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

I. EFFECT OF VARYING SUBSTRATE CONCENTRATION AND OF AZIDE

S. MURAOKA* AND E. C. SLATER

*Laboratory of Biochemistry, B. C. P. Jansen Institute**, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. Increasing amounts of substrate cause an increasing degree of reduction of nicotinamide nucleotides, cytochrome *b* and cytochrome *c* in rat-liver mitochondria in the presence of ADP (State 3). In State 4 (after consumption of ADP), however, the redox state is only slightly changed by varying the substrate concentration in the same range.

2. Increasing concentrations of azide cause increasing reduction of cytochromes *a*, *b* and *c* in State 3, but have no effect on the redox state in State 4, except at high concentration.

3. Oligomycin has no effect on the degree of reduction of NAD in State 4 with succinate, and only slightly increases it with NAD-linked substrates.

4. The effect of uncoupling on the redox state of the respiratory chain is best studied under conditions in which uncoupling and competition between uncoupler and substrate do not cause the redox state to move in the same direction. This is conveniently achieved by adding azide. Under these conditions, uncoupling leads to an increased reduction of cytochrome *c*, whereas competition for penetration of substrate leads to an increased oxidation.

5. It is concluded that the redox state of an electron carrier in State 3 represents a kinetic steady state governed by the relative activities of those portions of the chain responsible for reduction and oxidation of the carrier. In State 4, however, the respiratory chain approaches thermodynamic equilibrium with ADP, ATP and P_i.

INTRODUCTION

The requirement for ADP for maximal respiration by isolated mitochondria has been known since the pioneer studies of LARDY AND WELLMAN[1] in 1952. CHANCE AND WILLIAMS[2] showed that the rate of respiration declines sharply when ADP, added to a phosphate-containing suspension medium, is consumed by being phospho-

* Present address: Department of Microbial Chemistry, Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima, Japan.

** Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

rylated to ATP. This phenomenon is usually described under the name respiratory control. Mitochondria respiring in the presence of substrate, oxygen, ADP and P_i are said to be in State 3, and in State 4 when the added ADP is consumed.

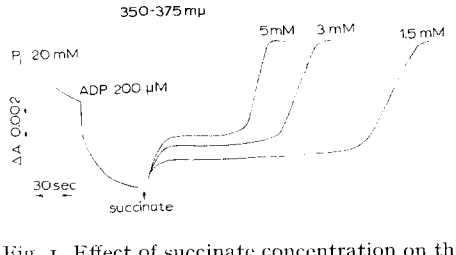
SLATER[3] pointed out in 1953 that there were two possible explanations for the respiratory control observed by LARDY AND WELLMAN[1], *viz.* (1) the ADP is completely phosphorylated so that oxidation fails for stoicheometric reasons since ADP is an essential requirement for the oxidation; (2) some of the phosphorylation reactions are readily reversible and the back reaction becomes important when [ATP]/[ADP] is high. For convenience, we shall refer to these two explanations as the kinetic and the thermodynamic, respectively. CHANCE AND WILLIAMS[2,4] subsequently emphasized the kinetic explanation, whereas KLINGENBERG AND SCHOLLMEYER[5] proposed that both respiratory control and reversal of the respiratory chain[6,7] are due to a thermodynamic equilibrium between redox and phosphorylation reactions at the first two phosphorylation sites.

In the present investigation, the redox state of respiratory-chain components has been measured, both in State 3 and State 4, under conditions in which the flow of electrons along the respiratory chain has been varied by changing the substrate concentration and by the addition of inhibitors.

RESULTS

Effect of varying succinate concentration

Fig. 1 shows the effect of varying the concentration of succinate on the redox state of the nicotinamide nucleotides in States 3 and 4. In the following paper[8], it will be shown that, under these conditions, NADP is completely reduced in both State 3 and 4, so that an increased absorbance at 350 *minus* 375 mμ reflects an increased reduction of NAD. It is clear that an increasing concentration of succinate causes an increasing degree of reduction of NAD in State 3. However, the degree of reduction in State 4 is largely independent of substrate concentration. Similar results were obtained with cytochrome *c* (see Fig. 3A) and cytochrome *b*, and also using glutamate or β-hydroxybutyrate as substrate. The concentration of succinate giving



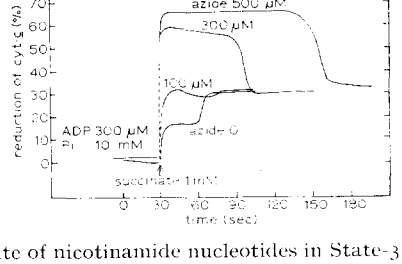


Fig. 1. Effect of succinate concentration on the redox state of nicotinamide nucleotides in State-3 and State-4 rat-liver mitochondria. Succinate added in State 2, induced by the addition of 20 mM P_i and 200 μM ADP, yields first State 3 and then State 4. 0.7 mg/ml mitochondria.

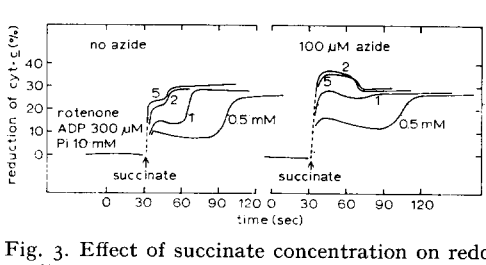
Fig. 2. Effect of azide concentration on the redox state of cytochrome *c* in State-3 and State-4 rat-liver mitochondria. Succinate added in State 2, obtained by adding 0.3 mM ADP and 10 mM P_i, yields first State 3 and then State 4. 100% reduction was determined by the absorbance difference between State 2 and the anaerobic state measured with $Na_2S_2O_4$. 0.1 μg/ml rotenone was present. 1.8 mg/ml mitochondria.

half-maximal reduction of NAD in State 3 was 1.5–3 mM, about the same as the K_m found for succinate oxidation under these conditions.

Effect of azide

CHANCE AND WILLIAMS[9] used azide to magnify the changes in the redox states of the cytochromes occurring on the transition from State 4 to 3. Azide inhibits cytochrome oxidase[10–13], uncouples[14–17] and inhibits energy transfer[18–22]. Fig. 2 shows the effect of various concentrations of azide on the redox state of cytochrome *c* in States 3 and 4, with a low concentration of succinate (1 mM). In the absence of azide, cytochrome *c* becomes more reduced when the system proceeds from State 3 to State 4. The inhibitory effect of azide on the rate of phosphorylation of the ADP is revealed by the longer period required before State 4 is reached. With increasing concentrations of azide, cytochrome *c* becomes increasingly reduced in State 3, but the redox state in State 4 is unaffected by azide*. In consequence, cytochrome *c* becomes more oxidized on the transition from State 3 to State 4, with concentrations of azide greater than 100 μM. Similar results were obtained with cytochrome *a* (with succinate as substrate) and with cytochrome *b* (with β-hydroxybutyrate as substrate). Since the effect of 0.1–0.5 mM azide is quite different from that of oligomycin or uncouplers described below, it is presumably due to inhibition of cytochrome oxidase, rather than to inhibition of energy-transfer reactions or to uncoupling. This conclusion is supported by the fact that other inhibitors of cytochrome oxidase, *viz.* cyanide[8] and hydroxylamine[23], gave similar results.

Fig. 3 shows that the degree of reduction of cytochrome *c* in State 3, in the presence and absence of azide, is dependent upon the concentration of succinate, being lower with the smaller succinate concentrations. The degree of reduction in State 4 is again largely independent of succinate concentration, increasing only slightly with a 10-fold increase in succinate concentration.



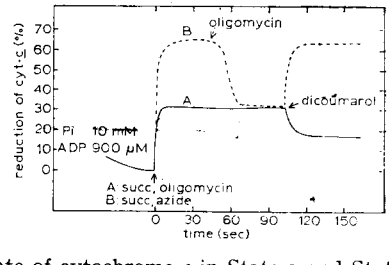


Fig. 3. Effect of succinate concentration on redox state of cytochrome *c* in State-3 and State-4 rat-liver mitochondria in the presence and absence of azide. Procedure as in Fig. 2. Concentrations of succinate (mM) are indicated against the trace. 0.1 μg/ml rotenone was present. 1.8 mg/ml mitochondria.

Fig. 4. Effect of dicoumarol on the redox state of cytochrome *c* in the presence of oligomycin with and without azide. Curve A: in the absence of azide, 10 mM succinate (succ) and 3 μg oligomycin added to State-2 rat-liver mitochondria (obtained with 0.9 mM ADP and 10 mM P_i) induce a reduction of cytochrome *c* and then addition of 50 μM dicoumarol results in an oxidation. Curve B: in the presence of 300 μM azide, 10 mM succinate induces a large reduction, and then 3 μg oligomycin induces an oxidation to the same level as Curve A before adding dicoumarol, while finally 50 μM dicoumarol gives a reduction of cytochrome *c*. 0.1 μg/ml rotenone was present. 1.8 mg/ml rat-liver mitochondria.

* With higher concentrations of azide, cytochrome *c* becomes more reduced in State 4. This is probably due to uncoupling.

Effect of oligomycin and uncouplers

When oligomycin is added in State 3, the redox state of the carriers rapidly changes to a level approaching that characteristic of State 4 (see Curve B of Fig. 4). With NAD-linked substrates, however, the degree of reduction of nicotinamide nucleotides after addition of oligomycin in State 3 is somewhat more than that obtained in State 4, and the addition of oligomycin in State 4 causes a small further reduction. Further reduction is obtained on addition of succinate. Oligomycin has no effect on the redox state in State 4 with succinate.

Uncouplers added to mitochondria in State 4 or inhibited by oligomycin change the redox state of the carriers towards a level approaching that of State 3. Using uninhibited mitochondria, it is difficult to differentiate between a true uncoupling effect and inhibition of penetration of substrate into the mitochondria[24,25], since both effects lead to oxidation of most of the carriers. Fig. 4, Curve A, illustrates this for cytochrome *c* and 50 μM dicoumarol. In the presence of azide, however, dicoumarol causes increased reduction of cytochrome *c* in oligomycin-inhibited mitochondria (Curve B). Clearly, at this concentration, the uncoupling activity of dicoumarol predominates over its inhibitory effect.

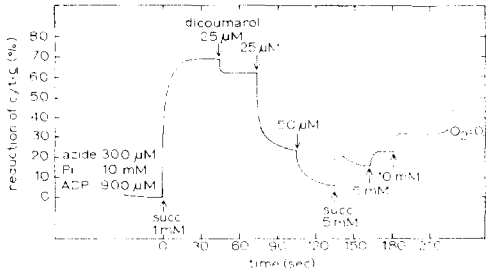


Fig. 5. Effect of different concentrations of dicoumarol and succinate on the redox state of cytochrome *c* in State-3 rat-liver mitochondria in the presence of azide. Succinate (succ) added in State 2 (obtained with 0.9 mM ADP and 10 mM P_i) in the presence of 0.9 mM azide yields a large reduction of cytochrome *c*, and increasing amounts of dicoumarol induce increasing oxidation after which the addition of increasing concentrations of succinate induces reduction. 0.1 μg/ml rotenone was present. 1.8 mg/ml mitochondria.

Fig. 5 illustrates the antagonistic effects of dicoumarol and succinate on the redox state of cytochrome *c* on azide-inhibited mitochondria in State 3. This antagonism is probably at the level of the mitochondrial inner membrane[24,25].

DISCUSSION

The effect of concentration of ADP, oxidizable substrate, uncouplers, and inhibitors of energy transfer and of electron transport on the redox state of respiratory carriers can best be discussed in terms of a simplified version of the respiratory chain, in which only one phosphorylation site is incorporated. This may be represented

$$SH_2 \longrightarrow A \rightleftharpoons B \longrightarrow O_2$$

where A → B is the phosphorylating site. This will be extended to multiple phosphorylating sites in the following paper.

Since State-3 respiration proceeds at a constant rate despite a continually increasing [ATP]/[ADP] ratio, it is clear that the redox state of the carriers in State 3 is not under the influence of the equilibrium of the phosphorylating reaction

$$AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP + H_2O \quad (1)$$

It is affected only by the activities of the reactions

$$SH_2 + A \longrightarrow S + AH_2 \quad (2)$$

and

$$BH_2 + \tfrac{1}{2}O_2 \longrightarrow B + H_2O \quad (3)$$

Thus, inhibition of Reaction 3 by azide, cyanide or hydroxylamine causes an increased redox state of the carriers, whereas slowing of Reaction 2 by lowering the substrate concentration causes the carriers to be less reduced. In other words, as already clearly demonstrated by KRÖGER AHD KLINGENBERG[26] for uncoupled respiration, the redox state of the carriers in State 3 represents a kinetic steady state governed by the relative activities of those portions of the respiratory chain responsible for reduction and oxidation of the carrier.

In State 4, however, the redox state of the carriers is independent of azide concentration and is only slightly dependent on the succinate concentration. It follows that the redox state in State 4 is governed by the reversible reaction (Eqn. 1). In other words, we conclude, in agreement with KLINGENBERG AND SCHOLLMEYER[5], that in State 4 the respiratory chain is near thermodynamic equilibrium with ADP, ATP and P_i, and that the transition from State 3 to State 4 is a transition from a kinetic steady state towards thermodynamic equilibrium (see also refs. 27 and 28). Indeed our results indicate that not only the first two sites are near thermodynamic equilibrium in State 4, as proposed by KLINGENBERG AND SCHOLLMEYER[5], but also the third site. Complete thermodynamic equilibrium is not reached, as is indicated by the slow residual respiration in State 4. This is probably due to slow side reactions leading to dissipation of the energy, or for its utilization in reactions other than the synthesis of ATP.

Since oligomycin prevents the reaction of a high-energy intermediate (*e.g.* $A \sim C$ (ref. 29)) or a high-energy state with ADP and P_i, the only slight effect of oligomycin (none with succinate as substrate) on the redox state suggests that the energy content of this intermediate or state must be rather close to the "phosphate potential", measured in State 4, *viz.* 15.6 (ref. 30)–15.8 (refs. 27 and 28) kcal/mole.

EXPERIMENTAL

Rat-liver mitochondria were isolated by the method of HOGEBOOM[31] as described by MYERS AND SLATER[32]. Protein was determined by the biuret method as described by CLELAND AND SLATER[33]. The degree of reduction of components of the respiratory chain was measured in an Aminco-Chance dual-wavelength spectrophotometer as described in the following paper[8]. All reactions were carried out at 25° in a reaction mixture containing 25 mM Tris–chloride buffer, 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl. Other components are indicated in the legends to the figures. The final volume was 3 ml and the pH 7.4.

Oligomycin, kindly supplied by Upjohn Chemical Co., and rotenone obtained from S. B. Penick and Co. were added in ethanol. Sodium azide was obtained from British Drug Houses.

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