH-linked respiration. Thus, this compound used in the millimolar range cannot be considered as a specific inhibitor of internal NADH respiration in intact rat heart mitochondria.

However, external NADH-linked respiration of the same rat mitochondrial fraction or beef heart mitochondrial fraction was fully inhibited by classical respiratory chain inhibitors like rotenone, antimycin A or potassium cyanide (not shown). The sensitivity to rotenone excludes the eventual involvement of the rotenone insensitive pathway for external NADH oxidation described previously [27].

Phosphorylation linked to external NADH-linked respiration. Since we found this activity sensitive to most of the respiratory chain inhibitors which are associated to proton translocation sites (i.e rotenone, antimycin and cyanide), the external NADH respiration should be associated to ATP synthesis. Table II shows that

TABLE II

ATP/O ratios of glutamate plus malate and added NADH-linked respirations in rat heart mitochondrial fractions

Mitochondrial fractions were isolated according to procedure 1. All the experiments were done in triplicate. Values are expressed as means ± SEM. ATP determination and respiratory assays were conducted as described in Materials and Methods.

Respiratory substrate	Oligomycin-sensitive ATP synthesis (nmol/min per mg protein)	Respiratory rate (natom O/min per mg protein)	*ATP/O* ratio
Glutamate-malate	294 ± 12 (3)	130 ± 7 (3)	2.3 ± 0.15 (3)
Added NADH	6 ± 3 (3)	202 ± 15 (3)	0.03 ± 0.02 (3)

TABLE III

Additivity of respiratory fluxes of rat and beef heart mitochondrial fractions

Respiratory substrate	Respiratory rate state 3 [a] (natom O/min per mg protein)			Respiratory rate state 4 [a] (natom O/min per mg protein)		
	beef heart	rat heart procedure 1	rat heart procedure 2	beef heart	rat heart procedure 1	rat heart procedure 2
NADH (*A*)	382	123	62	381	121	62
Glutamate-malate (*B*)	322	102	251	58	42	40
Succinate (*C*)	181	–	–	108	120	161
NADH, glutamate-malate (*A* + *B*)	695	221	310	440	161	100
NADH, succinate (*A* + *C*)	493	–	–	450	180	204
% recovery						
$(A+B)/(A)+(B)\cdot 100$	98.1	98.2	99.0	99.7	99.4	98.0
$(A+C)/(A)+(C)\cdot 100$	87.0	–	–	92.0	74.7	83.9

[a] The additivity results presented here were valid for three or more experiments obtained for each mitochondrial isolation procedure. Respiratory assays and isolation procedures were conducted as described in Materials and Methods.

ATP synthesis induced by external NADH-linked oxidation was close to zero, unlike glutamate plus malate respiration in rat heart mitochondrial fractions isolated according to protocol 1. More generally in our laboratory we have never found any significant ADP stimulation of external NADH-linked respiration in beef or rat heart mitochondrial fractions, whatever the isolation procedure used. An artifactual origin of external NADH oxidation in heart mitochondria may explain contradictory results in the literature and our laboratory concerning the phosphorylating properties of this activity (Table I). Tyler and Gonze [13] described a weak external NADH-linked respiration (pigeon and rat hearts) not stimulated by ADP. Rasmussen [14] did not find any ATP synthesis associated to external NADH linked respiration in pigeon heart mitochondrial fractions. Nohl [16] reported an external NADH-linked respiration strongly stimulated by ADP in rat heart mitochondrial fractions. The characteristics of contaminating particles may depend largely on the isolation procedure. Some submitochondrial particles, for instance, are able to phosphorylate ADP using external NADH with higher P/O values than intact mitochondria [28]. Conversely, altered mitochondria may be permeable to protons and NADH, and not efficient with regard to phosphorylation. Therefore the phosphorylating capacity of external NADH respiration can not be considered as an argument eitherfor or against the existence of an external NADH dehydrogenase activity.

Additivity of respiratory fluxes. According to the hypothesis of two separate respiratory chain pools supported by different particles, we should observe a total additivity of external NADH-linked respiration toward classic site I substrates. Table III clearly shows that with rat heart and different isolation procedures, or beef heart mitochondria, and in state 3 or 4 respiration, external NADH-linked respiration is additive (with more than 98% recovery) with glutamate plus malate respiration. The situation was quite different when succinate was used instead of glutamate plus malate: the sum of the two separate activities was significantly higher than the respiration in the presence of the two substrates simultaneously. Succinate interferes with external NADH linked respiration, probably by competing for oxidation in the same respiratory chain unit. However, the absence of interference between glutamate plus malate and external NADH-linked respiration clearly indicates that these two substrates are oxidized by two separate respiratory systems. Obviously the respiratory chain pool associated to external NADH-linked respiration is also able to oxidize succinate. But these results do not indicate whether these two separate systems are located together in intact heart mitochondria. External NADH and succinate oxidation do not need matricial cofactor and could occur in submitochondrial particles or in mitochondria which have been made permeable to NADH during the isolation procedure. Rasmussen and Rasmussen [15] proposed two alternate pathways for external NADH and glutamate plus malate respiration, related by a single cytochrome oxidase pool in intact heart mitochondria. From our results this would signify that this single cytochrome oxidase pool is kinetically sufficient to support the respiratory flux doubling in the presence of glutamate, malate, NADH and ADP compared to glutamate plus malate (state 3) alone. Moreover, it is relevant to note that cytochrome oxidase was shown to be a site of proton translocation [29], which is not compatible with the absence of energy coupling with external NADH as respiratory substrate in intact mitochondria.

Cytochrome aa_3 reduction. If we suppose that external NADH-linked respiration is confined to a subpopulation in the mitochondrial fraction, at least two distinct cytochrome oxidase pools should be differenciated. The choice of cytochrome aa_3 reduction study was directed by the following data: (i) cytochrome

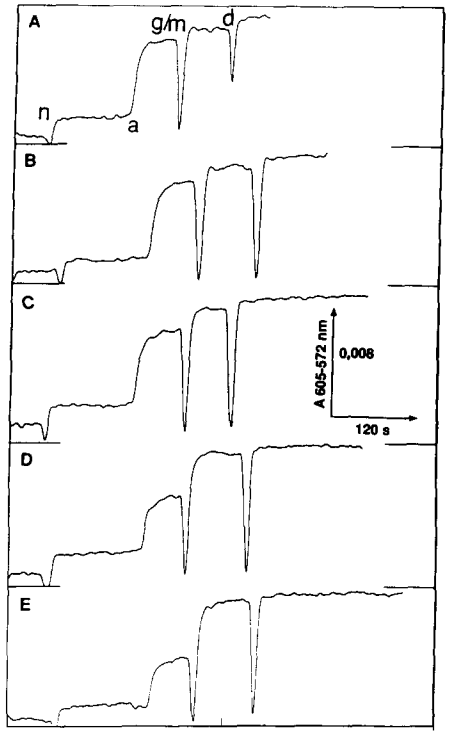

Fig. 3. Time-course of cytochrome aa_3 reduction with external NADH in bovine heart mitochondrial fractions preincubated in aerobiosis. Bovine heart mitochondria (0.3 mg protein/ml) were preincubated during: (A) 3 min; (B) 8 min; (C) 16 min; (D) 27 min; (E) 40 min. For compound additions, the following abbreviations were used: n: 2 mM NADH; g/m: 5/5 mM glutamate/malate; d: sodium dithionite (a few crystals). The time in which anaerobiosis was reached was determined in parallel measurements and corresponded to the first step of cytochrome reduction ('a' in the figure). Incubation procedure and spectrometric measurements were conducted as described in Materials and Methods.

oxidase is the last electron carrier before oxygen reduction; the detection of two distinct cytochrome aa_3 pools would then indicate a total separation between two respiratory chain pools linked either with external NADH or classical site I substrates; (ii) Rasmussen and Rasmussen [15] detected only one cytochrome aa_3 pool totally reducible by external NADH on pigeon heart mitochondria.

Beef heart mitochondrial fractions present a large respiration with external NADH as substrate (see Table I) and also respiratory additivity results similar to those obtained with rat heart mitochondria (see Table II). Fig. 3 shows that external NADH-induced cytochrome aa_3 reduction at anaerobiosis depends on the incubation time: a rapid and large reduction occurred when mitochondria were briefly incubated (respectively 78% and 72% of dithionite reducible cytochrome aa_3 at 3 min and 8 min., Fig. 3A, B and C). After 27 min incubation (Fig. 3D and E) only a third of the dithionite reducible cytochrome aa_3 was rapidly reduced when anaerobiosis occurred. In all cases glutamate plus malate addition after external NADH-induced anaerobiosis led to a 95% of the total reduction of the total pool. Parallel incubations were conducted under the same conditions: (i) the RCR on glutamate plus malate (8.0, 8.4, 8.5 and 8.0 at 3, 8, 27 and 40 min., respectively) and (ii) the external NADH-linked respiratory rate (420, 417, 428, 405 and 407 natom O/min per mg protein at 3, 8, 17, 27 and 40 min., respectively) remained constant during incubation. These results indicate the stability of the mitochondria during experiment.

Fig. 4 shows that under the same incubation conditions, cyanide-induced cytochrome aa_3 reduction at aerobiosis was also dependent on the incubation time; at short incubation time (Fig. 4A,B and C) a rapid and large reduction (about 80%) occurred due to reducing equivalents generated by endogenous metabolism. A very low steady state in reducing equivalent availability (around 1–5 natom O/min per mg protein) was sufficient to rapidly reduce the cytochrome aa_3 pool, estimated according to [21] at 1.2 nmol per mg protein. After 27 min incubation (Fig. 4D and E) cytochrome aa_3 reduction due to endogenous metabolism was largely decreased. This evolution gives an explanation to the results of Fig. 3 when anaerobiosis is attained. The cytochrome aa_3 pool which is reducible by external NADH appears only after enough incubation time to avoid the involvement of endogenous substrates.

The fact that in heart mitochondrial fraction at least two-thirds of the total cytochrome aa_3 pool are not accessible to external NADH is the most important result. This clearly indicates that: (i) two dictinct and functionally separate cytochrome oxidase pools cohabit in the same mitochondrial fraction; (ii) external NADH interacts only with one of them; (iii) internal NADH

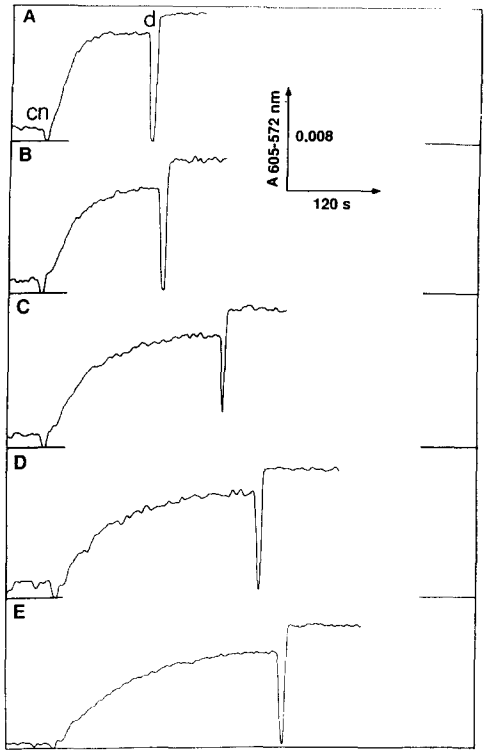

Fig. 4. Time-course of cytochrome aa_3 reduction with potassium cyanide in bovine heart mitochondrial fractions preincubated in aerobiosis. Bovine heart mitochondria (0.3 mg protein/ml) were preincubated for: (A) 3 min; (B) 8 min; (C) 16 min; (D) 27 min; (E) 40 min. For compound additions the following abbreviations are used: cn: 1 mM potassium cyanide, d: sodium dithionite (a few crystals). Incubation procedure and spectrometric measurements were conducted as described in Materials and Methods.

generated via glutamate plus malate oxidation reduces the other.

It may also be noted that the respiratory chain linked to the external NADH reducible cytochrome aa_3 pool (about a third of the total cytochrome aa_3 pool) is able to generate a respiratory rate of 400 natom O/min per mg protein. Glutamate plus malate state 3 respiration corresponds to 325 natom O/min per mg protein in the same mitochondrial fraction (for at least 70% of the cytochrome aa_3 pool). Unlike glutamate plus malate respiration which can be controlled by many factors (i.e., membrane transports, dehydrogenase velocity and steady-state concentration in substrates like matricial NADH/NAD^+ ratio), external NADH has a direct access to its oxidation site. External NADH-linked respiration is therefore around 3 times higher than glutamate plus malate state 3 respiration related to accessible respiratory chain units. Thus, the fact that external NADH-linked respiration is large cannot be considered as a relevant argument for confirming the existence of an authentic activity.

NAD^+ stimulation of glutamate plus malate respiration. An explanation of our results is the presence in the mitochondrial fraction of a variable number of

altered mitochondria which have been made permeable to protons and small cofactors during mechanical tissue disruption. The loss of mitochondrial cofactors is known to occur in damaged mitochondria [1]. According to this hypothesis, we should also theoretically be able to stimulate glutamate plus malate respiration after NAD^+ addition by regenerating an internal NADH pool in damaged mitochondria. We found in the same beef heart mitochondrial fraction: (i) a ratio of 1.7 between external NADH and glutamate plus malate respirations; (ii) glutamate plus malate respiration was stimulated by NAD^+ addition in state 4 (stimulation of 70 natom O/min per mg protein for a total of 132) and state 3 (stimulation of 80 natom O/min per mg protein for a total of 500). We also found this NAD^+ dependent stimulation of glutamate plus malate respiration in two other beef heart mitochondrial fractions. This NAD^+ dependent glutamate plus malate respiration is thus poorly coupled, to the same extent as external NADH-linked respiration. These two activities therefore seem localized on the same particles even if the added NAD^+ dependent glutamate plus malate respiration remains low compared to NADH-linked respiration (10–20%). These particles are probably mitochondria which have lost their cofactors but not their matricial enzyme equipment.

Conclusion

From a physiological point of view, the supposed existence of an external NADH oxidase raised the question of the nature of the mechanisms involved in the regulation between this activity and the well known malate-aspartate shuttle [30], both being assumed to lead to cytoplasmic NADH oxidation in the heart. The presence of such an oxidase in heart mitochondria and the knowledge of its coupling modalities to ADP phosphorylation appeared crucial for heart metabolism.

We characterize the external NADH-linked respiration in rat and beef heart mitochondria: (i) the respiratory fluxes associated with external NADH and classical site I substrates present an opposing evolution under the different isolation procedures; (ii) external NADH is oxidized by a classic respiratory chain system as shown by the effect of inhibitors like rotenone, antimycin A and cyanide; (iii) on the same mitochondrial fractions, external NADH-linked respiration does not lead to ADP phosphorylation while glutamate plus malate does; (iv) additivity experiments on respiration and cytochrome aa_3 reduction clearly indicates that external NADH and glutamate plus malate respiration are linked to two distinct and functionally separate respiratory chain pools; (v) glutamate plus malate respiration can be stimulated by NAD^+ addition, indicating the presence of damaged mitochondria in the fraction and respiratory chain pools accessible to external cofactors; (vi) unlike previously published experiments [16], we have found a similar sensitivity toward EFA of external NADH respiration and glutamate plus malate- or succinate-linked respiration.

Moreover, we show that a very careful procedure is essential for obtaining rat heart mitochondrial fractions which are well coupled and without significant external NADH-linked respiration.

Taken together, these results are therefore in contradiction with the previous hypothesis of an 'external' NADH dehydrogenase located on the outer face of the inner membrane. An explanation of our results could be a contamination of integral mitochondria free from external NADH-linked respiration by particles in which the internal NADH dehydrogenase has been made accessible to external NADH, and consequently has lost its internal cofactor pool. Obviously, the existence of a very low authentic external NADH dehydrogenase activity could be definitively discarded only by the prove which would clearly show that the 'external' NADH dehydrogenase activity is completely restricted to the damaged mitochondria, i.e. separation of two mitochondrial subfractions possessing high and no activities respectively.

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