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THE KINETICS OF CHANGES IN THE REDOX STATE OF UBIQUINONE ON THE TRANSITION FROM STATE 4 TO STATE 3 IN RAT-LIVER MITOCHONDRIA

R. D. VELDSEMA-CURRIE AND E. C. SLATER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)

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SUMMARY

1. When ADP is added to State-4 mitochondria at 10°, with β-hydroxybutyrate or succinate as substrate, Q becomes more reduced in the first 4 sec, followed by a slower oxidation.

2. The biphasic kinetics were not found when the energized state of the mitochondria was discharged by adding an uncoupler, instead of ADP.

3. It is suggested that phosphorylation Site III requires a higher concentration of ADP than the other sites.

INTRODUCTION

The synthesis of ATP on the addition of ADP to mitochondria or digitonin particles in State 4 (respiration limited by ADP concentration)[1] is biphasic, a rapid phase lasting about 15 sec at 10° being followed by a slower steady state lasting several minutes[2-4]. The rapid phase, originally believed to be due to the synthesis of ATP by reaction of P_i and ADP with high-energy intermediates of oxidative phosphorylation accumulated in State 4 (refs. 2 and 3), was shown by VAN DAM[4] to be accounted for by the rapid change in the steady-state redox level of NAD that occurs on the transition from State 4 to State 3 (respiration limited by activity of phosphorylating respiratory chain). Over the whole period of the rapid phase, $\Delta ATP:\Delta NAD^+:-\Delta O$ was close to the expected 3:1:1. (The Δ refers to the change above that due to the steady-state respiration.) The initial rate of ATP synthesis, however, was considerably less than that expected from the rate of NADH oxidation. VAN DAM[4] suggested as an explanation of this discrepancy that, during the early states of the reaction, some of the reducing equivalents from the NADH are temporarily stored in the pool of ubiquinone.

In this paper, evidence is presented supporting, by direct analysis, VAN DAM's suggestion. Immediately after the addition of ADP, the ubiquinone becomes more reduced followed, in less than 5 sec, by a slow oxidation. Factors affecting these kinetics have been investigated.

RESULTS

Fig. 1 shows the kinetics of the change in redox state of NAD, NADP and Q on the addition of ADP to State-4 mitochondria, preincubated exactly under the conditions used by VAN DAM[4]. In agreement with his results, NAD becomes rapidly more oxidized, whereas the change in redox state of NADP is much slower. In agreement with his prediction, Q becomes more reduced in the first 4 sec, followed by a slower oxidation. The biphasic Q kinetics were also found when arsenate replaced phosphate in the reaction mixture. Similar kinetics of Q reduction and oxidation were observed with succinate as substrate, in the presence of rotenone, under otherwise identical conditions (Fig. 2). Owing to the presence of rotenone, NAD did not become more oxidized.

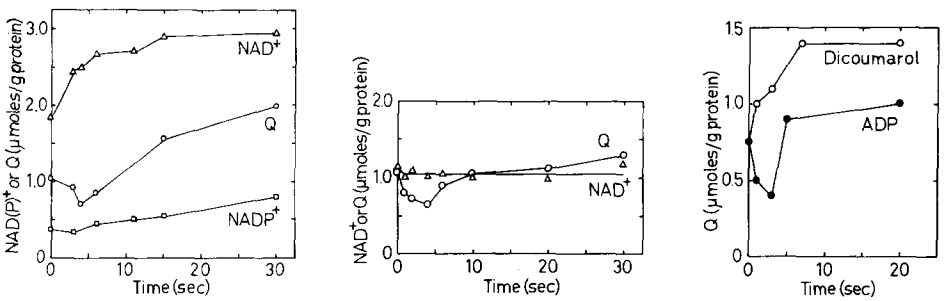

Fig. 1. Kinetics of changes of redox state of NAD, NADP, and Q on addition of ADP to State-4 mitochondria with β-hydroxybutyrate as substrate. Rat-liver mitochondria (18.8 mg protein/ml) were preincubated for 5 min at 10° in 0.45 ml of a solution containing 100 μmoles glycylglycine buffer (pH 7.4), 5 μmoles KCl, 6 μmoles KF, 3 μmoles $MgCl_2$, 25 μmoles sucrose, 5 μmoles sodium β-hydroxybutyrate and 5 μmoles potassium phosphate. At zero time, 0.05 ml 50 mM ADP was added.

Fig. 2. Kinetics of changes of redox state of NAD and Q on addition of ADP to State-4 mitochondria with succinate as substrate. Reaction conditions as in Fig. 1, except that 5 μmoles of succinate were used in place of β-hydroxybutyrate, the protein concentration was 12.2 mg/ml and 0.33 μg rotenone/mg protein was present.

Fig. 3. Kinetics of change in the redox state of Q on addition of dicoumarol or ADP to mitochondria in State 4 with succinate as substrate. Rat-liver mitochondria (10.9 mg protein/ml) were preincubated for 3 min at 10° in 0.45 ml of a solution containing 12.5 μmoles Tris chloride, 150 μmoles sucrose, 1 μmole EDTA, 15 μmoles KCl, 2.5 μmoles Sørensen phosphate buffer, 1 μg rotenone and 5 μmoles Tris succinate. The pH was 7.4. At zero time, 0.05 ml 50 mM ADP or 0.4 mM dicoumarol (pH 9.6) was added.

These experiments were carried out in a reaction mixture containing glycylglycine buffer, fluoride and magnesium, because this was used originally by SCHACHINGER *et al.*[2] and VAN DAM[4]. Similar results were obtained with succinate using the hypotonic reaction mixture used by MURAOKA AND SLATER[5], that contains no fluoride, and in a medium made isotonic with sucrose and free from Mg^{2+} (Fig. 3).

The biphasic kinetics of Q oxidation were not found when the energized state of the mitochondria was discharged by adding an uncoupler (dicoumarol) instead of ADP, as is shown in Fig. 3. Similar results were obtained with Ca^{2+} or valinomycin.

The biphasic kinetics were not found when P_i was added to mitochondria preincubated in the presence of ADP, nor when ADP or a mixture of ADP and P_i was added to mitochondria preincubated with substrate. Similarly, they were

not observed when substrate + ADP were added to mitochondria preincubated with P_i, nor when ADP was added to mitochondria preincubated with substrate, P_i and 1 mM ATP.

DISCUSSION

In rat-liver mitochondria in State 4, the redox reactions are near to equilibrium with the ATP, ADP and P_i system[5]. On the addition of ADP all the components become more oxidized when succinate is substrate[5] and all except cytochrome *a* become more oxidized when β-hydroxybutyrate is substrate[6,7]. As predicted by VAN DAM[4], however, Q becomes more reduced before it becomes more oxidized. It appears, then, that the reduction of Q by NADH can proceed more rapidly than the oxidation of QH_2 by oxygen. In other words, the 'pool' of Q (ref. 8) initially receives hydrogen atoms more rapidly than it delivers them, despite the fact that it is more oxidized in the steady-state State 3 than in the equilibrium State 4.

The Q 'pool' becomes temporarily more reduced even with succinate as substrate in the presence of rotenone, when the redox state of NAD is unchanged on the addition of ADP. In this case, the reducing equivalents must be supplied by the succinate. The kinetics of the initial burst of ATP synthesis could not be measured by VAN DAM[4] with succinate as substrate.

MURAOKA AND SLATER[6] have reported five separate 'cross-over points'[7] (four positive and one negative*) in rat-liver mitochondria on the transition from State 3 to State 4 under different conditions. The present kinetic studies have identified positive 'cross-over points' between NADH and Q, and succinate and Q. The first of these may be the same as that between NADH and flavoprotein previously identified[6,7], the latter is a new positive cross-over point.

It is significant that the biphasic Q kinetics are found only when the energized state is discharged with ADP. HELDT[9] and KEMP *et al.*[10] have shown that the rate-limiting reaction under these conditions is the translocation of ADP into the mitochondria, in exchange for ATP. Our results would be explained if Site III requires a higher concentration of ADP (or a higher [ADP]/[ATP] ratio) than the other sites. This is perhaps understandable on the basis of the chemical hypothesis[11] of respiratory-chain phosphorylation in which the oxidation of respiratory-chain components on addition of ADP to P_i-containing media is seen as being due to displacement of the equilibria of the Eqns. 1 and 2

$$AH_2 + B + C \leftrightharpoons A\sim C + BH_2 \quad (1)$$

$$A\sim C + ADP + P_i \leftrightharpoons A + C + ADP \quad (2)$$

to the right. If the reaction given by Eqn. 2 has a higher affinity for ADP in Sites I and II than in Site III, Q would first become more reduced and then more oxidized. This would not be the case when the energy in A ~ C is dissipated by an uncoupler or utilized for cation translocation, since the rate-limiting translocation of ADP is not involved in these reactions.

* A positive 'cross-over point' is defined as the point between which components of the chain on the substrate side become more oxidized and those on the oxygen side more reduced on transition from State 4 to State 3. Components on the substrate side of a negative 'cross-over point' become more reduced, and those on the oxygen side more oxidized.

The biphasic Q kinetics are less readily explained by the chemiosmotic hypothesis[12] as presently formulated, according to which three loops of the respiratory chain provide protons to a common proton-translocating ATPase working in the direction of synthesis of ATP.

METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[13] as described by MYERS AND SLATER[14].

Ubiquinone, NAD^+ and $NADP^+$ were determined in acid extracts of mitochondria prepared according to the method of KRÖGER AND KLINGENBERG[15]. To 0.5 ml of reaction mixture, containing 6–10 mg of mitochondrial protein, were injected 3 ml of light petroleum–methanol (3:1, v/v) containing 0.2 M $HClO_4$. The ubiquinone, present in the light-petroleum phase, was extracted as described by KRÖGER AND KLINGENBERG[14] and assayed in heptane–ethanol (1:4, v/v) by the difference in absorption at 275 nm before and after addition of KBH_4. An extinction coefficient of 12.2 $mM \cdot cm^{-1}$ (calculated from CRANE *et al.*[16]) was used. NAD^+ and $NADP^+$ were analysed in the acid layer as described by KLINGENBERG[17]. Protein was determined by the biuret method as described by CLELAND AND SLATER[18].

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