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THE REDOX STATES OF RESPIRATORY-CHAIN COMPONENTS IN RAT-LIVER MITOCHONDRIA

III. 'CROSS-OVER POINTS' IN SITE III

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SUMMARY

1. When ascorbate is used as a substrate for rat-liver mitochondria in the presence of rotenone, antimycin and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or phenazine methosulphate, a cross-over point is seen between cytochrome a_3 and oxygen.

2. This is shifted to between cytochromes *c* and *a* or below cytochrome *c* on adding varying amounts of terminal respiratory inhibitors.

3. With low substrate concentrations, positive cross-over points were observed between cytochromes *c* and *a* and between cytochromes a_3 and oxygen, and a negative cross-over between cytochromes *a* and a_3.

4. The cross-over data are consistent with a mechanism for Site-III phosphorylation that involves a high-potential form of one carrier (maybe copper) and a low-potential form of a_3, analogous to the mechanism of Site-II phosphorylation previously proposed.

INTRODUCTION

In a previous paper[1], it was proposed that in well coupled mitochondria in State 4 (ref. 2), the respiratory chain approaches equilibrium with ADP, P_i and ATP. With succinate or ascorbate (in the presence of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)) as substrate, in the absence of respiratory inhibitors, all carriers, including cytochromes *a* and a_3, become more reduced on the transition from State 3 to State 4 (ref. 3). Similar results have been obtained with glycerol 1-phosphate[4]. It was concluded that the reaction between a_3 and oxygen is inhibited in State-4 mitochondria[3].

With succinate or NAD-linked substrates, the cross-over point[2] (*i.e.* the point between adjacent carriers of the respiratory chain where, on the transition from State 3 to State 4, the component on the substrate side becomes more reduced and that on

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulphate.

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the oxygen side more oxidized) moved towards substrate on adding the inhibitors of cytochrome oxidase, cyanide, azide or hydroxylamine (*cf.* ref. 5). Since the number of cross-over points located with different substrates and varying concentration of inhibitor exceeds the number of phosphorylation sites, it was concluded that a cross-over point does not necessarily identify a site of inhibition in State 4.

Since the interpretation of the cross-over phenomenon is complicated by the presence of more than one phosphorylation site, and therefore of more than one site at which respiration is controlled in State 4, it was decided to examine the phenomenon in more detail in a system in which only one phosphorylation site is present, namely rat-liver mitochondria oxidizing ascorbate in the presence of TMPD, antimycin and rotenone.

RESULTS

Effect of respiratory inhibitors

As previously reported[3], all components of the respiratory chain become more reduced on the transition from State 3 to State 4 of rat-liver mitochondria oxidizing ascorbate in the presence of TMPD. In the presence of 0.2–1.0 mM azide, a cross-over between *c* and *a* is observed (Fig. 1). In the presence of rotenone and antimycin, similar results were obtained with 0.2 and 0.5 mM azide, but with 1.0 mM azide all three cytochromes concerned in the reaction between TMPD and oxygen become more oxidized on the transition from State 3 to State 4 (Fig. 2). Similar results were obtained when phenazine methosulphate (PMS) (1.3 μM) was used instead of TMPD as electron carrier between ascorbate and cytochrome *c*.

Cyanide, in a concentration between 5 and 60 μM (Fig. 3), and 0.5–7.5 mM hydroxylamine (Fig. 4) also shifted the cross-over to between cytochromes *c* and *a*. Sulphide (Fig. 5) behaved differently. Up to 60 μM had no effect on the cross-over.

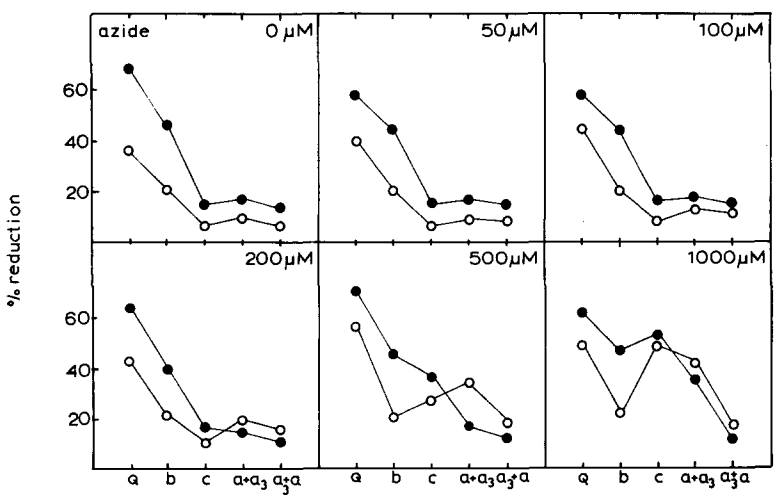


Fig. 1. Redox state of the respiratory chain of rat-liver mitochondria in State 3 and State 4 with TMPD and ascorbate as substrate, in the presence of increasing concentrations of azide. 7.2 mg/ml protein for assay of Q, 1.3 mg/ml protein for assay of cytochromes. 0.1 mM TMPD, 10 mM ascorbate, 0.1 mM ADP (except at 0.5 and 1.0 mM azide when a concentration of 40 μM was used), 10 mM P_i and 0.2 μg rotenone per mg protein. ●—●, State 4; ○—○, State 3.

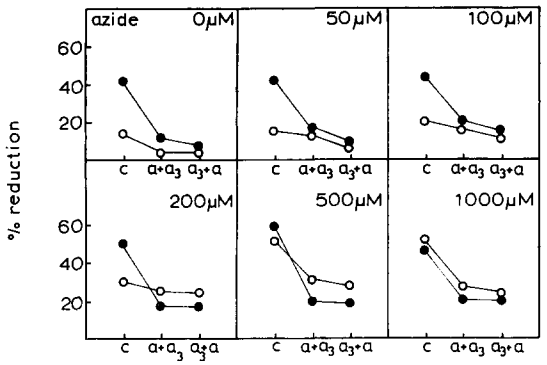


Fig. 2. Effect of increasing amounts of azide on Site III of the respiratory chain in State 3 and State 4. 0.1 mM TMPD, 10 mM ascorbate, 0.2 μg rotenone and 0.05 μg antimycin per mg protein, 10 mM P_i and 0.1 mM ADP (except with 500 and 1000 μM azide, when a concentration of 40 μM ADP was used), 1.2 mg/ml protein. ●—●, State 4; ○—○, State 3.

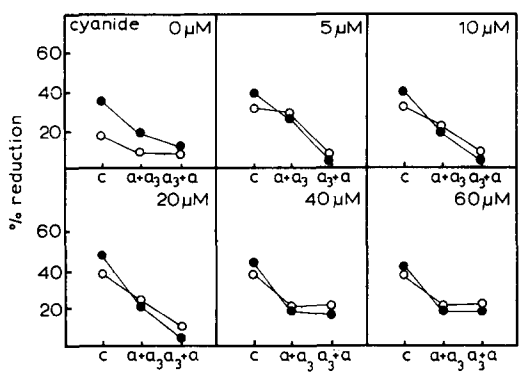


Fig. 3. Effect of increasing amounts of cyanide on the redox state of Site III of the respiratory chain in State 3 and State 4. 0.1 mM TMPD, 10 mM ascorbate, 10 mM P_i, 0.1 mM ADP, 0.2 μg rotenone and 0.05 μg antimycin per mg protein, 0.9 mg/ml protein. ●—●, State 4; ○—○, State 3.

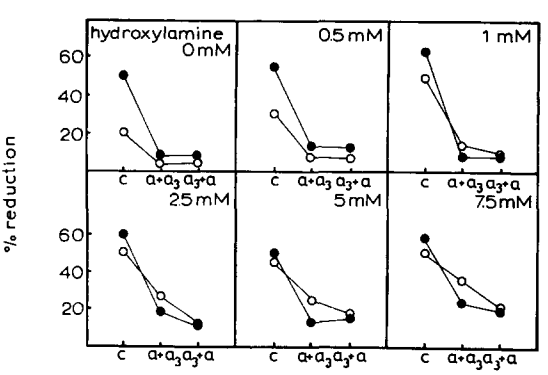


Fig. 4. Effect of increasing amounts of hydroxylamine on the redox state of Site III of the respiratory chain in State 3 and State 4. 1.3 μM PMS, 10 mM ascorbate. Other conditions as in Fig. 3. 1.3 mg/ml protein. ●—●, State 4; ○—○, State 3.

At 80–100 μM, however, all three cytochromes became more oxidized on the transition from State 3 to State 4.

When, in the absence of inhibitors, the concentration of TMPD is lowered to 25–50 μM a cross-over point appears between cytochromes *c* and *a*, with a 'negative' cross-over point[6] between cytochromes *a* and a_3 (Fig. 6). At all concentrations of TMPD, a_3 becomes more reduced in State 4. With 0.1–0.2 μM PMS, results were obtained similar to those found with 25–50 μM TMPD.

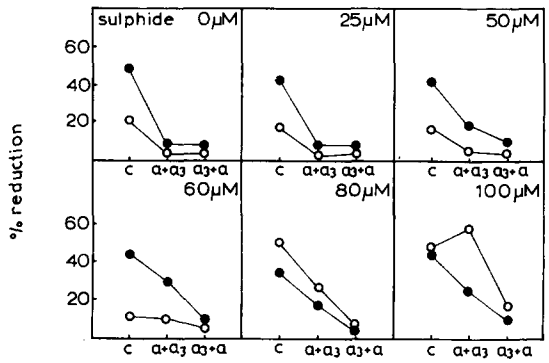


Fig. 5. Effect of increasing amounts of sulphide on the redox state of Site III of the respiratory chain in State 3 and State 4. 1.3 μM PMS, 10 mM ascorbate. Other conditions as in Fig. 3, except that with 80 μM and 100 μM sulphide a concentration of 40 μM ADP was used. 1.2 mg/ml protein. ●—●, State 4; ○—○, State 3.

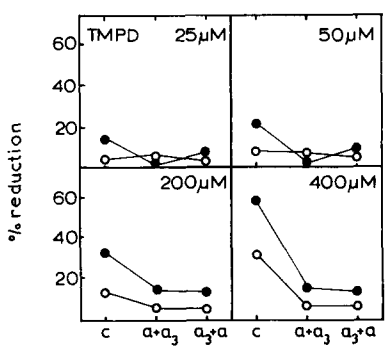


Fig. 6. Effect of varying concentration of TMPD on the redox state of Site III of the respiratory chain in State 3 and State 4. 10 mM ascorbate. Additions as in Fig. 3. 1.4 mg/ml protein. ●—●, State 4; ○—○, State 3.

Absorption spectra of cytochromes reduced on transition from State 3 to State 4

Fig. 7 shows that the components reduced on the transition from State 3 to State 4 of mitochondria respiring with ascorbate and 0.1 mM TMPD have absorption peaks at 550 nm (reference wavelength at 540 nm), 605 nm (reference wavelength at 590 nm) and 445 nm (reference wavelength 455 nm), characteristic of cytochromes *c*, *a*, and aa_3, respectively.

Fig. 8 shows absorption peaks of cytochromes *a* (A), aa_3 (B) and *a* (C) oxidized on the transition from State 3 to State 4 in the presence of 0.2 mM azide (Figs. 8A and 8B) and with low concentrations of TMPD (Fig. 8C).

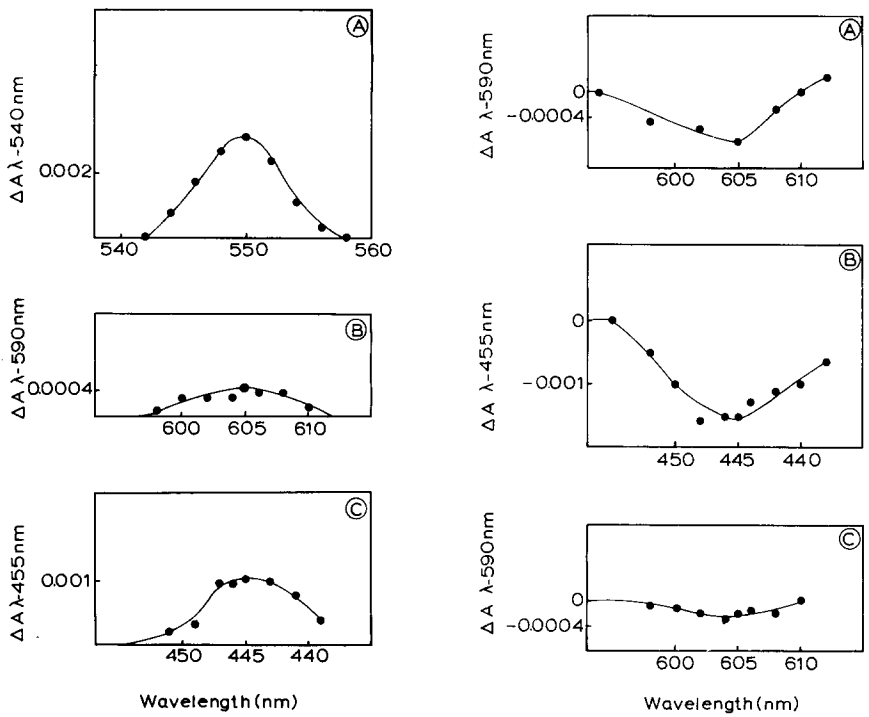


Fig. 7. State 4 *minus* State 3 difference spectra of cytochromes in Site III of the respiratory chain. For each wavelength separate traces were made. 0.1 mM TMPD, 10 mM ascorbate. Further additions as in Fig. 3. A. The spectrum of cytochrome $c + c_1$ with 540 nm as reference wavelength. 1.1 mg/ml protein. B. The spectrum of cytochrome aa_3 with 590 nm as reference wavelength. 1.1 mg/ml protein. C. The spectrum of cytochrome aa_3 with 455 nm as reference wavelength. 1.3 mg/ml protein.

Fig. 8. State 4 *minus* State 3 difference spectra of cytochromes in Site III of the respiratory chain. Conditions as in Fig. 3. A. The spectrum of cytochrome aa_3 with 590 nm as reference wavelength, in presence of 200 μM azide, 0.1 mM TMPD, 10 mM ascorbate, 0.1 mM ADP and 10 mM P_i. 1.9 mg/ml protein. B. The spectrum of cytochrome aa_3 with 455 nm as reference wavelength under the same conditions as spectrum A. 1.0 mg/ml protein. C. The spectrum of cytochrome aa_3 with 590 nm as reference wavelength in presence of 40 μM TMPD, 10 mM ascorbate, 0.1 mM ATP and 10 mM P_i. 1.7 mg/ml protein.

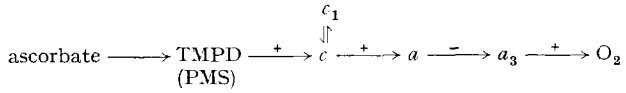
Fig. 9 shows the absorption peaks of cytochrome *c* oxidized on the transition from State 3 to State 4 in the presence of 0.55 mM azide (A) and 80 μM sulphide (B).

Fig. 10, a spectrum at the temperature of liquid nitrogen, shows that the *c* band that disappears on the transition from State 3 to State 4 in the presence of 0.55 mM azide is symmetrical, indicating that cytochrome c_1 makes little contribution to the spectral changes. The oxidation of cytochrome *a* is also clearly seen in Fig. 10.

DISCUSSION

Since all experiments in this paper, except that described in Fig. 1, were carried out in the presence of rotenone and antimycin, Site III was isolated from the rest of

the respiratory chain, and we may represent the path of electron transfer schematically



The positive signs indicate where we have demonstrated positive cross-over points, and the negative sign the site of a negative cross-over. The demonstration of three positive points in a segment of the chain in which there is only one phosphorylation site is an extreme demonstration of the thesis[3,7] that the cross-over point need not be identical with a phosphorylation site.

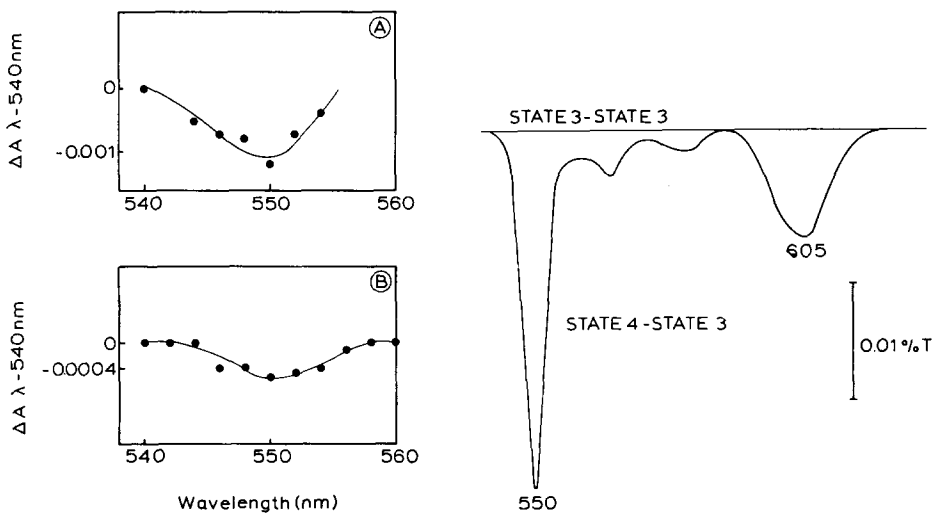


Fig. 9. State 4 *minus* State 3 difference spectra of cytochromes $c + c_1$ under conditions that a cross-over point arises between this cytochrome and substrate. A. 0.1 mM TMPD, 10 mM ascorbate, 30 μM ADP, 10 mM P_i and 550 μM azide. 1.7 mg/ml protein. B. 0.1 mM TMPD, 10 mM ascorbate, 30 μM ADP, 10 mM P_i and 80 μM sulphide. 0.8 mg/ml protein.

Fig. 10. Low-temperature spectrum of the α bands of cytochromes c, c_1 and aa_3. Same reaction medium as in Fig. 9A. 3 mg/ml protein.

In our previous paper[3], we have concluded that the reaction between cytochrome a_3 and O_2 is the site of inhibition in State 4 mitochondria, in the absence of respiratory inhibitor. This may be because a low-potential form of cytochrome a_3 accumulates in energized mitochondria[8]. In the presence of respiratory inhibitors that, more drastically than the transition from State 3 to State 4, inhibit the reaction of a_3 with oxygen, additional inhibitory sites, even between substrate and cytochrome c become manifest. The most direct interpretation of a cross-over between substrate and c would be that a high-energy form of cytochrome c is present in energized mitochondria. Since, however, no effect on the energy state of the redox potential of cytochrome c has been detected[9], this is unlikely.

An alternative explanation is that cytochrome c is brought into equilibrium

with a high-potential species (*e.g.* copper) in energized mitochondria, by mediation of TMPD, thus:

$$\begin{aligned} X_{ox} + ATP &\leftrightharpoons X_{ox}\sim + ADP + P_i \\ X_{ox}\sim + TMPD &\leftrightharpoons X_{red} + TMPD_{ox} \\ TMPD_{ox} + c^{2+} &\leftrightharpoons TMPD + c^{3+} \\ \hline \text{Sum: } X_{ox} + c^{2+} + ATP &\leftrightharpoons X_{red} + c^{3+} + ADP + P_i \end{aligned} \quad (1)$$

Thus the transition from the steady state (State 3) to equilibrium (State 4)[1] would be accompanied by oxidation of cytochrome *c*, and also by oxidation of cytochromes *a* and a_3 which are in equilibrium with cytochrome *c*. This explanation implies that the high-potential species cannot be cytochrome *a*.

In the absence of respiratory inhibitor, the ATP-induced reduction of cytochrome a_3, with consequent reduction of cytochromes *a* and *c*, dominates over the TMPD-mediated reaction proposed.

The cross-overs between TMPD and cytochrome *c* and between cytochrome a_3 and oxygen are consistent with a mechanism for Site-III phosphorylation that involves a high-potential form of one carrier (maybe copper) and a low-potential form of a_3, analogous to the mechanism of Site-II phosphorylation that has recently been proposed[10]. The cross-over between cytochromes *c* and *a* observed at intermediate concentrations of respiratory inhibitor remains, however, unexplained.

METHODS

Rat-liver mitochondria were isolated as described by MYERS AND SLATER[11]. Protein was determined by the biuret method as described by CLELAND AND SLATER[12].

All experiments were carried out at room temperature in a reaction medium containing 50 mM Tris–HCl buffer, 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$ and 60 mM sucrose. Other components were added as described in the legends of the figures. The final volume was 3.0 ml and the pH 7.4.

The degree of reduction was determined in an Aminco-Chance dual-wavelength spectrophotometer. For cytochromes the following wavelength pairs were chosen: 430 *minus* 410 nm for cytochrome *b*, 550 *minus* 540 nm for cytochromes cc_1, 605 *minus* 590 nm for cytochromes $a + a_3$ and 445 *minus* 455 nm for cytochromes $a_3 + a$. The absorbance at these wavelengths was measured in the sequence State 2 → 3 → 4 → anaerobic[2]. In calculating the percentage of reduction in State 3 and State 4 it was assumed that the cytochromes in State 2 are fully oxidized and after addition of dithionite are fully reduced.

Split-beam spectra were measured in the same spectrophotometer with use of the low-temperature accessory.

The degree of reduction of ubiquinone was determined by the extraction method of KRÖGER AND KLINGENBERG[13].

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