AN EFFECT OF MALATE ON THE REDOX STATE OF A CYTOCHROME B COMPONENT IN MITOCHONDRIA FROM VARIOUS SOURCES

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During a study of the respiratory chain of mitochondria from the spadix of Arum maculatum, it was noticed that the degree of reduction of the cytochrome b components in the anaerobic state varied with the substrate used and furthermore that L-malate appeared to cause a specific reoxidation of a b-type cytochrome on addition to mitochondria which had been allowed to go anaerobic with succinate as substrate or, alternatively, had been reduced by sodium dithionite. We report these findings here, and also some preliminary observations on mitochondria from other sources, which indicate that the effect of malate on cytochrome b components may be a widespread phenomenon.

Materials and Methods

Aroid spadices were harvested from their natural habitat just before the spathes opened. The outer pigmented layer of the spadix was removed by wiping with cotton wool before the tissue was homogenised.

Mung beans (Phaseolus aureus) and peas (Pisum sativum var. Meteor) were soaked for 1 min. in a 25% solution of sodium

hypochlorite and then overnight in running tap water. The seeds were planted in well-moistened Vermiculite and incubated for 6 days at 25°C in a dark room. The etiolated shoots were then harvested.

Mitochondria from these sources were prepared by the method of Ikuma and Bonner (1967) except for two minor modifications. The wash medium contained 10mM HEPES (Good et al. 1966) adjusted to pH 7.2 with potassium hydroxide and the final mitochondrial pellet was resuspended in a small volume of this wash medium. Mitochondria isolated in this way from mung beans and peas have been shown to be tightly coupled when oxidising malate in accordance with the observations of Ikuma and Bonner.

Rat liver mitochondria were prepared from the livers of Wistar hooded rats by the method of Hogeboom, Schneider and Palade (1948), using 0.25M sucrose in 5mM Tris chloride buffer (pH 7.2) as the homogenising medium.

The protein concentration of mitochondrial preparations was determined by the method of Gornall, Bardawill and David (1949) after clarification with Triton X-100. Bovine serum albumin was used as a standard.

Redox changes in cytochrome b components were measured on an Aminco-Chance dual wavelength spectrophotometer using the wavelength pair 562-575mμ. Difference spectra were recorded on a Perkin-Elmer Model 450 double beam spectrophotometer.

D(+) and L(-) malate (free acids) and sodium pyruvate

were obtained from Sigma Chemical Corp. Succinic acid and trisodium citrate were the AnalaR products of Hopkin and Williams.

Results

The effect of the addition of small quantities of L-malate to Arum spadix mitochondria which have been allowed to go anaerobic with succinate as substrate is shown in Fig. 1a. There is an apparent reoxidation of cytochrome b to the extent of about 35% of the total. A similar effect is seen in mitochondria from this source in which the cytochromes have been reduced by the addition of sodium dithionite (Fig. 1b). That these results

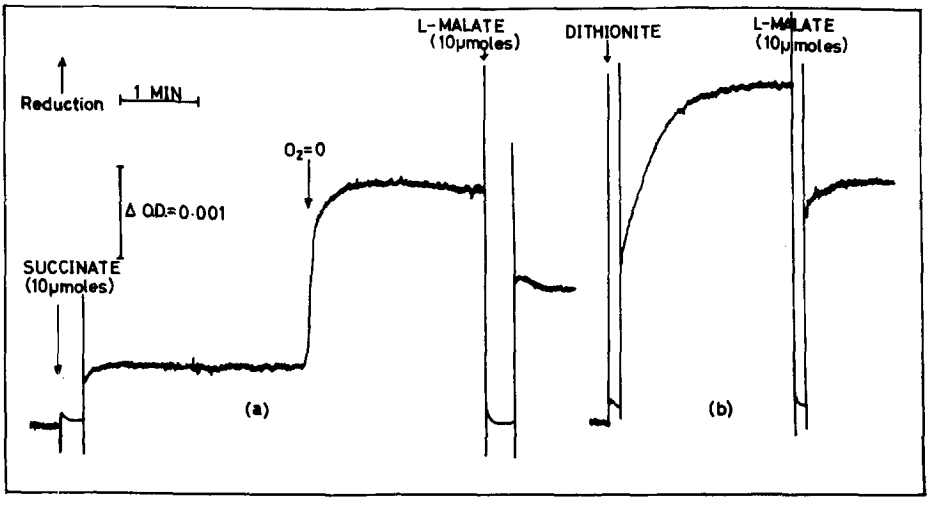

Fig. 1. Reduction of cytochrome b in Arum spadix mitochondria. The wavelength pair was 562-575mμ. Each cuvette contained initially in a volume of 3ml. :- 1.6mg. mitochondrial protein, 0.9mmoles mannitol, 30μmoles KCl, 15μmoles $MgCl_2$, and 30μmoles potassium phosphate buffer, pH 7.2. Other additions were as shown.

are due to a change in the redox state of cytochrome b rather than an artifact due to mitochondrial swelling or contraction can be seen from Fig. 2 which shows difference spectra for these two conditions. In both cases there appears to be a well-

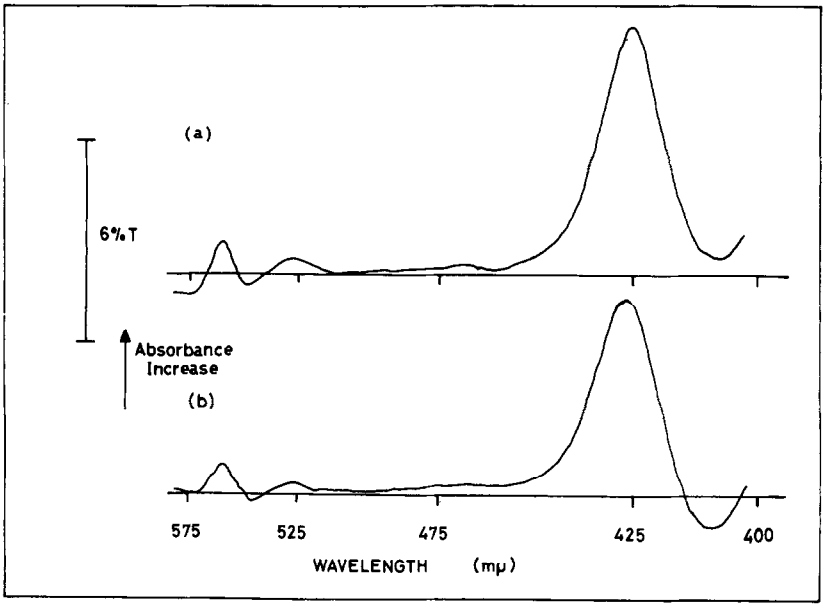

Fig. 2. Difference spectra for Arum spadix mitochondria. Each cuvette contained in a volume of 3ml. :- 1.6mg. mitochondrial protein, 0.9mmoles mannitol, 30μmoles KCl, 15μmoles $MgCl_2$ and 30μmoles potassium phosphate buffer, pH 7.2. In addition, (a) was anaerobic and contained 10μmoles succinate in the sample and 10μmoles succinate and 10μmoles L-malate in the reference. (b) contained dithionite in both sample and reference cells and 10μmoles L-malate in the reference.

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defined difference spectrum of the cytochrome b type, with an α-band peak at 559mμ and a γ-band peak at 427.5mμ. There is a weak β-band at 527mμ. None of the other substrates or potential substrates for these mitochondria which have so far been investigated could reproduce the effect of L-malate in causing a decrease in the degree of reduction of cytochrome b reduced either by succinate or dithionite. These included pyruvate (10 μmoles), citrate (10 μmoles) and NADH (1.0 μmoles) added in a similar manner to malate in Fig. 1.

Fig. 3 shows difference spectra of dithionite reduced

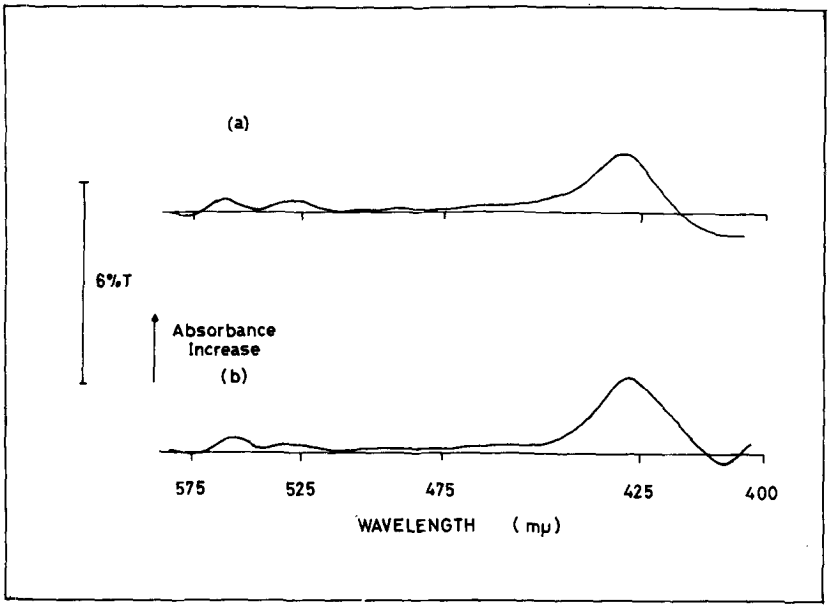

Fig. 3. (a) Difference spectrum for pea mitochondria. Each cell contained 1.9mg. mitochondrial protein in a volume of 3ml. Other experimental details, as in Fig. 2 b.
(b) Difference spectrum for mung bean mitochondria. Each cell contained in a volume of 3ml. 2.4mg. mitochondrial protein. Other experimental details as in Fig. 2b.

minus dithionite plus malate reduced mitochondria from peas and mung beans. In both cases the presence of L-malate causes a decrease in the amount of cytochrome b reducible by dithionite. In pea mitochondria the difference spectrum absorption maxima are 429mμ, 528mμ and 559mμ. For mung bean mitochondria, the absorption maxima of this component are at 426mμ and 558mμ, the β-band being too weak to locate with any degree of accuracy.

Tightly coupled rat liver mitochondria show a similar phenomenon. Fig. 4a shows that addition of L-malate to dithionite-reduced mitochondria causes a decrease in absorption at 562mμ relative to 575mμ. In this case the cytochrome b component which is non-reducible in the presence of L-malate

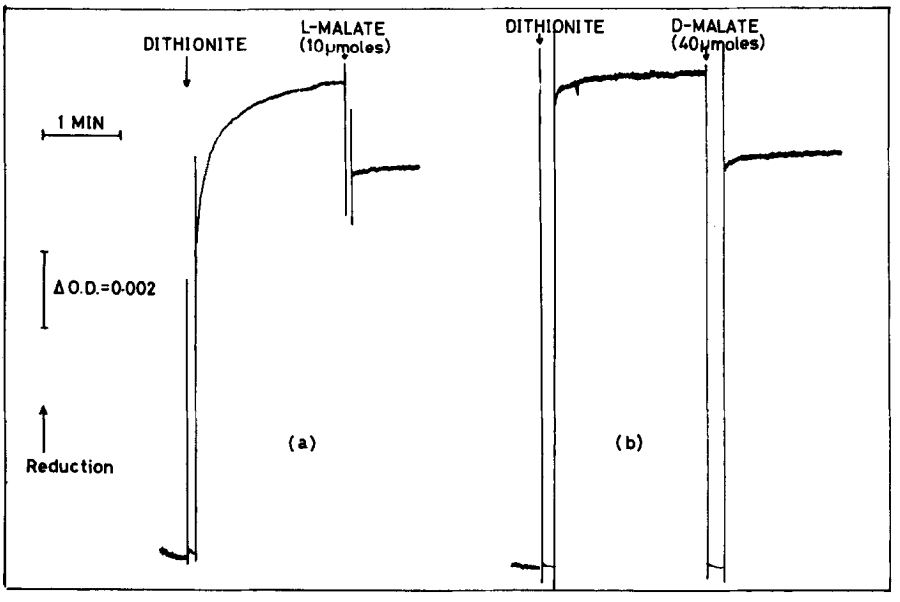

Fig. 4. Reduction of cytochrome b in rat liver mitochondria. The wavelength pair was 562-575mμ. In each experiment, the cell contained initially in a volume of 3ml. :- 4.5mg. mitochondrial protein, 240μmoles KCl, 15μmoles $MgCl_2$ and 60μmoles Tris-HCl buffer, pH 7.5. Subsequent additions were as shown.

accounts for approximately 20% of the total cytochrome b reducible by dithionite. The difference spectrum of dithionite reduced minus dithionite plus malate reduced mitochondria (Fig. 5a) once again shows a b-type cytochrome spectrum with absorption maxima at 427mμ and 558mμ. In this case, however, the situation is complicated by the appearance of a small shoulder on the γ-band at 440mμ (Fig. 5a, Curve 1). On incubation for 10 mins. at 30°C this shoulder disappears, leaving a symmetrical peak with a maximum at 427mμ (Fig. 5a, Curve 2). As in the case of Arum spadix mitochondria, addition of pyruvate (10 μmoles) or citrate (10 μmoles) caused no reoxidation of the dithionite-reduced cytochrome b. Reoxidation of a cytochrome b component can be achieved, however, by relatively large quantities (40 μmoles) of D-malate (Fig. 4b and 5b).

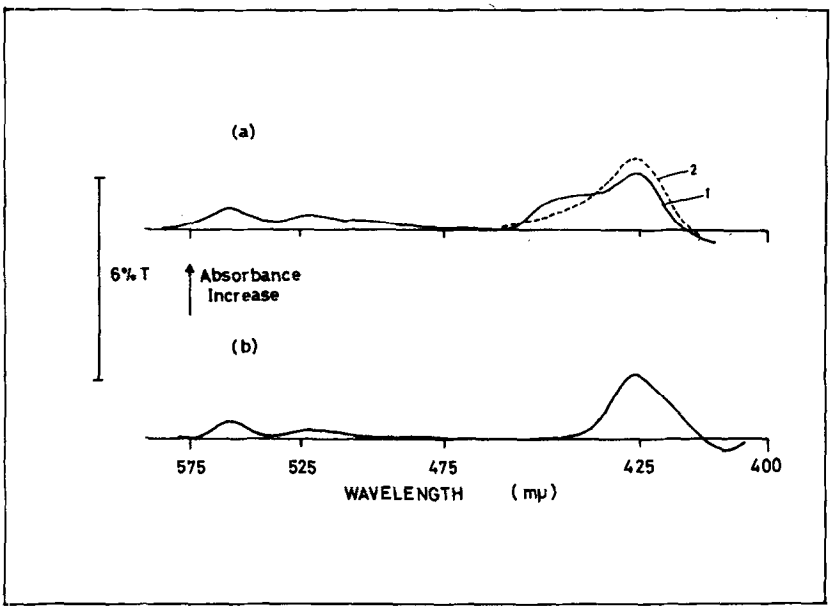

Fig. 5. Difference spectra for rat liver mitochondria. Each cell contained in a volume of 3ml. :- 9.0mg. mitochondrial protein, 240μmoles KCl, 15μmoles $MgCl_2$, and 60μmoles Tris-HCl buffer, pH 7.5. In addition, (a) contained dithionite in the sample cell and dithionite and 10μmoles L-malate in the reference. Curve (1), 2 mins after L-malate addition. Curve (2), 10 mins after L-malate addition. (b) contained dithionite in both sample and reference cells and 40μmoles of D-malate in the reference cell. Spectrum taken 2 mins after D-malate addition.

The mechanism of the reoxidation process in all these cases appears to be that a small quantity of air is introduced into the anaerobic cuvette during the addition of malate which causes a temporary reoxidation of the respiratory chain components. This oxygen is quickly exhausted, either by direct reaction with the excess of dithionite present or by respiration, and re-reduction of the respiratory chain takes place, except, in the presence of malate, for a component of the cytochrome b pool. Evidence for this comes from the observation that the effect can be much reduced by using deoxygenated malate solutions and gassing the spectrophotometer with nitrogen during malate addition.

Discussion

In all mitochondria examined so far, there appears to be an absorbing component of the cytochrome b type which is not reducible by the respiratory chain or by dithionite in the presence of malate. In rat liver the effect can be produced by D-malate, which is not utilised by malic dehydrogenase (Straub, 1942). A direct effect due to the oxidation of malate would therefore seem to be unlikely, but cannot be entirely ruled out due to the presence of a D-α-hydroxy acid oxidising enzyme in liver (Tubbs and Greville, 1961) which will utilise D-malate. Even if this enzyme is involved, it is difficult to account for this anomalous cytochrome b component remaining oxidised.

A possible explanation of the effect appears to be that it is connected with the passage of malate across the mitochondrial membrane. Chappell and Haarhof (1967) have shown that in rat liver mitochondria both D and L-malate can cross the membrane via a permease system, although such systems have not been well documented for plant mitochondria. If cytochrome b is involved in the utilisation of energy for driving transport processes, then a reversal of the energy requirement, i.e. by passage of malate down an electrochemical gradient, could alter the redox state of cytochrome b. Chance and Schoener (1966) observed a cytochrome b with similar spectral properties to the one described here, in pigeon heart mitochondria in which electron flow was minimal, when the mitochondria were in a high energy state. Tyler, Estabrook and Sanadi (1965) have described a similar pigment in submitochondrial particles using TMPD and ascorbate as substrate, when ATP is present. They suggest that reduction of this component takes place by reversed electron transport.

The observations described here, and those of Chance & Schoener and Tyler et al., suggest that at least part of the mitochondrial pool of cytochrome b is not on the direct electron transport pathway and may fulfil some other function.

Acknowledgements

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