The NADH oxidase system (external) of muscle mitochondria and its role in the oxidation of cytoplasmic NADH

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1. An exo-NADH oxidase system [NADH oxidase system (external)], effecting intact-mitochondrial oxidation of added NADH, was studied in pigeon heart mitochondria. Breast muscle mitochondria showed an equal specific activity of the system. 2. The exo-NADH oxidase activity (200 μ mol of NADH/min per g of protein) equalled two-thirds of the State-3 respiratory activity with malate + pyruvate or one-seventh of the total NADH oxidase activity of heart mitochondria. 3. The activity was not caused by use of proteinase in the preparation procedure and all measured parameters were very reproducible from preparation to preparation. The activity is therefore most likely not due to preparation artefacts. 4. The exo-NADH oxidase system is present in all mitochondria in the preparation and is not confined to a subpopulation. The system reduced all cytochrome anaerobically and direct interaction with all cytochrome oxidase was demonstrated by interdependent cyanide inhibition. 5. The exo-NADH oxidase system seems to be located at the outer surface of the mitochondrial inner membrane because, for instance, only this system was rapidly inhibited by rotenone, and ferricyanide could act as acceptor in the rotenone-inhibited system (reductase activity = 20 times oxidase activity). 6. In the presence of antimycin, added NADH reduced only a part of the *b*-cytochromes. Freezing and thawing the mitochondria, one of the methods used for making them permeable to NADH, destroyed this functional compartmentation. 7. The characteristics of the exo-NADH oxidase system and the malate–aspartate shuttle are compared and the evidence for the shuttle's function in heart *in vivo* is re-evaluated. It is proposed that oxidation of cytoplasmic NADH in red muscles primarily is effected by the exo-NADH oxidase system.

Thermodynamic considerations exclude free permeability of mitochondria to NAD^+ and NADH because very different NAD^+/NADH ratios are maintained in the cytoplasmic and mitochondrial compartments of the cell (e.g. Newsholme & Start, 1973). As the methods of preparations were improved, the ability of isolated

Special nomenclature used: exo-NADH oxidase, enzyme system oxidizing NADH added to intact mitochondrial [systematic name NADH oxidase system (external)]; endo-NADH oxidase, enzyme system oxidizing NADH formed inside the mitochondria [systematic name NADH oxidase system (internal)]; the NADH dehydrogenases of the two systems are referred to as exo-NADH dehydrogenase and endo-NADH dehydrogenase, respectively.

liver mitochondria to oxidize added NADH decreased, and it was therefore extrapolated that the intact liver mitochondria would show no such catalytic activity (Lehninger, 1951). It has, however, been widely accepted that mitochondria from plants and micro-organisms possess special systems for oxidation of cytoplasmic NADH. Such systems have been described for yeast by Ohnishi *et al.* (1966) and von Jagow & Klingenberg (1970), for *Neurospora* by Weiss *et al.* (1970), and for plants by, for instance, Storey (1970), Palmer & Passam (1971), Douce *et al.* (1973), and, in a review, Palmer & Møller (1982).

Ability of muscle mitochondria to oxidize added NADH has been reported by, for instance, Aviad *et al.* (1958) (monkey and rat heart), Des-

phande *et al.* (1961) (rabbit heart), Hedman *et al.* (1962) (rat and human skeletal muscle), and Wrogemann & Blanchaer (1968) (hamster heart and skeletal muscle). Most investigators have, however, drawn a parallel between muscle and liver mitochondria and considered the observed oxidation of added NADH with suspicion. Minakami *et al.* (1962) assumed that the activity of beef heart mitochondria was not related to the respiratory chain. Blanchaer and coworkers ascribed the activity of pigeon heart mitochondria to permeability to NADH but did not regard it as an artefact because the mitochondria otherwise behaved as if they were of high integrity. A function *in vivo* of the activity was not considered (Blanchaer *et al.*, 1966; Blanchaer & Griffith, 1966; Griffith & Blanchaer, 1967; Blanchaer, 1968). Lee & Slater (1972) suggested that the alkaline proteinase treatment of the preparation procedure induced permeability to NADH in pigeon heart mitochondria.

One of us, however, considered the ability of pigeon heart mitochondria to oxidize added NADH as due to an integral and authentic activity of the mitochondria (Rasmussen, 1969, 1977). The activity was large, additive to the normal substrate respiratory activities, and more sensitive than these to respiratory chain inhibitors. Its distribution in subfractions obtained by differential centrifugation was different from that of the normal respiratory activities. Added NADPH was not oxidized. No oxidative phosphorylation accompanied the oxidation of added NADH and no substance interfering with this process, affected the activity. It was proposed that a separate respiratory chain effected the oxidation of added NADH and that this chain was accessible to NADH without permeation of the inner mitochondrial membrane.

This paper describes further studies of the oxidation of added NADH by pigeon heart mitochondria. We suggest the name 'exo-NADH oxidase system', and the systematic name 'NADH oxidase system (external)', for the system that is responsible for this activity. Our results demonstrate that the system most likely is not due to preparation artefacts, that it is present in all heart mitochondria, and that it seems to be located at the outer surface of the mitochondrial inner membrane. Except at the level of cytochrome oxidase, the system is isolated from the normal respiratory chain. Its activity and other characteristics make it very well suited to oxidize cytoplasmic NADH in the intact cell. This, and a re-evaluation of the evidence for operation of the malate-aspartate shuttle *in vivo*, lead us to propose that oxidation of cytoplasmic NADH in muscles primarily is effected by the exo-NADH oxidase system.

Experimental

Preparations

Normal mitochondria were prepared by a modified Hagihara-Chance procedure described previously (Rasmussen *et al.*, 1982). The homogenate was incubated for 10 min at 0°C with bacterial proteinase, and the mitochondria were isolated and washed in four centrifugations (integrated field times 50 000, 2000, 44 500, and 44 500 g·min respectively at r_{av} 8.1 cm). Incubation and first centrifugation were omitted when preparing without proteinase treatment. The mitochondria were kept at 0°C for maximally 6 h at a concentration of 5–10 mg of protein/ml in the preparation medium (225 mM-mannitol/75 mM-sucrose/1 mM-Tris/50 μ M-EDTA, pH 7.35).

Frozen-thawed mitochondria were usually obtained by freezing suspensions of normal mitochondria at –20°C, at least overnight, and thawing at 0°C. These preparations showed reproducible NADH oxidase activity. The oldest preparation used had been kept frozen for 14 months. Freezing at lower temperatures (–80 or –196°C) or for less than 2 h at –20°C resulted in less than optimal activity, probably because size or transformations of the ice crystals were critical. Except after freezing once at –196°C, respiratory control was completely lost by the treatment.

Triton-treated mitochondria were obtained by diluting a 200 μ l suspension of normal mitochondria with 2500 μ l of medium containing 0.4% (w/v) Triton X-100. The rate of ferricyanide reduction by NADH was unaffected by the concentration of Triton in the range from 0.2 to at least 1.0%.

Sonication was carried out with a 150 W ultrasonic disintegrator equipped with 9.5 mm ($\frac{3}{8}$ inch) diameter titanium probe (MSE, London, U.K.). The instrument was 'tuned' for maximum energy input and operated for 2 $\times$ 20 s with an intermittent period of 20 s. The mitochondrial suspension (0.15–0.3 mg of protein/ml) was cooled in an ice bath during sonication.

Assays

The NADH oxidase reaction has an NADH : O₂ stoichiometry of 2 : 1 (Rasmussen, 1969) and may be followed by measurement of NADH or oxygen, or both.

NADH was measured with a microfluorimeter having front surface geometry (Ultropak objective from E. Leitz, Wetzlar, Germany). Optical filters selected the excitation (365 nm) and the emission (max. at 450 nm). The mercury arc lamp (90 W, type 93136; Philips, Eindhoven, The Netherlands) was cooled by a stream of air and housed in a water-cooled compartment. The lamp was fed

from a stabilizing transformer via the auto-leak transformer supplied by the manufacturer. The signal from the photomultiplier (type 9524S; EMI, Hayes, Middx., U.K.) was amplified by an AC-coupled current amplifier, rectified by a full wave precision circuit [see, for instance, Fig. 8.19, Clayton (1971)] and demodulated and amplified for registration on graphic recorders.

Fluorescence intensity was not linearly related to NADH concentration because of the inner filter effect due to absorption of the excitation illumination by the fluorophore [for a review, see Lloyd (1981)]. Simple theoretical considerations lead to the equation:

$$F = a \cdot c \cdot 10^{-bc}$$

where F is the fluorescence intensity, a and b are constants, and c is the NADH concentration. The value of a is determined by several factors, e.g. the excitation intensity at the surface of the cell. The equation permits calculation of c from F by iteration, or, in the differentiated form, calculation of dc/dt from measured values of dF/dt , i.e.:

$$dc/dt = (1 - 2.30 \cdot b \cdot c)^{-1} \cdot F^{-1} \cdot c \cdot (dF/dt)$$

The constant b (about 2000 M^{-1} in our system) was, however, dependent on the concentration of mitochondria. It was therefore measured for each different reaction mixture: NADH was added stepwise to the reaction mixture, containing $1 \mu\text{M}$ -rotenone, and $\log(F/c)$ was plotted against c .

Measurements of respiration by means of modified Clark-type oxygen electrodes were carried out with instrumentation described elsewhere (Rasmussen, 1979). Endogenous respiration was measured in a $200 \mu\text{l}$ thermostatted glass vessel, which was closed with a ground glass stopper holding the oxygen electrode. An important feature of this apparatus was that diffusion of O_2 from the atmosphere into the vessel was below the limit of detection.

The NADH:ferricyanide stoichiometry in the reductase reaction was found to be 1:2. The reaction was followed by dual-wavelength spectrophotometry of ferricyanide ($\epsilon_{420-460} 950 \text{ M}^{-1} \cdot \text{cm}^{-1}$) with the instrument described previously (Rasmussen *et al.*, 1982). Rotenone ($1 \mu\text{M}$) was added to inhibit NADH oxidase activity. Measurement of the reaction by fluorimetry of NADH was possible, but less advantageous because of the fluorescence quenching exerted by ferricyanide.

All assays were performed at room temperature (about 21°C) using an air-saturated medium of 225 mM -mannitol / 75 mM -sucrose / 20 mM -Tris / 0.5 mM -EDTA / 10 mM -phosphate, pH 7.35.

Cytochrome measurements (spectra and reduction kinetics)

Dual-wavelength spectra were measured at room temperature and at 77 K with the instrument described previously (Rasmussen *et al.*, 1982). The kinetics of cytochrome reduction on anaerobiosis were measured in a cylindrical cell with two sockets (1 cm pathlength, parallel windows; Thermal Syndicate, Wallsend, Northumberland, U.K.). An oxygen electrode was placed in one socket and a glass stopper in the other socket. This stopper was constructed of heavy-walled capillary tubing in a design that allowed introduction of a microsyringe needle without simultaneous transfer of O_2 from the atmosphere to the cell content. The cell was completely filled with reaction mixture. Anaerobiosis at a known concentration of NADH was obtained by adding NADH in two increments. The first amount equalled about 90% of the O_2 in the reaction mixture, and when respiration of this amount had ceased, the second amount was fixed on the basis of the steady oxygen electrode signal, measured with increased sensitivity.

Protein analysis

Protein was determined by a method based on staining with Amido Schwartz (Schaffner & Weissmann, 1973) with bovine serum albumin as standard (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.). The mitochondrial suspension was diluted with the Tris/sodium dodecyl sulphate reagent to a concentration of about $50 \mu\text{g}$ of protein/ml and stored frozen for later determination. Two independent dilutions were made of each preparation. The mean of the highest value relative to the lowest value for each set of two determinations was 1.020 ± 0.015 (s.d., $n = 101$). The method gave analytical value close to 80% of those obtained by a biuret method (Gornall *et al.*, 1949), corrected for turbidity and interference from medium components.

Chemicals

NADH (disodium salt, grade I) was from Boehringer Mannheim, antimycin from Sigma, and rotenone (analytical grade) from Serva. Succinate, glutamate, 2-oxoglutarate, pyruvate and malate were obtained as free acids from Sigma and used neutralized. Triton X-100 was of scintillation grade from BDH. The proteinase (subtilisin A) was obtained by courtesy of NOVO.

Results

Two functionally different NADH dehydrogenases in heart mitochondria

The respiratory rate of normal mitochondrial preparations supplied with NADH was $200 \mu\text{mol/}$

min per g of protein (Table 1). This activity, called the exo-NADH oxidase activity, was comparable with the other respiratory activities of the mitochondria. It exceeded all State-4 activities and amounted to two-thirds of the State-3 rate with malate + pyruvate or one-third of the rate with succinate + glutamate.

The exo-NADH oxidase activity was most likely limited by the activity of the system's first enzyme, the exo-NADH dehydrogenase, because the succeeding components exhibited very low steady state reduction and NADH, as described in a later section, in all probability reached the enzyme without permeating the mitochondrial membrane. Replacing O_2 with ferricyanide as acceptor increased the rate of NADH oxidation 20-fold (Table 1). The kinetics of the reductase reaction in intact mitochondria showed the characteristics of a ping-pong mechanism with NADH inhibition, a mechanism very similar to that proposed for isolated Type-I NADH dehydrogenase by Doijewaard & Slater (1976). The kinetics of the exo-NADH oxidase reaction showed no substrate inhibition and deviations from the Michaelis-Menten equation were only detected at NADH concentrations below $1.5 \mu M$. The mechanism that is consistent with all experimental findings involves that NADH and ferricyanide react at sites close to each other, whereas the reoxidation of the enzyme by the natural acceptor occurs at a site distant from the NADH-binding site (U. F. Rasmussen & H. N. Rasmussen, unpublished work). This mechanism agrees well with the knowledge about the catalytic properties of NADH dehydrogenase [for a review, see Ragan (1976)].

Respiration of tricarboxylic acid cycle substrates was previously observed to be additive to the exo-NADH oxidase activity (Rasmussen, 1969). This means that the mitochondria must contain NADH dehydrogenase activity in excess of that measured in the exo-NADH oxidase reaction or in the reductase reaction in intact mitochondria. Different treatments of the mito-

chondria to make them permeable to NADH were therefore investigated in order to obtain an estimate of the total oxidase and reductase activities. We have used freezing and thawing, treatment with Triton X-100, and sonication.

Freezing and thawing the mitochondria as described proved to be very satisfactory, especially for measuring the NADH oxidase activity. Respiratory control was lost, but the State-4 rate of succinate + glutamate respiration was not altered by the treatment, and the rate of NADH oxidation was increased with very little change of K_m^{NADH} . The maximal rate of NADH oxidation that could be obtained was dependent on the condition of the mitochondria at the time of freezing (Table 1). Freezing and thawing also increased the reductase activity. The preparation appeared, however, insufficiently permeable to sustain the high rate of NADH oxidation by ferricyanide because both substrates showed pseudo-first-order kinetics.

Treatment of the mitochondria with Triton X-100 resulted in complete loss of respiratory activity. The reductase activity was increased 7-fold with a change in K_m^{NADH} that might indicate some modification of the enzyme. The turbidity of the mitochondria disappeared instantaneously on addition of Triton and the reductase activity, obviously, did not depend on mitochondrial integrity.

The sonicated preparation appeared fully permeable to NADH and ferricyanide. But sonication was inferior to the other methods in producing complete and reproducible increase of activity, and no improvement was obtained by varying the time of exposure. Freezing and thawing a sonicated preparation caused no further increase of the oxidase activity and the reductase activity was not increased by treating the preparation with Triton either. Thus, some activity was probably lost during sonication, by foaming or by contact with the metal probe.

The preferred methods for making the mitochondria permeable to NADH were freezing and thawing, leading to a 7.2-fold increase of oxidase

Table 1. *NADH oxidizing activities of pigeon heart mitochondria and derived preparations*

Data are mean values $\pm$ s.d.

Preparation . . .	NADH oxidase		NADH-ferricyanide reductase	
	Normal	Frozen-thawed	Normal	Triton-treated
Number of preparations	44	14	6	4
K_m^{NADH} (μM)	5.2 ± 1.2	7.0 ± 1.8	70 ± 20	34 ± 8
V (μmol of NADH/min per g of protein)	200 ± 40	$1035 \pm 152^*$	4235 ± 1037	30138 ± 1315

* These preparations were obtained by freezing mitochondria that had been kept at $0^\circ C$ for 5–6 h. Frozen-thawed preparations made from freshly prepared mitochondria showed $V = 1446 \pm 154 \mu mol$ of NADH/min per g of protein (S.D., 31 preparations).

activity, and Triton treatment, leading to a 7.1-fold increase of the reductase activity. The methods might not have led to complete exposure of the enzyme and some modification or inactivation might have occurred. But the identity of the two ratios is striking.

Heart mitochondria are, in contrast with liver mitochondria, isolated from a homogenate incubated with proteinase. Liver mitochondria are generally accepted to be impermeable to NADH (Lehninger, 1951), and the exo-NADH oxidase activity of heart mitochondria might be suspected to be caused by the proteinase treatment. We have examined the effect of proteinase treatment on isolation of mitochondria from different tissues (Table 2). In no case did proteinase treatment increase the specific activity of the exo-NADH oxidase.

Proteinase treatment increased the yield of mitochondrial protein about 60% with heart and liver and improved the reproducibility of the preparations. With breast muscle, the yield of protein was lowered about 20% by using proteinase because large, fluffy layers in this case were removed in the washings. The slightly lower specific activity of all mitochondria prepared with proteinase was therefore independent of yield of protein and for that reason probably not caused by increased contaminating material. More likely, some loss of activity occurred during the longer preparation procedure with proteinase.

Mitochondria from heart and breast muscle oxidized added NADH with identical specific activity (Table 2). It was not examined whether the low activity of pigeon liver mitochondria possessed the characteristics of the exo-NADH oxidase system. Freezing and thawing increased the activity of all preparations.

The exo-NADH oxidase system and the phosphorylating respiratory chain

Even though the exo-NADH oxidase activity of heart mitochondria was large and most likely not due to preparation artefacts, the possibility existed that the system was confined to a subpopulation of

damaged mitochondria. Such a subpopulation could be expected to possess characteristics that might account for, in particular, two observations, namely that the highest specific activity was found in the slowest sedimenting mitochondria and that oxidation of added NADH led to no phosphorylation of ADP (Rasmussen, 1969). But the possibility of confinement to a subpopulation was ruled out by the experiments to be described. They demonstrated that the exo-NADH oxidase system interacts with other mitochondrial systems in ways that implicate its presence in all mitochondria of the preparation.

The entire pool of mitochondrial cytochromes was reduced in State 5 (anaerobiosis) obtained by addition of NADH to normal mitochondria. This is shown in the difference spectrum (dithionite-reduced minus NADH-reduced mitochondria) presented in the lower part of Fig. 1. Succinate + glutamate also reduced all cytochrome in State 5.

The rate of cytochrome reduction in the aerobic-anaerobic transition was highly dependent on the concentration of NADH. Fig. 2 shows the time course of cytochrome *aa*₃ reduction at anaerobiosis, in the presence of 40 μ M-NADH (Fig. 2a) and

Fig. 1. Reduction of cytochromes by added NADH. Upper curve, room temperature spectrum of mitochondria (0.56 mg of protein/ml; 1 cm pathlength) in State 5 with added NADH (645 μ M). Lower curve, difference between the spectrum of dithionite-reduced mitochondria and the upper spectrum.

Table 2. NADH oxidase activities of mitochondria from different pigeon tissues

The activity of normal mitochondria represents the exo-NADH oxidase activity and the activity of the frozen-thawed mitochondria the total NADH oxidase activity. Data are mean values $\pm$ s.d. with the number of preparations in parentheses.

Preparation . . .	NADH oxidase activity (μ mol of NADH/min per g of protein)		
	Heart	Breast muscle	Liver
Normal + proteinase	200 $\pm$ 40 (44)	170 $\pm$ 31 (3)	17.3 $\pm$ 3.8 (3)
Normal - proteinase	254 $\pm$ 130 (7)	247 $\pm$ 39 (3)	28.6 $\pm$ 9.0 (3)
Frozen-thawed	1035 $\pm$ 152 (14)	733 $\pm$ 99 (4)	372 $\pm$ 23 (6)

Fig. 2. Time course of cytochrome aa_3 reduction at anaerobiosis

(a) NADH was added in an excess of $40\ \mu\text{M}$ to O_2 ; (b) NADH was added with an estimated deficit to O_2 of less than $1\ \mu\text{M-O}_2$. Reduced cytochrome aa_3 was measured by $A_{605} - A_{572}$ and the rate of reduction in (a) was determined from a simultaneous high-speed record. The protein concentration was $0.76\ \text{mg/ml}$.

with the estimated excess of O_2 over NADH of less than $1\ \mu\text{M-O}_2$ (Fig. 2b). The rate of reduction in Fig. 2(b) represented the maximal rate that could be ascribed to reduction by endogenous respiration. It might have been overestimated because of the technical difficulties in balancing the NADH addition to the O_2 content. The maximal rate of reduction by endogenous respiration amounted to only 3% of the maximal rate with NADH present. Endogenous respiration was furthermore never observed to effect reduction of the b -cytochromes in State 5. These large differences in reducing ability of endogenous substrates and added NADH ruled out the possibility that endogenous respiration could account for the State-5 reduction of cytochrome observed with added NADH. Experiments like those of Fig. 2 showed that the rate of endogenous respiration must be very low. Direct measurements of the oxygen consumption of undiluted mitochondria verified this: a rate of $0.17 \pm 0.04\ \mu\text{mol}$ of O_2/min per g of protein (S.D., three preparations) was obtained.

While all cytochrome was reduced in State 5 with NADH, only a fraction of the b -cytochromes was reduced by added NADH in antimycin-inhibited mitochondria. Furthermore, the size of this fraction was controlled by the integrity of the mitochondria. Fig. 3 shows the correlation between reduction of the b -cytochromes (antimycin present) and NADH oxidase activity (antimycin absent) in preparations subjected to different freezing-thawing procedures. The amount of b -cytochromes reduced by succinate + glutamate in the presence of antimycin (filled symbols) was unaffected by the treatment of the mitochondria and insensitive to supplementary addition of NADH. As the NADH oxidase activity was increased by freezing and thawing, an increasing part of the b -cytochromes became available to reduction by added NADH. The correlation

Fig. 3. Correlation between cytochrome b reduction (antimycin present) and NADH oxidase activity (antimycin absent) in mitochondria subjected to different freezing-thawing procedures

Two preparations, represented by circles and triangles, were subjected to either short freezing at 77K for a number of times or to one freezing at 253K for different periods of time. ●, ▲, Cytochrome b reduction in the presence of succinate + glutamate ($7.8 + 8.7\ \text{mM}$) and antimycin ($1.2\ \mu\text{mol/g}$ of protein) plotted against the relative NADH oxidase activity of that preparation. ○, △, As for closed symbols, but with NADH ($640\ \mu\text{M}$) as substrate. The total NADH oxidase activity (100%) was 1293 (circles) and 1693 (triangles) μmol of NADH/min per g of protein. The filled symbols at abscissa 100% are slightly displaced to allow reading of the drawing. Cytochrome b reduction was measured from the 77K spectra as the area between 543 and 567nm and given as % of the mean reduction with succinate + glutamate. The treatments were, in order of ascending NADH oxidase activity, circles; (1) none; (2) 77K , once; (3) 77K , twice; (4) 253K , 2.5h ; (5) 253K , 14days . Triangles; (1) none; (2) 253K , 1h ; (3) 77K , once; (4) 77K , twice; (5) 77K , three times; (6) 253K , 4h ; (7) 253K , 20h . The broken lines indicate mean values obtained with a number of normal mitochondrial preparations and the oblique line is the calculated regression curve for the NADH data.

between cytochrome b reduction and respiratory rate was apparently linear. Different treatments most likely made different numbers of mitochondria permeable to NADH and in these mitochondria, added NADH could also be oxidized via the endo-NADH oxidase system and reduce the b -

cytochromes that otherwise only were reduced by the tricarboxylic acid cycle substrates.

The broken lines in Fig. 3 indicate the values obtained with normal mitochondria: the exo-NADH oxidase activity amounted to 14% of the total NADH oxidase activity (Table 1) and, added to antimycin-inhibited mitochondria, NADH reduced 44% (s.d. 4%, $n = 40$) of the amount of *b*-cytochromes reduced by succinate+glutamate. The correlation between cytochrome *b* reduction and oxidase activity in normal mitochondria was very different from the correlation in frozen-thawed mitochondria. This was taken as evidence for the notion that the exo-NADH oxidase of normal mitochondria did not involve permeation as obtained by freezing and thawing.

Interaction of the exo-NADH oxidase system with systems present in all mitochondria was also observed in more functional relations. Added NADH and succinate+glutamate are oxidized without mutual influence (Rasmussen, 1969), but the development of cyanide inhibition of succinate+glutamate was strongly affected by the presence of added NADH (Fig. 4). The control experiment (curve *a*) was a normal State 4–3–4 transition in uninhibited mitochondria oxidizing succinate+glutamate. In the two other experiments (curves *b* and *c*), four times as much ADP was added to ensure that State-3 respiration persisted during the registration. Cyanide was in these experiments added in a concentration that inhibited exo-NADH oxidation completely and

succinate+glutamate respiration only partially. The inhibition of succinate+glutamate State-3 respiration developed slowly after addition of ADP (curve *b*). But in the presence of added NADH, the fully inhibited State-3 rate was immediately attained (curve *c*). Establishment of cyanide inhibition of succinate+glutamate State-3 respiration obviously depended on turnover of the system unless added NADH was present. The mechanism of this effect must involve a direct contact between the exo-NADH oxidase system and the system oxidizing succinate+glutamate, i.e. the two systems must be present within the same mitochondria and exo-NADH oxidase cannot be confined to a subpopulation of the mitochondria.

The results presented in this section fit well with the notion that two respiratory systems, the exo-NADH oxidase system and the matrix-faced respiratory chain, exist in all heart mitochondria. Added NADH was unable to reduce the components of the matrix-faced respiratory chain except by reaction through cytochrome oxidase, as for instance under anaerobiosis (Fig. 1), or in case of deliberately induced permeability of the mitochondria (Fig. 3). The functional compartmentation of the *b*-cytochromes is similar to the compartmentation of ubiquinone described by Jørgensen *et al.* (1985).

Location of the exo-NADH dehydrogenase in the mitochondrial membrane

Some of the experiments already described strongly indicate that added NADH is oxidized in normal mitochondria without permeating the inner mitochondrial membrane. Subfractionation of the mitochondria by differential centrifugation showed that the exo-NADH oxidase activity is rather distributed according to particle surface, whereas the other respiratory activities are distributed according to particle mass (Rasmussen, 1969). Further observations indicating that the exo-NADH dehydrogenase must be readily accessible are presented below.

The mitochondrial inner membrane is usually assumed to be impermeable to ferricyanide, which is an accepted probe for sidedness of membrane-bound dehydrogenases (e.g. Klingenberg, 1979). In the absence of antimycin, some ferricyanide reduction may occur from internally located dehydrogenases via cytochrome *c*. In the presence of antimycin, ferricyanide reduction reveals external location of the dehydrogenase. The present mitochondria exhibited the expected characteristics: very slow rate of reduction of ferricyanide by the substrates 2-oxoglutarate and succinate in the presence of antimycin and reduction by succinate in the presence of rotenone (Fig. 5). But, irrespectively of inhibitor used, added NADH reduced

Fig. 4. Effect of added NADH on establishment of cyanide inhibition of succinate+glutamate respiration

(a) Control experiment: succinate+glutamate (5.7+6.8 mM) added, followed by ADP (286 μ M); (b) as (a), but cyanide (7.2 μ M) was added prior to succinate+glutamate and the ADP concentration was increased to 1.14 mM; (c), as (b), but NADH (40 μ M) was added between addition of cyanide and addition of succinate+glutamate. The protein concentration was 0.36 mg/ml.

Fig. 5. Ferricyanide reduction by tricarboxylic acid cycle substrates and added NADH

—, antimycin (6 μM) present; ----, rotenone (1.7 μM) present. Reaction curves are superimposed and the numbers on the curves are rates (μM -ferricyanide/min) determined from simultaneous high-speed records. The initial concentrations were: 5.5 mM-2-oxoglutarate, 4.5 mM-succinate, 34.7 μM -NADH, 277 μM -ferricyanide and 0.16 mg of protein/ml.

ferricyanide very fast. The ferricyanide reaction site of the exo-NADH dehydrogenase is therefore in all probability accessible from the outside of the inner mitochondrial membrane, and so is the NADH reaction site, because the mechanism of reaction (ping-pong type with substrate inhibition) implies that NADH and ferricyanide reacts at sites close to each other.

The notion of an external location of the exo-NADH dehydrogenase is also supported by the observation that rotenone inhibition of this enzyme was established much faster than that of the endo-NADH dehydrogenase. Rotenone inhibited exo-NADH dehydrogenase completely in its oxidase reaction but did not affect its reductase reaction. The time course of establishment of rotenone inhibition is shown in Fig. 6. Substrates were absent during the incubation of mitochondria with a suboptimal concentration of rotenone. The endo-NADH dehydrogenase of normal mitochondria, assayed by State-3 respiration of malate + pyruvate, was very slowly inhibited (upper curve). Inhibition might even have been slower than indicated by the curve, as the rates were not corrected for loss of phosphorylating activity during the preincubation. The combined activities of the NADH dehydrogenases in frozen-thawed mitochondria (middle curves) were inhibited almost as fast as that of exo-NADH dehydrogenase (lowest curve). An obvious interpretation of these results is that the endo-NADH dehydrogenase, but not the exo-NADH dehydrogenase, is located behind a permeability barrier to rotenone and that

Fig. 6. Establishment of rotenone inhibition, in normal and frozen-thawed mitochondria

The time of preincubation is the time from addition of rotenone (90 nmol/g of protein) to addition of substrate; the rates of respiration are the maximal rates recorded after substrate addition. The points at abscissa ∞ indicate rates at fully developed inhibition (obtained in the presence of substrate). Symbols used: ●, normal mitochondria with malate + pyruvate + ADP (5.7 + 6.4 + 1.9 mM); ○, normal mitochondria with added NADH (3.3 mM); Δ, ▲, once and twice frozen-thawed (-20°C) mitochondria with added NADH (3.3 mM). The uninhibited respiratory rates (100%) were ●, 339; ○, 223; Δ, 1230; ▲, 1061 μmol of O/min per g of protein.

this barrier largely is destroyed by freezing and thawing the mitochondria.

The exo-NADH dehydrogenase and the combined activities of the twice-frozen preparation were inhibited to the same degree by rotenone (Fig. 6). This probably indicates identical affinity of the two NADH dehydrogenases for rotenone. Other similarities between the endo-NADH dehydrogenase and the exo-NADH dehydrogenase were observed. The value of K_m^{NADH} in the oxidase reaction differed only little in normal and in frozen-thawed mitochondria and the ratio between reductase and oxidase activity was identical in normal mitochondria and in the preparations assumed to exhibit total activities (Table 1). The pH dependence of the exo-NADH oxidase activity resembled that of total NADH oxidase activity very much and differed, at high pH values, significantly from that of succinate + glutamate respiration (Fig. 7). These observations of similarities were of course not conclusive, but they suggest

Fig. 7. *pH-dependence of respiratory activities*
 Symbols used: ○, ●, exo-NADH oxidase activity (two experimental series); △, total NADH oxidase activity in frozen-thawed mitochondria; ▲, succinate + glutamate respiration in normal mitochondria.

that the two NADH dehydrogenases probably are chemically identical and different only with respect to orientation in the inner mitochondrial membrane.

Discussion

The exo-NADH oxidase activity of pigeon heart mitochondria is in our opinion so large, possesses so special characteristics, and may execute so fundamental a function in the metabolism of the intact cell that it should not be ignored. It is, of course, difficult to prove the system's existence in the intact cell by studying isolated mitochondria. No single experiment can prove its authenticity and the evidence presented here has been obtained by collating many observations. On the other hand no experimental data indicate that the system is not a natural constituent of intact mitochondria. The proteinase treatment in the preparation procedure did obviously not induce the exo-NADH oxidase activity. Substantial variation among preparations would be expected if the activity was caused by preparation artefacts. The specific exo-NADH oxidase activity and the relative size of the cytochrome *b* pool associated with exo-NADH oxidase were, however, very reproducible. It is therefore concluded that the activity most likely is not due to preparation artefacts.

Added NADH may be assumed to be oxidized in a subpopulation of, for instance, damaged, permeable, or 'inverted' mitochondria. If so, added NADH should communicate only with the part of the cytochromes that resides in the subpopulation. But added NADH reduced all cytochromes in State 5, and inhibition of the exo-NADH oxidase system's cytochrome oxidase by cyanide conveyed to all remaining cytochrome oxidase the ability to

become rapidly inhibited by cyanide. Thus, the exo-NADH oxidase system must be present in all mitochondria and cannot be confined to any subpopulation.

The frozen-thawed mitochondria appeared sufficiently permeable to NADH and still sufficiently intact in their respiratory systems to provide a basis for assay of the total NADH oxidase activity. The notion that freezing and thawing induced permeability was also supported by the experiments with rotenone. In intact mitochondria, rotenone inhibition was established slowly, except in the case of exo-NADH oxidase. In frozen-thawed mitochondria, inhibition was established almost as fast as that of exo-NADH oxidase in intact mitochondria. Rotenone probably had free access to the exo-NADH oxidase system in intact mitochondria and so had ferricyanide as indicated by the high NADH:ferricyanide reductase activity. With respect to oxidation of succinate and oxoglutarate, ferricyanide, however, behaved as an impermeable acceptor. It may therefore be inferred that the exo-NADH oxidase system most likely is located at the outer side of the inner mitochondrial membrane.

Freezing and thawing of the mitochondria also affected the functional compartmentation of *b*-cytochromes observed in the presence of antimycin. One pool of the *b*-cytochromes was reduced by added NADH (and by succinate + glutamate). The other pool, normally reduced by succinate + glutamate, became available to reduction by added NADH only by freezing and thawing the mitochondria. In experiments with different freezing-thawing treatments, maximal cytochrome *b* reduction by added NADH was obtained simultaneously with maximal NADH oxidase activity. Ubiquinone exhibited a similar functional compartmentation (Jørgensen *et al.*, 1985). We believe that the inner membrane of heart mitochondria contains two respiratory chains, an internal and an external. The internal chain effects oxidations in connection with oxidative phosphorylation. The external chain is the exo-NADH oxidase system. This chain has none of the characteristics of oxidative phosphorylation processes: the exo-NADH oxidase activity is unaffected by ADP, uncouplers, and oligomycin; it leads to no ATP formation and is accompanied only by stoichiometric proton consumption and no translocation. We may thus infer that the two chains have no common step at the substrate side of any phosphorylation site. They interact, however, at cytochrome oxidase as seen from the interdependent cyanide inhibition. The State-5 reduction of all cytochrome by added NADH as well as by normal substrates may also be accounted for by this interaction. The internal chain can

reduce the external chain by leakage at some point at the substrate side of the antimycin block. From the ubiquinone studies, we know that this leakage is slow and therefore without physiological significance. Leakage from the external to the internal chain at the substrate side of the antimycin block has not been detected. But it cannot be excluded because the antimycin inhibition of the internal chain may not prevent the very slow flux in question.

The exo-NADH oxidase system is well suited to oxidize cytoplasmic NADH in the intact heart cell. Its low K_m^{NADH} ensures high rate of oxidation at realistic, cytoplasmic NAD^+/NADH ratios. The rate of complete oxidation of pyruvate equals the maximal rate of the slowest process in the tricarboxylic acid cycle. In isolated mitochondria the State-3 rates of malate + pyruvate and succinate + glutamate respiration were respectively 1.5- and 3-fold the exo-NADH oxidase rate. At least two oxidative processes were included in each of these State-3 rates. The steady state rate of the tricarboxylic acid cycle is therefore most likely lower than or equal to the exo-NADH oxidase rate, i.e. this system appears sufficiently active to secure formation of pyruvate from glucose or lactate. This statement may be checked in another way: Taegtmeier *et al.* (1980) measured the maximal rate of aerobic glucose oxidation in perfused rat hearts to be $1070 \mu\text{mol/h}$ per g dry wt. To maintain this, oxidation of cytoplasmic NADH must proceed at a rate of $36 \mu\text{mol/min}$ per g dry wt. The data are probably also valid for pigeon hearts as the major part of metabolism is determined by the mechanical work, which is likely to be similar on a dry-weight basis. The exo-NADH oxidase activity of pigeon heart mitochondria can account for the oxidation of cytoplasmic NADH if more than 18% of the dry weight is mitochondrial protein. This appears to be so: Safer *et al.* (1971) used the figure 29% of rat hearts.

Of several possible shuttle mechanisms, the malate-aspartate shuttle (Borst, 1963) is the one that usually is assumed to oxidize cytoplasmic NADH in the heart. The other shuttle mechanisms have insufficient capacity in this tissue (e.g. Safer *et al.*, 1971). The malate-aspartate shuttle is firmly coupled to the tricarboxylic acid cycle. When glucose or lactate are metabolized, the turnover at malate dehydrogenase must be twice the turnover at the other enzymes of the cycle. A very subtle control of the relative turnover at aminotransferase and at citrate synthase is therefore needed. This complex metabolic system may well function in a narrow range of rates, but it appears most unlikely that it can adjust to very different rates such as is required in the metabolism of the heart. The exo-NADH oxidase system is actually better suited to

oxidize cytoplasmic NADH than is the malate-aspartate shuttle. Its only function is apparently to oxidize extramitochondrial NADH, and it is completely independent of all other mitochondrial processes including respiratory control. This independence is acquired at the expense of only about 10% of the energy yield obtainable from oxidation of glucose.

The evidence for an operating malate-aspartate shuttle in the heart cell stems from interpretation of the changes in metabolites that were measured when isolated, perfused hearts were subjected to various transitions, e.g. from starvation to substrate administration, or to the aminotransferase inhibitor amino-oxyacetate (Safer *et al.*, 1971; Safer & Williamson, 1973; Williamson *et al.*, 1973). The perfusion techniques at that time were less than optimal and the experimental data may therefore be of limited value to the situation *in vivo* (cf. Taegtmeier *et al.*, 1980). Moreover the exchange reactions predicted by the model have repeatedly been observed to operate at much lower rates than required if cytoplasmic NADH is oxidized via the malate-aspartate shuttle [for a review, see Ottaway (1983)]. A major function of shuttle mechanisms in the metabolism of the heart therefore appears less likely. The exo-NADH oxidase system of heart and breast muscle mitochondria seems to possess the characteristics and capacity required for the oxidation of cytoplasmic NADH in the cell. Hence, we propose that this system effect the greater part of the oxidation of cytoplasmic NADH, in the heart and probably also in other red muscles.

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