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RETARDED REDUCTION OF CYTOCHROME b_5 FOLLOWING THE AEROBIC-ANAEROBIC TRANSITION OF INTACT RAT LIVER MITOCHONDRIA

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SUMMARY

1. We measured the absorbance at 560–575 nm, using a dual wavelength spectrophotometer, of a suspension of rat liver mitochondria during depletion of dissolved oxygen. The absorbance, predominantly due to the cytochromes *b* and b_5, underwent a transition consisting of two rapid phases of increase separated in time by a slower phase of increase.

2. The retarded phase of absorbance increase disappeared when NADH was added, and the total amplitude of absorbance increase in the aerobic–anaerobic transition was reduced by an amount corresponding to the amplitude of the retarded phase of absorbance increase. This indicates that the first rapid phase of absorbance increase is due to the reduction of cytochrome *b*, and the retarded phase is due to the reduction of cytochrome b_5.

3. Liquid nitrogen temperature difference spectra of two samples with and without added NADH identified the pigment undergoing the retarded reduction as mitochondrial cytochrome b_5. The concentration of this mitochondrial cytochrome is 0.4 moles per mole of cytochrome *c* oxidase.

4. The retarded termination of the steady state of oxidation of mitochondrial cytochrom b_5 indicates the presence of a pool of oxidizing equivalents.

INTRODUCTION

When a suspension of mitochondria is allowed to consume dissolved oxygen, it is usually found that the absorbance of the mitochondrial cytochromes remains constant over a wide range of oxygen concentrations. Only when the oxygen tension reaches a very low value does the absorbance of the cytochromes begin to increase monophasically to concomitantly with that of the reduced cytochromes[1].

In a study of the aerobic–anaerobic transition in rat liver mitochondria, using a dual wavelength spectrophotometer, we found that at wavelengths corresponding to absorbance maxima of cytochromes a_3, *a*, and *c*, a monophasic transition in the cyto-

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chrome reduction occurs. However, at 560–575 nm the transition is not monophasic[2]. When the transition occurs, the absorbance at first increases rapidly to about one-third of its value in the fully reduced state; it then levels off and after several minutes an acceleration of the absorbance change takes place. In the present work we have investigated this phenomenon. We identify the acceleration in the absorbance change with a retarded termination of the steady-state oxidation of cytochrome b_5.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to the method of PARSONS *et al.*[3]. The low-speed–high-speed washes were repeated 5 times when a minimal contamination by microsomal cytochrome b_5 was essential. Rat liver microsomes were prepared according to the method of REMMER *et al.*[4]

The assay medium was 0.22 M mannitol, 0.07 M sucrose, 200 μM Na_2-EDTA, and 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES); the pH was adjusted to 7.4 with 1 M KOH. The uncoupler 5-Cl,3-*t*-butyl,2′-Cl,4′-NO_2-salicylanilide (S-13)[5,6] was generously provided by the agricultural division of Monsanto Chemical Co., St. Louis, Mo. TES was obtained from Sigma Chemical Co., St. Louis, Mo.

The concentration of mitochondria was determined on the basis of their cytochrome *c* oxidase content[7]. Cytochrome b_5 concentration was determined by using the difference (reduced *minus* oxidized) mmolar extinction coefficient ($\varepsilon^{557-575}_{mM^{-1}cM^{-1}}$) of 20.0[8].

No preincubations beyond the time required to add all components were carried out. The start of the optical trace marks the last addition to the suspension.

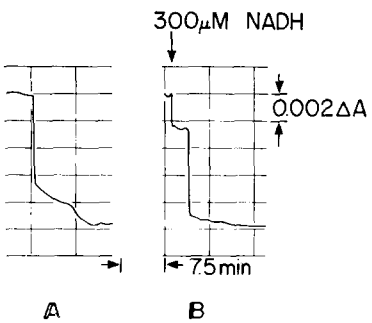

Fig. 1. Retarded cytochrome b_5 reduction. Mitochondria were suspended in aerobic mannitol–sucrose–EDTA–TES medium, pH 7.4, at a concentration of 0.26 μM cytochrome *c* oxidase in the presence of 10 mM sodium glutamate, 10 mM sodium malate and 1.8 moles of S-13 per mole of cytochrome *c* oxidase. The absorbance was measured at 560–575 nm. It increases towards the bottom. Time runs from left to right. A and B are identical experiments except for the addition of NADH in B.

RESULTS

Non-monophasic absorbance change associated with the aerobic–anaerobic transition

Mitochondria were suspended in aerobic mannitol–sucrose–EDTA–TES medium in the presence of glutamate, malate and S-13. Fig. 1A shows the absorbance

trace at 560–575 nm. A sudden large increase occurs in the absorbance during the aerobic–anaerobic transition. This change is primarily due to the reduction of mitochondrial cytochrome *b*. Then the rate of change of the absorbance trace decreases significantly. However after several minutes the rate of absorbance change increases again before it levels off to zero. The absorbance which contributes to the slow changes is about 15–20 % of the total absorbance change which occurs at 560–575 nm with these carefully washed and uncoupled mitochondria. The amplitude of the retarded phase of reduction is the same in coupled and uncoupled mitochondria. Thus, its relative contribution to the total change at 560–575 nm is larger with coupled mitochondria where the cytochrome *b* steady state oxidation is smaller[9].

The effect of exogenous NADH on the retarded absorbance change

When we add NADH during the steady oxidation state (Fig. 1B), there is a sudden increase of absorbance, and the retarded absorbance change after the aerobic–anaerobic transition does not occur. The sudden absorbance change which occurs upon the addition of NADH to the aerobic mitochondria is of about the same magnitude as the retarded absorbance change which occurs in the absence of exogenous NADH. Figs. 1A and B also show that exogenous NADH does not increase the respiration rate of the mitochondria in suspension because in both experiments it takes the same time before the dissolved oxygen is consumed. These experiments suggest that cytochrome b_5 is the slowly reduced cytochrome, since it is known that cytochrome b_5 is readily reduced with NADH even under aerobic conditions[10], due to its slow rate of auto-oxidation[11].

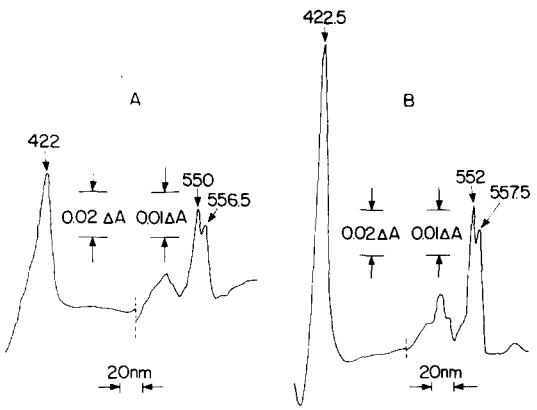

Fig. 2. Liquid nitrogen temperature difference spectra of mitochondrial and microsomal cytochrome b_5. Both spectra were recorded with 2 mm light path cuvettes. A, Mitochondria (2.5 μM cytochrome *c* oxidase) were suspended in oxygenated mannitol–sucrose–EDTA–TES medium in the presence of 10 mM sodium glutamate, 10 mM sodium malate, and 2 moles of S-13 per mole of cytochrome *c* oxidase. The sample cuvette contained also 300 μM NADH. B, Microsomes (1.6 μM cytochrome b_5) were suspended in mannitol–sucrose–EDTA–TES medium. The sample cuvette contained also 500 μM NADH.

Spectral properties of mitochondrial and microsomal cytochrome b_5.

In order to identify spectroscopically the pigment undergoing the retarded reduction, we took spectra at liquid nitrogen temperature of carefully washed mito-

chondria in the steady oxidation state *minus* mitochondria in the steady oxidation state in the presence of exogenous NADH. Two α-bands at 550 and 556.5 nm and a Soret band at 422 nm were found (Fig. 2A). The separation of the α-bands is 6.5 nm. Spectra of rat liver microsomes under identical conditions show α-bands at 552 and 557.5 nm and a Soret band at 422.5 nm (Fig. 2B). While the absolute accuracy of the spectrophotometer is within $\pm$ 0.5 nm, the accuracy with which one can determine the separation of two spectral peaks within a single spectrum is much higher. It is of interest to note that the α-band of the mitochondrial cytochrome is more split than that of the microsomal cytochrome b_5. This fact, together with the position of the Soret bands, agrees qualitatively with the liquid nitrogen spectra of outer-mitochondrial membrane cytochrome b_5 and microsomal cytochrome b_5 reported by PARSONS *et al.*[3]. Differences between the positions of the α-bands of mitochondrial cytochrome b_5 in their preparation (551 and 558 nm) and ours (550 and 556.5 nm) may be due to differences in the animal species or to the fact that they solubilized their preparation with sodium cholate and reduced it with dithionite.

It is very probable that the carefully washed mitochondria still contain some microsomal cytochrome b_5. The superposition of the microsomal cytochrome b_5 spectrum over that of the mitochondrial cytochrome b_5 will result in a smaller splitting of the α-bands. Thus, the highly purified outer mitochondrial membranes of PARSONS *et al.*[3] may indeed have contained less microsomal cytochrome b_5 contamination than our preparation of intact mitochondria.

We calculated the concentration of mitochondrial cytochrome b_5 from the low temperature spectra by assuming that the extinction coefficient, as well as the low temperature amplification factor[12], is the same as that for microsomal cytochrome b_5. This yields a concentration of 0.4 moles of cytochrome b_5 per mole of cytochrome *c* oxidase.

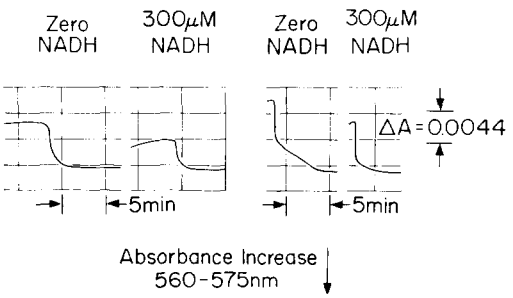

Fig. 3. Cytochrome b_5 reduction in coupled and uncoupled mitochondria. Mitochondria (0.35 μM cytochrome *c* oxidase) were suspended in aerated mannitol–sucrose–EDTA–TES medium in the presence of 10 mM sodium glutamate and 10 mM sodium malate. No uncoupler was present in the two experiments on the left, while 0.7 mole of S-13 per mole of cytochrome *c* oxidase was present in the other experiments. Absorbance changes were measured at 560–575 nm.

Effects of substrate and uncoupler on the retardation of the cytochrome b_5 reduction.

The acceleration in the 560–575 nm absorbance trace was completely absent when well-coupled mitochondria were allowed to reach anaerobiosis in the presence of glutamate and malate. The absorbance change associated with the aerobic–anaerobic transition was monophasic (Fig. 3). Exogenous NADH decreased the extent of the monophasic absorbance change (Fig. 3). This demonstrates that cytochrome b_5

in this type of experiment is reduced as rapidly as cytochrome *b*. Sometimes, however, we did observe a slight retardation in the cytochrome b_5 reduction even in the presence of glutamate and malate, suggesting that other factors besides good energy coupling may be involved.

Coupled mitochondria that became anaerobic in the presence of succinate or pyruvate and malate always showed a retardation in the cytochrome b_5 reduction (Fig. 4). The only correlation between the degree of uncoupling of a mitochondrial suspension and the retardation in the cytochrome b_5 reduction was that, for any given substrate, the retardation in the presence of uncoupler was larger than in its absence.

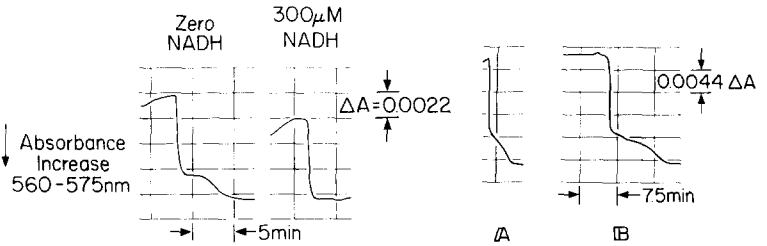

Fig. 4. Retardation of cytochrome b_5 reduction in the presence of succinate. Mitochondria (0.3 μM cytochrome *c* oxidase) were suspended in aerated mannitol–sucrose–EDTA–TES medium in the presence of 1 mole of S-13 per mole of cytochrome *c* oxidase and 10 mM sodium succinate.

Fig. 5. Increase in the retardation of the cytochrome b_5 reduction by increasing the initial oxygen concentration of the medium. Mitochondria (0.52 μM cytochrome *c* oxidase) were suspended in aerated (A) and oxygenated (B) mannitol–sucrose–EDTA–TES medium in the presence of 10 mM sodium glutamate, 10 mM sodium malate and 3 moles of S-13 per mole of cytochrome *c* oxidase. The absorbance was measured at 560–575 nm and increases towards the bottom.

The retardation of the cytochrome b_5 reduction was extended farther in the presence of any of the above substrates by allowing uncoupled mitochondria to reach anaerobiosis in oxygenated buffer instead of aerated buffer (Figs. 5A and B). This means that the mitochondria have to respire at least five times as much to reach anaerobiosis. Absorbance changes at 340–380 nm (reflecting the redox state of NADH and NADPH) showed that when the retardation was longer, the half-time for pyridine nucleotide reduction after the aerobic–anaerobic transition was longer. This suggests an inverse correlation between the concentration of endogenous NADH or NADPH and the extent of retardation.

Titration of the retardation of cytochrome b_5 reduction with NADH.

The final question that we attempted to answer was, why is there an acceleration in the cytochrome b_5 absorbance increase? Since cytochrome b_5 has a very low autoxidation rate and since the acceleration occurs in the anaerobic suspension, we concluded that it must be a direct reflection of the availability of NADH to the oxidized cytochrome b_5. We repeated most of the above mentioned experiments in a 90° light scattering spectrophotometer. Cytochrome *a* (605–630 nm) was monitored, while the light scattering changes were detected at 90° with a photomultiplier that was masked with a number 92 Kodak Wratten filter (Eastman Kodak Company). All the light scattering changes which occurred after the aerobic–anaerobic transition were monophasic. There was no detectable sudden change in the light scattering which

could be interpreted as an NADH-releasing structural change of the mitochondria.

We then titrated the aerobic–anaerobic transition with exogenous NADH. If the retardation is a reflection of a pool of oxidizing equivalents, then small amounts of exogenous NADH should shorten the retardation without changing the amplitude of the retarded absorbance change. This is indeed what we observed when smaller than μM amounts of NADH were added (Fig. 6). In this experiment an NADH concentration of only about 10 times that of the cytochrome b_5 concentration is sufficient to eliminate the retarded b_5 reduction.

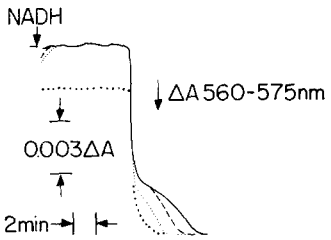

Fig. 6. Titration of the retarded cytochrome b_5 reduction with exogenous NADH. Experimental conditions as in Fig. 5A, except that the concentration of cytochrome *c* oxidase was 0.36 μM. The following amounts of NADH were added: none (———); 0.2 μM (– – – –); 0.4 μM (· · · · · ·) and 4.0 μM (●●●).

CONCLUSION

When mitochondria reach anaerobiosis and their absorbance is monitored at 560–575 nm, we observe an initial monophasic increase in absorbance which levels off and is followed several minutes later by an acceleration in the absorbance increase. This behavior is contrary to the generally accepted monophasic absorbance increase associated with the aerobic–anaerobic transition of mitochondria[1]. The fact that the retarded reduction disappears when NADH is added indicates that cytochrome b_5 is responsible. The liquid nitrogen temperature difference spectrum of two samples with and without added NADH identifies the pigment as cytochrome b_5 of the outer mitochondrial membrane.

There is a general inverse correlation between the endogenous NADH or NADPH concentration and the retardation in the cytochrome b_5 reduction. This inverse correlation is supported most clearly by the observation that the retardation in the cytochrome b_5 reduction is always larger with uncoupled than with coupled mitochondria, provided the same substrate is used.

An inverse correlation exists also between the degree of energization of the mitochondria and the retardation of the cytochrome b_5 reduction. It has been demonstrated recently that cytochrome oxidase shows an energy-linked retarded reduction after the aerobic–anaerobic transition in the presence of glutamate–malate but not in the presence of succinate. This retarded reduction of cytochrome oxidase does not occur in the presence of an uncoupler[13]. In our present experiments we observe essentially no retarded cytochrome b_5 reduction in the presence of glutamate and malate while a significant retardation occurs with succinate with coupled mitochondria. Both anaerobic systems have essentially completely reduced pools of NADH and NADPH. The higher degree of energization of the anaerobic mitochondria thus

makes reducing equivalents more readily available for the reduction of cytochrome b_5.

The acceleration in the absorbance change is due to the termination of a steady oxidation state of cytochrome b_5. An NADH concentration of about 10 times the concentration of cytohcrome b_5 present eliminates the retardation in cytochrome b_5 reduction (Fig. 6). It appears as if this retardation can be an extremely sensitive indicator of leakage of NADH or other reducing equivalents out of the inner mitochondrial membrane vesicle.

It is important to mention that the cytochrome b_5 reduction is not always retarded. In the case of coupled mitochondria with glutamate and malate (Fig. 3) it is not possible to distinguish between cytochrome b and b_5 on the basis of their reduction rates. The addition of NADH during the aerobic steady state, however, yields an absorbance change during the aerobic–anaerobic transition which is not due to cytochrome b_5.

Our observations should be useful in the study of the function of cytochrome b_5 and its relation to the energy-conserving processes in the mitochondria.

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