RAPIDLY OXIDIZABLE EQUIVALENTS IN THE TERMINAL PORTION OF THE RESPIRATORY CHAIN OF UNCOUPLED RAT LIVER MITOCHONDRIA

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Received April 15, 1969

Terminal portions of the respiratory chains of uncoupled rat liver mitochondria were kinetically isolated either by Antimycin A inhibition or by cytochrome c extraction and then titrated with buffers of varying oxygen concentrations. We found from the kinetic titrations that there are about four reducing equivalents stored on the oxygen side of cytochrome c and about seven on the oxygen side of the Antimycin A block. The four terminal reducing equivalents can be oxidized about as fast as cytochrome a.

Some details of the mechanism and control of electron transport in the respiratory chain remain unknown. Heme oxidation reduction centers (cytochromes) have been elegantly shown to react rapidly as members of a primary sequence in uncoupled and coupled electron transport [Chance et al. (1967)]. Much effort has been made to show that non-heme oxidation-reduction centers can participate in the electron transport in the terminal portion of the respiratory chain [reviews by Beinert (1966) and Slater et al. (1965)]. We present evidence in this paper that the oxidation state of two non-cytochrome oxidation-reduction centers in the terminal portion of the respiratory chain can change at rates similar to that of cytochrome a.

Attempts to present direct kinetic evidence for the participation of non-cytochrome oxidation-reduction centers have been in the form of the EPR signal of cupric ion of isolated cytochrome c oxidase [Beinert and Palmer (1963), Atherton et al. (1963)] and phosphorylating submitochondrial particles [Beinert and Palmer (1965)]. Detergent isolated oxidase studies are open to criticisms of an in vitro preparation while the EPR studies on submitochondrial particles are open to criticisms as presented by Chance (1965). Chance and coworkers [Chance and Yonetani (1959), Chance (1961)] constructed a flow apparatus for mixing isolated oxidase with molecular oxygen and found a balance between added oxidizing equivalents and the heme a concentration. Yonetani (1960) titrated the magnetic susceptibility change of reduced, isolated cyto-

chrome c oxidase with oxygen and concluded that copper ions in the oxidase are unlikely to donate electrons to oxygen. Gibson et al. (1963) employed a stopped-flow apparatus for the titration of reduced, isolated cytochrome c oxidase by molecular oxygen and found that about two oxidizing equivalents per heme a are required to oxidize cytochrome c oxidase. We used molecular oxygen to titrate succinate-reduced, uncoupled rat liver mitochondria which were inhibited by Antimycin A or by the extraction of cytochrome c. The fast oxidation maximum (P_{max}) of cytochrome c and of cytochrome a was plotted as a function of the oxidizing equivalents (four times the molecular oxygen concentration) added per cytochrome a*). We found that about four oxidizing equivalents per cytochrome a were needed to completely oxidize cytochrome a in the cytochrome c extracted mitochondria, and that about seven oxidizing equivalents were needed to completely oxidize cytochrome c in Antimycin A inhibited mitochondria.

Methods and Materials

Rat liver mitochondria were prepared in MSE solution (0.22 M manitol, 0.07 M sucrose, and 200 μM Na_2EDTA, pH 7.2) essentially according to the methods of Schneider (1948) and Lardy and Wellman (1952). Respiratory control ratios were routinely determined immediately before each experiment [Chance and Williams (1955)] and were found to be between 4.5 and 6.0 using succinate and rotenone.

The titrations were carried out with the 20 ml regenerative flow apparatus [Chance et al. (1968)], using a Tektronix storage oscilloscope for the data read out. The observation chamber of the flow apparatus had a 6 mm optical path. Oxygen concentrations were determined with a Clark type oxygen electrode in the side arm reservoir of the flow apparatus which was stirred to prevent diffusion limited electrode currents. The mixing ratio of the regenerative flow apparatus was 1:67, permitting the oxygen measurements to be made with the Clark electrode; i.e. a final oxygen concentration of 0.5 μM is at 33.5 μM in the side arm reservoir and thus readily measurable by the Clark electrode. The side arm buffer reservoir was deoxygenated with argon gas for the lowest oxygen concentration attainable with the experimental set-up. Turbidity changes of the mitochondrial preparation due to the small dilutions occurring during each titration experiment were determined and found to be linear for the number of mixings of one experiment.

The cytochrome a concentration was determined from difference spectra

*) The cytochrome a concentration refers to one half of the total enzymatically reducible heme a concentration of the mitochondria [van Gelder (1966)].

of deoxycholate solubilized mitochondria which were reduced with succinate. The addition of dithionite caused no further increase in the Δ A 605-630 nm. Cytochrome c was extracted according to the method of Jacobs and Sanadi (1960) to retain maximum intactness of the mitochondrial structure. Difference spectra of the mitochondria upon treatment (a) with detergent (Hitachi Double Beam) or (b) without detergent (Johnson Foundation split beam spectrophotometer) and then reduction with succinate showed 100 % cytochrome a reduction upon anaerobiosis since dithionite caused no further increase in the Δ A 605-630 nm. The milimolar extinction coefficient of 26.4 of cytochrome c oxidase for Δ A 605-630 nm of the difference spectra of the mitochondria was used [van Gelder (1966), Slater and van Gelder (1966)].

Results and Discussion

Instead of using detergent solubilized and purified cytochrome c oxidase and titrating the oxidase with molecular oxygen [Chance and Yonetani (1959), Chance (1961), Gibson et al. (1963)], we decided to use a more physiological oxidase preparation. Rat liver mitochondria were uncoupled with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and their cytochrome c oxidase was kinetically isolated either by Antimycin A inhibition or cytochrome c extraction [Jacobs and Sanadi (1960)].

Figure 1 shows a titration of Antimycin A inhibited mitochondria. The

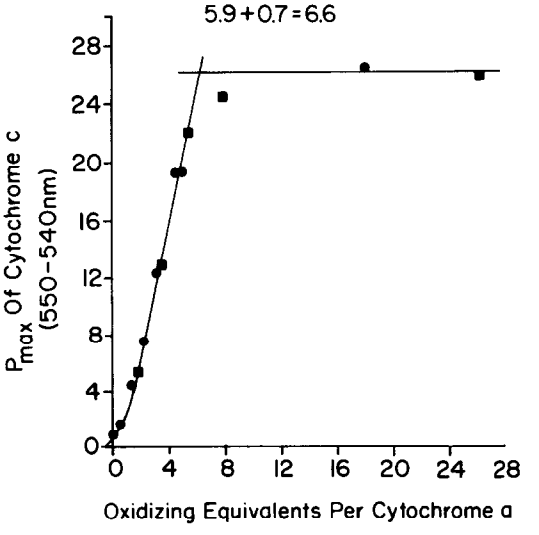

Fig. 1. Titration of Antimycin A (2 μg Antimycin A per mg protein) inhibited and FCCP (1 μM) uncoupled rat liver mitochondria with molecular oxygen. The substrate was 5 mM succinate. ■——■ refers to titration from high to low oxygen. ●——● refers to titration from low to high oxygen. Initial cytochrome a concentration was 0.97 μM.

fast rise to maximum oxidation (P_{max}) of cytochrome c (550-540 nm) with a half time of about three miliseconds is plotted as a function of oxidizing equivalents (four times the molecular oxygen concentration) added per cytochrome a. The extrapolated values of two experiments come to 6.6 and 6.8. The curvature of the titration plot near the origin can be interpreted in terms of an oxygen control model of the respiratory chain [Chance and Pring (1968)]; at very low oxygen concentrations we have a very slow interaction between cytochrome c and cytochrome c oxidase, thus the steady state oxidation level of cytochrome c is lower in the slow chain configuration than in the fast (high oxygen) configuration. The figure also shows that our method yields the same extrapolated value whether going from high oxygen to low oxygen or vice versa. Constant measurement errors due to "dead" volumes in the flow apparatus can thus be eliminated.

Figure 2 shows the titration curve for the cytochrome c extracted mitochondria. Extrapolated values of 3.7 and 3.8 were obtained. Gibson et al.

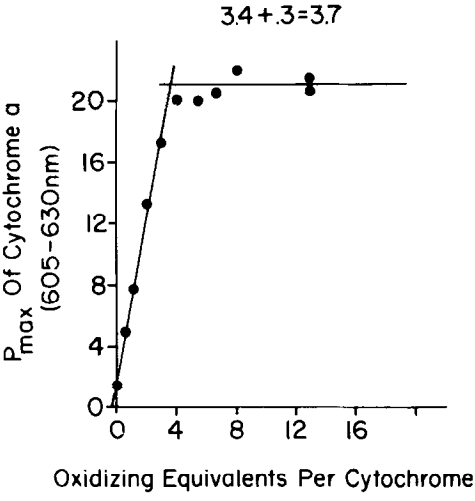

Fig. 2. Titration of cytochrome c extracted, FCCP uncoupled rat liver mitochondria with molecular oxygen. The substrate was 5 μM succinate. The initial cytochrome a concentration was 1.8 μM.

(1963) concluded from their titrations on isolated oxidase that one molecule of oxygen could oxidize 1.8 molecules of heme a. Thus their and our results are in good agreement. This agreement may, however, be fortuitous since we used the difference millimolar extinction coefficient $\Delta\epsilon_{mM}^{605\text{-}630\ nm}$ (reduced minus oxidized) of 26.4 of van Gelder (1966) while Gibson and Greenwood (1963) used the difference millimolar extinction coefficient $\Delta\epsilon_{mM}^{445\ nm}$ (reduced minus oxidized) of 55 instead of van Gelder's (1966) value of 82.

The data can be interpreted to mean that molecular oxygen can oxidize four reducing equivalents on the oxygen side of cytochrome c as fast or faster than it can oxidize cytochrome a. The three equivalents found between the Antimycin A block and cytochrome c oxidase support the conclusion reached by Wilson and Epel (1968) who suggest that there are two cytochrome c molecules per cytochrome c oxidase in the respiratory system of sea urchin sperm and rat liver. Our data are consistent with an uncoupled cytochrome chain on the oxygen side of the Antimycin A block consisting of cytochromes c_1, c, a, a_3, and copper occurring in ratios of 1:2:1:1:2.

Acknowledgements

The author is grateful to Professor Britton Chance for his guidance and encouragement and to Dr. D. F. Wilson for many stimulating discussions. This work was in part supported by U. S. Public Health research grant GM 12 202.

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