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Steady-state changes in loosely coupled submitochondrial particles induced by ADP and uncouplers

CHANCE AND WILLIAMS[1] have described the steady-state changes in mitochondria attendant upon the change in electron flux produced by the addition of phosphate acceptor (State 4 to 3 transition). It was not proven, however, that such changes were purely a function of the changes in flux induced at the three sites of phosphorylation. Changes in steady state resulting from the State 4–State 3 transition may also be postulated to occur as a result of either a change in electron transfer pathway or a change in concentration of some spectroscopically detectable high-energy intermediate (see ref. 2). Such changes would be independent of changes in flux and could therefore occur in the 'loosely coupled' system, showing no overall kinetic response to ADP addition. Digitonin particles represent a near approximation to such a system. Observations of steady-state changes in such particles[3] are also of interest because of their optical superiority to mitochondrial suspensions, the absence of large scattering errors increasing the accuracy of the possible observations. Responses to both ADP and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) can therefore be readily observed and compared.

Digitonin fragments from beef heart mitochondria were prepared as described by ELLIOTT AND HASS[4]. O_2 uptake and the changes in the cytochromes were monitored simultaneously by the O_2 electrode and Aminco-Chance dual wavelength spectrophotometer, respectively[5]. NADH (grade III) was obtained from Sigma Chemical Co. CCCP was obtained from Calbiochem.

Digitonin fragments so prepared have ratios $a+a_3:c+c_1:b$ of approx. 1.0:0.4:0.35 by succinate or NADH reduction. This compares with a ratio of 1.0:0.7:0.4 for Keilin–Hartree particles. Some 60 % of the cytochrome *c* is thus absent and the total cytochrome *b* is slightly less reducible than in the Keilin–Hartree case. Succinate dehydrogenase activity is some 30 % of the NADH dehydrogenase activity (Table I).

TABLE I

STEADY-STATE CHANGES INDUCED IN DIGITONIN PARTICLES BY THE ADDITION OF ADP

0.06 M phosphate buffer (pH 7.4), 25°. Total volume of the reaction medium 6.0 ml. ADP added to 160 μM in State 3. 0.35 mg protein per ml particle concentration, equivalent to 0.35 μM cytochrome *b* (563–575 mμ), 0.42 μM cytochrome $c + c_1$ (551–540 mμ), 0.8 μM cytochrome $a + a_3$ (605–625 mμ).

Substrate	*State*	*Rate of respiration* ($\mu M\ O_2 \cdot min^{-1}$)	*Reduction of electron carriers* (%)		
			Cyt. b	*Cyt. c*	*Cyt. a*
NADH	4	44	73	44	15
	3	45	30	30	20
Succinate	4	14	14	17	12
	3	17	0	11	15

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; State 3, the normal state of respiration in presence of O_2, substrate, ADP and inorganic phosphate; State 3_u, the uncoupled state, respiration in presence of O_2, substrate and CCCP; State 4, the acceptor-limited state, respiration in presence of O_2, substrate and phosphate without ADP.

The rate of respiration increased in State 3_u (Table II) but only very slightly in State 3 (Table I) in digitonin fragments respiring on NADH. In both States 3 and 3_u, however, there was less reduction of cytochromes *b* and *c* as compared to State 4, while cytochrome *a* was more reduced. When anaerobiosis occurred in State 3_u, cytochrome *b* was only 70% reduced as compared to anaerobiosis from State 3.

TABLE II

STEADY-STATE CHANGES IN DIGITONIN PARTICLES INDUCED BY UNCOUPLER (CCCP)

0.06 M phosphate buffer (pH 7.4), 25°. Total volume of the reaction medium 6.0 ml. 830 μM NADH, CCCP added to 1.7 μM in State 3_u. 0.7 mg protein per ml particle concentration, equivalent to 0.5 μM cytochrome *b*, 0.85 μM cytochrome $c + c_1$, 1.6 μM cytochrome $a + a_3$ (as in Table I).

Substrate	*State*	*Rate of respiration* ($\mu M\ O_2 \cdot min^{-1}$)	*Reduction of electron carriers* (%)		
			Cyt. b	*Cyt. c*	*Cyt. a*
NADH	4	64	($\geqslant$100)*	50	19
	3_u	80–90	38	41	26

* From anaerobiosis in State 3_u. Approx. 70% if compared to State 3 (Table I).

This failure of cytochrome *b* to become completely reduced, a phenomenon especially noticeable in the presence of very high ($\geqslant$10 μM) amounts of CCCP, may perhaps be interpreted as indicating an energy requirement for the reduction of a certain fraction of the cytochrome *b*. It is also evident that the changes in reduction of cytochrome *b*, both on the addition of ADP (Table I) and of CCCP (Table II), are much greater than the changes of flux would seem to demand. This may be contrasted with the increase in reduced cytochrome *a*, a change approximately consonant with the rate change both in coupled and uncoupled systems.

If we employ the simplified scheme of Eqns. 1 and 2, and assume that the flavoprotein remains completely 'reduced' and the cytochrome a_3 completely oxidized throughout, we may calculate the changes in the apparent second order rate constants on going from one state to another.

$$AH_2 \longrightarrow fp \xrightarrow{k_1} b \xrightarrow{k_2} c \xrightarrow{k_3} a \xrightarrow{k_4} a_3 \longrightarrow O_2 \tag{1}$$

$$v = k_1{}^1\,[b]_{ox} = k_2\,[b]_{red}\,[c]_{ox} = k_3\,[c]_{red}\,[a]_{ox} = k_4{}^1\,[a]_{red} \tag{2}$$

where $k_1{}^1 = k_1\,[fp]$ and $k_4{}^1 = k_4\,[a_3]$.

As the concentration of oxidized cytochrome *b* increases 2- or 3-fold on entering State 3, and by an even larger proportion on entering State 3_u (Tables I and II), yet the respiratory activity is almost unchanged, the apparent value of k_1 has diminished. Evidently oxidized cytochrome *b* is not behaving simply as an electron acceptor from the flavoproteins. On the other hand, the products $[b]_{red}\,[c]_{ox}$ and $[c]_{red}\,[a]_{ox}$ decrease by factors of about 2, while the rate of respiration increases slightly. They therefore represent kinetically orthodox interaction sites, both for ADP and for CCCP; that is, the values of k_2 and k_3 are acceptor dependent. In each case, also, the quantity of reduced cytochrome *a* varies in the same direction as the slight changes of flux; the velocity constant k_4 is thus unchanging, and the behavior of cytochrome *a* is consistent with its being a member of the electron transport chain with no interaction sites lying between it and oxygen.

The unorthodox behavior of cytochrome *b*, contrasted with the orthodoxy of cytochromes *c* and *a*, suggests a direct role in energy conservation as proposed for mitochondria by CHANCE AND SCHOENER[2]. Alternatively, the high reduction of cytochrome *b* in State 4 may reflect reversed electron transfer to molecules of cytochrome *b* not reducible directly. This is also indicated by the incomplete reduction of cytochrome *b* upon anaerobiosis in presence of CCCP. Cytochrome *a* in the digitonin fragments seems to differ from that in the mitochondrion[1] by being more reduced in State 3 or State 3_u.

Higher concentrations of CCCP (10–15 μM) had very little effect on electron transport in digitonin particles. In mitochondria, an inhibited state involving further oxidation of all the cytochromes supervenes[6]. Perhaps in the digitonin fragments we have two differences from the intact mitochondrion: (a) considerably less overall respiratory control in spite of large steady-state changes, (b) increased substrate accessibility. The structural correlates of these differences are discussed elsewhere[7].

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