Deterioration of Rat Liver Mitochondria Under Conditions of Metabolite Deprivation

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In a previous study [Parce, Cunningham & Waite (1978) *Biochemistry* **17**, 1634–1639] changes in mitochondrial phospholipid metabolism and energy-linked functions were monitored as coupled mitochondria were aged in iso-osmotic sucrose solution at 18°C. The sequence of events that occur in mitochondrial deterioration under the above conditions have been established more completely. Total adenine nucleotides are depleted early in the aging process, and their loss parallels the decline in respiratory control. Related to the loss of total adenine nucleotides is a dramatic decrease in ADP and ATP translocation (uptake). The decline of respiratory control is due primarily to a decrease in State-3 respiration; loss of this respiratory activity can be related to the decline in ADP translocation. Mitochondrial ATPase activity does not increase significantly until State-4 respiration has increased appreciably. At the time of loss of respiratory control the ATPase activity increases to equal the uncoupler-stimulated activity. The H^+/O ratio and P/O ratios do not decrease appreciably until respiratory control is lost. Similarly, permeability of the membrane to the passive diffusion of protons increases only after respiratory control is lost. These observations reinforce our earlier conclusion that there are two main phases in mitochondrial aging. The first phase is characterized by loss of the ability to translocate adenine nucleotides. The second phase is characterized by a decline in the ability of the mitochondrion to conserve energy (i.e. maintain a respiration-driven proton gradient) and to synthesize ATP.

Previous studies *in vivo* (Buffa *et al.*, 1970; Gaja *et al.*, 1973; Trump *et al.*, 1974) have demonstrated clearly that hepatic mitochondria deteriorate rapidly when liver tissue is made ischaemic, thereby being deprived of nutrients and oxygen. The mitochondria first assume a condensed conformation, and if the ischaemia is not soon corrected, they swell irreversibly. At this stage of tissue trauma, respiratory control and the oxidative phosphorylation capacity of the mitochondria are lost (Mergner *et al.*, 1972). Moreover, the increase in the amount of fatty acids occurring during an ischaemic episode (Boime *et al.*, 1970) implies the possibility of mitochondrial phospholipid hydrolysis. Trump *et al.* (1976) have suggested that the mitochondrial phospholipase may participate in an autocatalytic degradation of hepatic mitochondria *in vivo* by hydrolysing membrane phospholipids.

The processes accompanying deterioration of

mitochondria *in vivo* appear to correlate well with those occurring when mitochondria are allowed to age or deteriorate in systems *in vitro* where nutrient supplies and O_2 are limiting (Scarpa & Lindsay, 1972; Parce *et al.*, 1978; Chan & Higgins, 1978). In a previous study (Parce *et al.*, 1978) the changes in mitochondrial phospholipid metabolism and energy-linked functions were followed as tightly-coupled mitochondria were allowed to deteriorate in an incubation system *in vitro*. Based on those studies we proposed two main stages through which the mitochondrion passes as it progressively loses the ability to carry out oxidative phosphorylation and other energy-linked processes. The changes that occur initially, such as loss of respiratory control and the progressive decline in endogenous ATP concentrations, appear to be reversible (Siliprandi *et al.*, 1973; Ozelkok & Romani, 1974). In this early stage reacylation of monoacyl phospholipids and fatty-acid-oxidation activity are also lost due to lowered ATP levels (Parce *et al.*, 1978). The loss of

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these properties is followed by the apparently irreversible loss in energy-linked functions, such as the ability of the membrane to produce an energized state as measured by the energy-linked fluorescence response of 8-anilinoanthracene-1-sulphonate. This irreversible phase is facilitated by the action of the endogenous phospholipase A_2 on membrane phospholipids.

In this paper we have established more completely the sequence of events that occur as mitochondria progressively deteriorate. In addition the interrelationships between these events have been defined more clearly. A preliminary report of this work has appeared elsewhere (Cunningham *et al.*, 1979).

Experimental

Materials

Most of the materials used were obtained from the sources listed previously (Parce *et al.*, 1978). Valinomycin, *N*-ethylmaleimide, phosphoenolpyruvate, and hexokinase (type C-130) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. $[8-^{14}\text{C}]\text{ADP}$ and $[8-^{14}\text{C}]\text{ATP}$ were purchased from New England Nuclear, Boston, MA, U.S.A., and $^{32}\text{P}_i$ from ICN Pharmaceuticals, Irvine, CA, U.S.A. Pyruvate kinase was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.

Methods

Preparation of mitochondria, their incubation in 0.25 M-sucrose at 18°C, and the assays for respiratory control ratios were carried out as described previously (Parce *et al.*, 1978). Total adenine nucleotides (ATP, ADP, and AMP) in mitochondria were measured as described by Williamson & Corkey (1969) after aged mitochondria (2.5 mg of protein) were centrifuged at 46000 *g* for 5 min and resuspended in 0.2 ml of 0.25 M-sucrose.

Membrane permeability to protons, and H^+/O ratios, were measured at 25°C by a modification of the oxygen pulse technique of Mitchell & Moyle (1967). The assay mixture contained mitochondria (3 mg of protein) and 3.0 ml of an oxygen-free buffer containing 150 mM-KCl, 25 mM-sucrose, 3 mM-glycylglycine, 0.33 μM -rotenone, 1.6 mM-succinate and 0.6 μg of valinomycin. The buffer was deaerated with N_2 before use and the assay mixture was maintained under an N_2 atmosphere during all steps. *N*-Ethylmaleimide (120 nmol) was added to inhibit phosphate transport (Brand *et al.*, 1976). The H^+/O ratio and permeability to proton back-diffusion were calculated as described by Hinkle & Horstman (1971). Proton back-diffusion is expressed as the half-life for the pH change as protons diffuse from outside to inside the mitochondria after respiration ceases.

The mitochondrial ATPase activity measurements were carried out as described by Spach *et al.* (1979). An iso-osmotic assay mixture (Spach *et al.*, 1979) was utilized to ensure that the mitochondria were not disrupted during the assay, since such disruption would result in an increasing ATPase activity as the assay proceeded.

Adenine nucleotide translocase activity was assayed at 0°C using a slight modification of the method of Wojtczak & Zaluska (1967). The assay mixture contained 120 mM-KCl, 20 mM-Tris/HCl, pH 7.4, 1.1 mM-MgCl₂, 25 mM-sucrose and either 60 μM - $[^{14}\text{C}]\text{ATP}$ or 60 μM - $[^{14}\text{C}]\text{ADP}$ (20000 c.p.m.). The assay was initiated by the addition of 0.2 ml of mitochondrial suspension (30 mg of protein/ml). Assays were terminated at 0, 30, 60 and 120 s by the addition of 60 nmol of atractyloside. All assays were performed in duplicate. The tubes were centrifuged for 5 min at 40000 *g*. The pellets were resuspended in 2.2 ml of 0.25 M-sucrose and centrifuged at 40000 *g* for 15 min; this washing procedure was repeated. The final pellets were solubilized in 10% (w/v) potassium deoxycholate and counted for radioactivity in 10 ml of Aquasol.

The P/O ratio measurements were carried out by the procedure described by Christiansen *et al.* (1969) with slight modifications. Toluene was substituted for benzene in the phosphomolybdate extraction procedure. Analyses utilizing $^{32}\text{P}_i$ and D- $[U-^{14}\text{C}]\text{glucose 6-phosphate}$ demonstrated that separation of phosphomolybdate from glucose 6-phosphate was quantitative and that recovery of glucose 6-phosphate was above 95%.

Results

Fig. 1 illustrates the effect on four energy-linked parameters of aging mitochondria at 18°C. The respiratory control ratio (Fig. 1a) declined rapidly, reaching a value of 1 in about 4 h. Fig. 1(b) (see also Fig. 4) demonstrates that a significant portion of the loss in respiratory control is due to a decrease in the State-3 rate (in Fig. 1b $\Delta\mu\text{g}$ -atoms of O/min per mg of protein = 0.18). As seen in Fig. 1(b) and Fig. 4, State-4 respiration increased 1.8- and 1.6-fold, respectively, in the period during which respiratory control is lost (in Fig. 1b $\Delta\mu\text{g}$ -atoms of O/min per mg of protein = 0.05). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-stimulated succinate oxidation decreased to 82% of the initial State-3 respiratory rate in the period during which respiratory control is lost. The permeability of the membrane to H^+ (Fig. 1c) decreased until State-4 respiration had increased appreciably (respiratory control ratio approx. 2); at that point proton permeability began to increase dramatically. The H^+/O ratio (Fig. 1d) decreased very slowly up to 6 h, after which it began to decline rapidly.

Fig. 1. Effects of mitochondrial aging on (a) respiratory control, (b) State-3 and State-4 respiration, (c) membrane permeability to protons and (d) proton translocation

A mitochondrial suspension was incubated at 18°C and aliquots were removed for the various assays at the times indicated. The t_1 in (c) is the half life for the decay of the proton gradient established in the oxygen-pulse experiment. A larger value indicates lowered permeability, and vice versa.

Fig. 2. Effect of aging *in vitro* on ADP uptake into mitochondria

ADP translocation was measured at the times indicated as described in the Experimental section. Maximal ADP incorporation/2 min for the freshly prepared mitochondria was 3.2 nmol/mg of protein. (a) Shows the respiratory control ratio with the times of ADP translocation assays marked ($\downarrow$); (b) shows the rates of ADP translocation in mitochondria 0.6 h ($\circ$) and 3.1 h ($\triangle$) after preparation.

Proton translocation activity was maintained at or above the initial level until respiratory control is lost.

The observation that State-3 respiration is more sensitive to perturbation by aging than is State-4 or uncoupler-stimulated respiration suggests that adenine nucleotide transport is declining because of the aging process. In experiments where adenine nucleotide uptake was measured in freshly prepared mitochondria, and in preparations that had been aged until the respiratory control ratio had decreased to slightly below 2, both ADP translocation (Fig. 2) and ATP translocation (data not shown) had decreased appreciably. These observations appear to account for the loss in State-3 respiration demonstrated in Fig. 1. Translocation of exogenously added adenine nucleotides might be decreased in aged mitochondria because of a loss in matrix adenine nucleotides, since the translocase is an adenine nucleotide antiport. We have established that both ATP (Parce *et al.*, 1978) and total adenine nucleotides (Fig. 3) are lost from the matrix of the mitochondrion at a rate equivalent to the rate of decline in respiratory control. These observations indicate that decreased adenine nucleotide uptake is

Fig. 3. Effect of aging *in vitro* on the total adenine nucleotide content of mitochondria

The assays were performed as described in the Experimental section. $\blacktriangle$, Total adenine nucleotide content; $\bullet$, respiratory control ratio.

Fig. 4. Effect of aging in vitro on mitochondrial ATPase activity

The assays were performed as described in the Experimental section. The assay in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was carried out on a portion of freshly prepared mitochondria. All other assays were carried out in the absence of uncoupler. ■, State-3 respiration; ▲, State-4 respiration; ●, ATPase activity; ○, ATPase activity in presence of uncoupler.

due to the progressive loss of matrix adenine nucleotide.

Under conditions where energy conservation is maintained (a low State-4 respiration) the ATPase activity of coupled mitochondria would be expected to be low. Fig. 4 demonstrates that in the time period during which respiratory control was lost there was a slow increase in ATPase activity. In this experiment, ATPase activity was measured at an osmolarity equal to that used in respiratory-control-ratio measurements, to minimize disruption of mitochondria during the assay. After an initial lag period the ATPase activity began to increase dramatically, and reached maximal activity within 6 h. The period of slow increase in ATPase activity appeared to correspond closely to the interval during which there was little change in State-4 respiration. The rapid increase in ATPase activity occurred when the rate of State-4 respiration approached that of State-3 respiration.

The kinetics of the decrease in the P/O ratio are shown in Fig. 5. The P/O ratio decreased with a decay profile similar to those seen for the increase in

Fig. 5. Effect of aging in vitro on the P/O ratio of mitochondria

Succinate (20 mM) was the oxidizable substrate. See the Experimental section for the assay procedure.

permeability to protons and for the decline in the H^+/O ratio (Fig. 1). The rate of decrease of the P/O ratio was much slower than that observed for respiratory control (State-3 respiration). Similar decreases in the P/O ratio were also observed when glutamate or 2-hydroxybutyrate were used as energy sources (data not included).

Discussion

From this and our previous work (Parce *et al.*, 1978) we can describe a sequence of events that occurs as mitochondria age under controlled conditions. A summary of the aging process is shown in Table 1. Endogenous adenine nucleotide amounts decrease at a rate equivalent to the rate of loss of respiratory control. As expected, adenine nucleotide translocation activity is progressively lost. This results in lowered State-3 respiration, and hence, a decreased rate of ATP synthesis. Accompanying the loss of these functions is a decline in monoacyl phospholipid reacylation and in β -oxidation; these two activities are lost due to the lowered ATP levels. This eliminates the possibility of phospholipid repair

Table 1. *Events occurring during aging in vitro of rat liver mitochondria*

The number of arrows indicate the relative rates of change when one property is compared to another. ↑, Increase; ↓, decrease.

Property	Change
(a) Time period during which respiratory control is lost	
Endogenous adenine nucleotides	↓↓↓
Adenine nucleotide transport	↓↓↓
State-3 respiration	↓↓↓
State-4 respiration	↑
ATPase activity	↑
Monoacyl phospholipid reacylation	↓↓↓
β-Oxidation activity	↓↓↓
Proton permeability	↓↓
(b) Time period during which phospholipase A ₂ activity increases	
Membrane phospholipid hydrolysis	↑↑
ATPase activity	↑↑↑↑
Proton permeability	↑↑
Energy-linked 8-anilino-naphthalene-1-sulphonate fluorescence response	↓↓
H ⁺ /O ratio	↓↓
P/O ratio	↓↓

and also results in a slow accumulation of unesterified fatty acids (Parce *et al.*, 1978).

During this early phase of the aging process State-4 respiration and mitochondrial ATPase activity increase only slightly. At the time of complete loss of respiratory control, ATPase activity increases dramatically, emphasizing that the capability of the mitochondria to conserve energy is decreasing. When respiratory control is lost and ATP levels are depleted the endogenous phospholipase A₂ begins to hydrolyse membrane phospholipids, dramatically altering their chemical composition during this second stage of the aging process. This degradation of the membrane is accompanied by a steady decrease in the ability of the mitochondrion to conserve energy, as is indicated by the loss of the energy-linked fluorescence response of 8-anilino-naphthalene-1-sulphonate and a decrease in H⁺/O ratios, increased back-diffusion of ejected protons, and a steady drop in P/O ratio. It must be emphasized that declines in these latter mitochondrial properties are not appreciable until after the phospholipase begins to hydrolyse membrane phospholipids. This study therefore emphasizes that to maintain the functional integrity of the mitochondrion the activity of the mitochondrial phospholipase A₂ must be maintained at low levels.

There appears to be no relationship between State-4 respiration and permeability to protons in the early stage of aging. While State-4 respiration is increasing at a slow rate, membrane permeability to protons is decreasing dramatically (compare Fig. 1b with Fig. 1c). Clearly, in these experiments, net proton back-diffusion is not the rate-limiting factor for State-4 respiration. The membrane becomes

increasingly permeable to protons at the time that the rate of State-4 respiration approaches the rate of State-3 respiration. After respiratory control has declined, the increase in proton permeability parallels the decrease in proton-pumping ability, which suggests that the magnitude of the H⁺/O ratio is influenced by proton back-diffusion. This is expected in oxygen-pulse experiments where some back-diffusion can occur as protons are being translocated out of the mitochondrial matrix by the electron transport process.

In our study, all the events occurring concomitantly with, or after, the loss of adenine nucleotides from the mitochondrion would appear to be related to this initial loss of adenine nucleotide. We have not determined the reason for the leakage of adenine nucleotide from the mitochondrion, and do not know whether this event occurs in ischaemic liver tissue *in vivo*. It will, therefore, be of major interest to determine if total adenine nucleotide concentrations drop in mitochondria during tissue ischaemia. If such were the case, it would strengthen the postulate that the deterioration of liver mitochondria under our aging conditions parallels many of the events that occur *in situ*.

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