REDOX CHANGES OF CYTOCHROME *a*-607 AND NAD(P)H IN RAT LIVER MITOCHONDRIA INDUCED BY L-MALATE UNDER ANAEROBIC CONDITIONS*

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1. Introduction

Dawson et al. [1] demonstrated that L- and D-malate induced the oxidation of *b*-type cytochrome absorbing at 559 nm in *Arum spadix* mitochondria and rat liver mitochondria. The present work showed that L-malate induced oxidation of cytochrome *a*-607 in anaerobic rat liver mitochondria. The effect of L-malate on cytochrome *a*-607 was abolished in four ways: by respiratory inhibitors, such as rotenone and antimycin A, by redox dyes, such as menadione and phenazine methosulfate, by alkaline pH and by uncouplers. Malate caused similar changes of nicotinamide nucleotides to those of cytochrome *a*-607 and the reason for the anomalous changes of cytochrome *a*-607 in the presense of L-malate is discussed.

2. Materials and methods

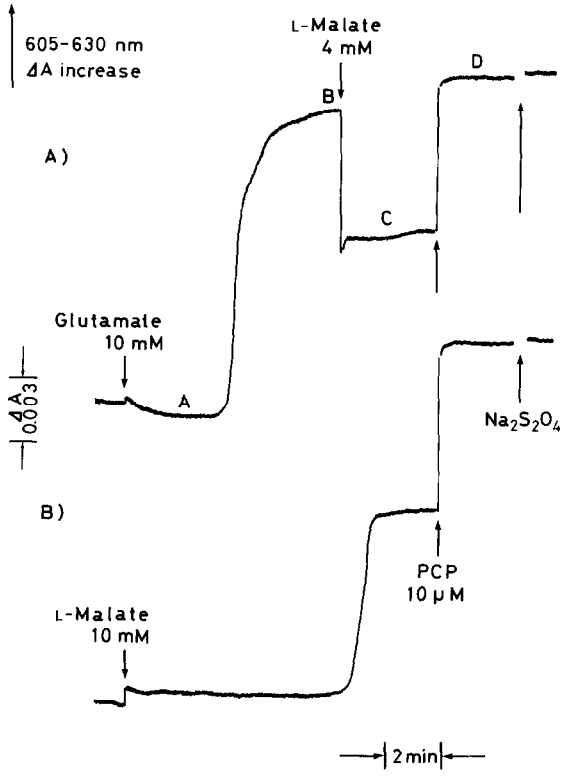
Rat liver mitochondria were isolated by the method of Hogeboom [2], as described by Myers and Slater [3]. Submitochondrial particles were prepared by the method of Gregg [4]. Protein was determined by the biuret method as described by Cleland and Slater [5]. All reactions were carried out in the medium containing 25 mM Tris–HCl buffer (pH 7.4), 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl. The other components used are indicated in the legends to the figures. The final volume of the mixture was 3 ml and pH was 7.4. Measurements of the absorbancy changes of respiratory components were made with a Hitachi, Model 356, two-wavelength spectrophotometer using the following wavelength pairs: cytochrome *a*-607 and cytochrome *a*-603, 605 nm minus 630 nm; cytochrome $c_1 + c$, 550 nm minus 540 nm; *b*-type cytochromes, 566 nm or 558 nm minus 575 nm; NAD(P)H, 340 nm minus 375 nm. Fluorescence changes due to nicotinamide nucleotides were measured with a Hitachi, Model MPF 3, spectrofluorometer using the wavelength of 450 nm for fluorescence and of 365 nm for excitation. In all experiments nitrogen gas was passed through the reaction mixture for 1 min before addition of mitochondrial suspension to shorten the time required to attain anaerobiosis. In order to minimize oxygen contamination, the surface of the reaction mixture was covered with liquid paraffin. All measurements were carried out at room temperature (20–25°C).

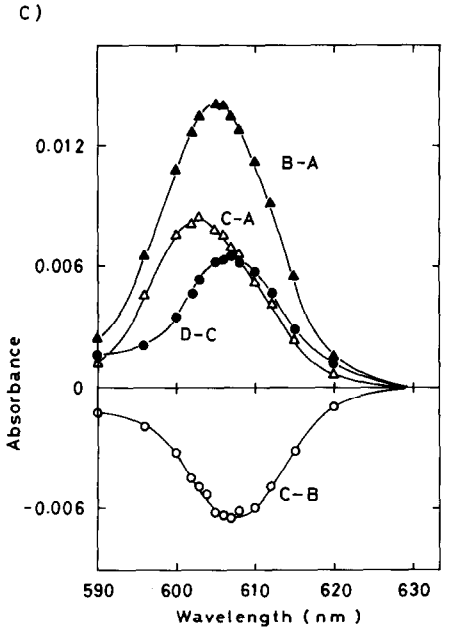
Hypotonic treatment of mitochondria was carried out by the method of Fukushima et al. [6] in a medium of 10 mM Tris–phosphate buffer (pH 7.2) for 10 min at 0°C. Then the suspension was subjected to high-speed centrifugation (105 000 *g* for 30 min). Tryptic digestion of mitochondria was performed with 0.1% trypsin in 0.25 M sucrose containing 5 mM potassium phosphate buffer (pH 7.5) for 10 min at 25°C, as described by Kuylenstierna et al. [7]. The supernatant was removed by centrifugation (105 000 *g* for 30 min).

3. Results and discussion

Fig. 1A shows that the reduction of cytochromes *a* proceeds in a biphasic way with fast and slow phases

* For practical reasons the terms of cytochrome *a*-607 and cytochrome *a*-603 were used, based on the absorption maxima of these components at room temperature.





in the transition from State 4 (nomenclature is as in ref. [8] but no inorganic phosphate was used in this experiment) to the anaerobic State 5 in rat liver mitochondria oxidizing glutamate, as already demonstrated in submitochondrial particles [9] and in intact mitochondria [10]. Addition of L-malate to anaerobic mitochondria resulted in oxidation of cytochromes *a* to a level corresponding to that attained in the fast phase of reduction. With L-malate as substrate, the reduction level in anaerobiosis became approximately one half that in State 5 mitochondria induced by glutamate (fig. 1B). The effect of malate was abolished by the further addition of 10 μM pentachlorophenol. The effect was also abolished by 6 μg rotenone, 6 μg antimycin A (Fig. 2A), 50 μM menadione, 50 μM phenazine methosulfate or an alkaline pH (pH change from 7.4 to 8.3 with 1*N* NaOH) under the similar conditions to those for Fig. 1A or B. The effect of alkaline pH was reversible, neutralization by HCl inducing the reoxidation of cytochromes *a*. Oligomycin did not reverse the effect of malate in this system. As indicated in Curve C–B and Curve D–C of Fig. 1C, cytochrome *a*-607 was oxidized by L-malate and re-reduced by pentachlorophenol, while cytochrome *a*-603 remained largely reduced on addition of malate (Curve C–A, Fig. 1C). The effect of L-malate was very similar to the ATP-linked oxidation of cytochrome *a*-607 reported by Wikström [10], who concluded that the component with ab-

Fig. 1. (A) Effect of L-malate on the redox state of cytochromes *a* in rat liver mitochondria oxdizing glutamate. State 5 was induced by 10 mM glutamate. 4 mM L-malate was added to State 5 mitochondria and then 10 μM pentachlorophenol (PCP) was added. 3.9 mg/ml of rat liver mitochondria were used. (B) Effect of pentachlorophenol on the redox state of cytochromes *a* in mitochondria oxidizing L-malate. State 5 was induced by 10 mM L-malate. 10 μM pentachlorophenol was added in State 5. 3.9 mg/ml rat liver mitochondria were used. (C) Absorption spectra of cytochromes *a*. Conditions were the same as for (A) except that 3.1 mg/ml of rat liver mitochondria were used. Difference spectra were obtained by the following procedure. The redox change was measured at various wavelengths between 590 nm and 630 nm with a constant reference at 630 nm. A separate incubation was performed for each point. Curve B–A, cytochrome *a*-603 and cytochrome *a*-607 were reduced in State 5 (phase B in (A)); Curve C–B, cytochrome *a*-607 absorbing at 607 nm was oxidized after addition of L-malate in phase B; Curve D–C, cytochrome *a*-607 was re-reduced after addition of pentachlorophenol; Curve C–A, cytochrome *a*-603 remained reduced in phase C.

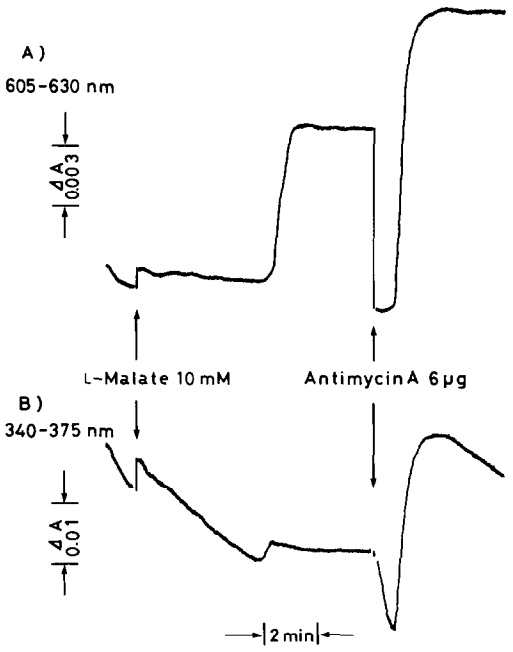


Fig. 2. Effect of antimycin A on the redox state of cytochromes *a* (A)) and of nicotinamide nucleotides (B)) in the presence of L-malate. State 5 was induced by 10 mM L-malate and then 6 μg of antimycin A were added. The transient oxidations of both components after addition of antimycin A are due to a trace of oxygen in the alcoholic solution of antimycin A. 3.9 mg/ml of rat liver mitochondria were used.

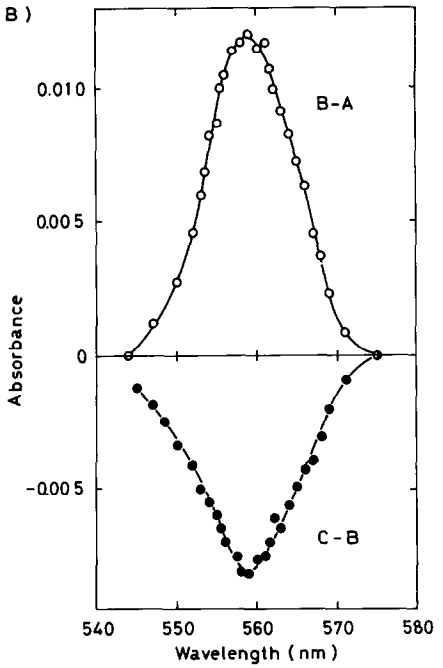
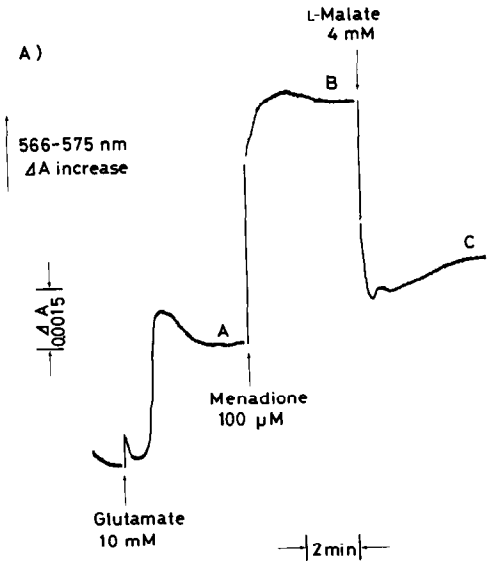


Fig. 3. (A) Effect of menadione and L-malate on the redox state of cytochrome *b* components. State 5 was induced by 10 mM glutamate. 100 μM menadione was added in phase A and 4 mM L-malate was added in phase B. 3.3 mg/ml of rat liver mitochondria were used. (B) Absorption spectra of cytochrome *b* components. Conditions were as for (A) except that 6.6 mg/ml of rat liver mitochondria were used. Difference spectra were obtained by scanning the measuring wavelengths, taking 575 nm as a reference wavelength, using a single cuvette. Curve B–A, the cytochrome *b* component absorbing at 559 nm was reduced after addition of menadione in phase A; Curve C–B, the cytochrome *b* component absorbing at 559 nm was oxidized after addition of L-malate in phase B.



sorption at 607 nm is cytochrome *a*, and that with absorption at 603 nm is cytochrome a_3. Biphasic reduction in anaerobiosis and the oxidizing effect by L-malate were also observed in mitochondria with other NAD-linked substrates such as pyruvate, 2-oxoglutarate and β-hydroxybutyrate, but neither were clearly observable when succinate was used as substrate at pH 7.4.

An anomalous effect of L-malate on nicotinamide nucleotide was observed when absorbance was measured at 340 nm minus 375 nm or measured by the fluorometric method. Fig. 2B shows that an extremely low level of reduced NAD(P) was obtained in anaerobiosis in the

presence of L-malate. Further addition of antimycin A induced simultaneous reduction of NAD(P) and cytochromes *a* as shown in Fig. 2A and B. Like antimycin A, rotenone, uncoupler and menadione also had reversing effects.

Somewhat different results were obtained with *b*-type cytochromes. We observed an oxidizing effect of L-malate (but not of D-malate, in contrast to ref. [1]) on *b* cytochromes absorbing mainly at 559 nm in the presence of menadione using glutamate as substrate as indicating in Fig. 3A and B. The effect by L-malate was not observed in the absence of menadione, even under the conditions where both cytochrome *a*-607 and NAD(P)H were oxidized on addition of L-malate. The effect of L-malate in the presence of menadione was not reversed by the various agents which reversed the effect of malate on cytochromes *a*.

Next studies were made on the relationship between the effect of malate on cytochrome *a*-607 and on *b*-type cytochromes absorbing at around 558 nm, located out of the inner membrane. Two kinds of preparation were used, since it is known that tryptic digestion causes release of b_5-like cytochrome located in the outer membrane [6,7,11] while hypotonic treatment causes solubilization of *b* cytochrome in the intermembrane space [6,11]. Fig. 4 shows that tryptic digestion resulted in loss of the L-malate effect on cytochrome *a*-607, while hypotonic treatment did not. Neither treatment altered the effect of L-malate on the NAD(P) region (not shown). Thus it is likely that b_5-like cytochrome, located in the outer membrane, is important in a control of the redox change of cytochrome *a*-607 in the inner membrane and that its release results in disappearance of the L-malate effect on cytochrome *a*-607. This conclusion was also supported by the results indicating a disappearance of L-malate effect on cytochrome *a*-607 in mersalyl-treated mitochondria, in which rotenone-insensitive NADH-cytochrome b_5 reductase system, located in the outer membrane, could be inhibited as reported by Archakov et al. [12]. Results on cytochromes *b* will be described in more detail in a subsequent paper.

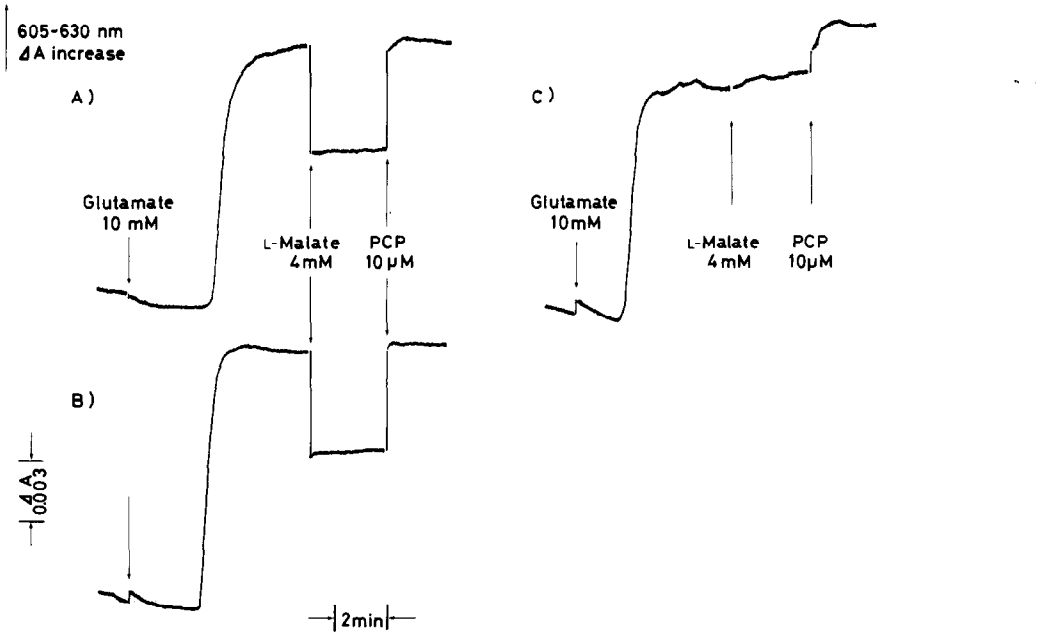


Fig. 4. Effect of L-malate on the redox state of cytochromes *a* in intact mitochondria (A)), and those subjected to hypotonic treatment (B)) and trypsin (C)). State 5 was induced by 10 mM glutamate and then L-malate and pentachlorophenol were added, successively. The absorbance at 605 nm minus 630 nm of each preparation was adjusted to the same level, taking that of State 5 mitochondria with $Na_2S_2O_4$ as fully reduced and that of mitochondria with uncoupler in the absence of substrate as fully oxidized. Protein content: intact mitochondria, 3.4 mg/ml; hypotonic treated mitochondria, 3.6 mg/ml; tryptic digested mitochondria, 3.8 mg/ml.

The malate effect on cytochromes *a* was not observed in submitochondrial particles from rat liver mitochondria with NADH as substrate. This indicates that L-malate does not affect the electron flow between NADH and cytochrome *a*-607 via the respiratory chain located in the inner membrane.

The four types of reversal observed in this work, seemed to affect different sites from that attacked by L-malate, although the site of L-malate action is still unknown. Further investigations are required on the multiple points of interaction in intermembrane electron flow between the outer and inner membranes and the influence on these sites of various factors, such as redox dyes and morphological changes induced by changes in the energy state of the mitochondrial membranes.

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