TOPOGRAPHY OF THE CRISTAE MEMBRANE AS ELUCIDATED BY A NEW INHIBITOR, TRIFLUOROFURYLBUTANEDIONE

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Received September 10, 1973

SUMMARY

The oxidation of ferrocyanide in ETP from beef heart mitochondria is insensitive to inhibition by antimycin A but partially sensitive to 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione. This inhibitor appears to inhibit electron transport at locations of non-heme iron proteins. Succinate based ferricyanide reduction in electron transport particles and mitochondria is strongly inhibited by antimycin and is inhibited by cytochrome c in mitochondria. These findings suggest the location of the ferro/ferricyanide sites, supposedly non-heme iron III and cytochrome c_1, on the M-side of the cristae.

INTRODUCTION

The use of impermeant electron donors and acceptors has facilitated the study of the topography of the mitochondrial inner membrane. The use of ferricyanide, to which the cristae are impermeable (1,2), has shown the location of succinic dehydrogenase (2,3) and NADH dehydrogenase (2) on the M-side of the membrane. Previous investigations have indicated that ferricyanide reduction in ETP is antimycin insensitive while that of mitochondria is sensitive (2,3,4), although inhibition has been observed in sub-mitochondrial particles (5).

On the basis of ferricyanide reduction in rat liver mitochondria, Estabrook (6) concluded that cytochrome c and c_1 were located on the outer (C-side) of the intact membrane. Data obtained from studies of antibody binding and inhibition have also indicated that c_1 may be located on the C-side (7).

The location of cytochrome c on the outside of the intact mitochondrial membrane and on the inside of the ETP membrane has been shown in studies involving detergents (8), extraction with salt (9), antibodies (10), and radioactive surfactants (11).

The elucidation of topography with ferri/ferrocyanide is dependent upon the number of reactive sites in the electron transport system and the availability of suitable inhibitors to verify the location of these sites. Because of the inability of the ferricyanide couple to permeate the cristae membrane, any reactive site must be exposed to the medium. The discovery of a new inhibitor of electron transport provides an approach to topography of the sites between cytochromes b and c.

MATERIALS AND METHODS

Beef heart mitochondria were obtained from fresh heart trimmed of excess connective tissue, minced, and homogenized in 0.25 M sucrose-0.01 M $MgCl_2$. The pH was adjusted with 1.0 M Trizma base to acheive 7.1-7.2 after homogenization. The homogenate was then centrifuged at 300 X g for 20 minutes in a swinging bucket rotor. The decanted supernatant is filtered through four layers of cheese cloth and centrifuged at 27,000 X g for 15 minutes. The resulting crude mitochondria are then washed, resuspended in minimal volume and either used immediately or frozen.

ETP were isolated from beef heart mitochondria by the alkaline treatment method of Crane et al. (8).

The reaction mixture for NADH oxidase and succinoxidase was 83.4 mM PO_4, 83.4 uM EDTA at pH 7.4, and either 3.89 mM NADH or 5.56 mM succinate for each 0.1 mg of protein assayed. When indicated, 0.2 mg cytochrome c (Sigma, Type III) was added. Oxygen uptake was measured polarigraphically on a Gilson oxygraph at 37° C.

Succinate-$Fe(CN)_6^{3-}$ or NADH-$Fe(CN)_6^{3-}$ reductase assays were performed according to Crane et al. (8) at 37° C, except that 0.2 µmole ferricyanide was used. Succinate was 50 uM, while NADH was 7.0 uM. The rate of reaction was monitered by measuring the change in absorbancy at 410 nm on a Gilford spectrophotometer.

The reaction mixture for measuring ferrocyanide oxidation polarigraphically was the same as that used for succinoxidase except that 2.78 mM ferrocyanide was substituted as substrate. 0.25 mg of durohydroquinone (DH_2Q) was used in duroquinol oxidase assays (16). All assays were performed at pH 7.4.

TABLE I

Effect of Inhibitors on Ferricyanide Reduction in ETP

Inhibitor	% Inhibition	
	Succinate→$Fe(CN)_6^{3-}$	NADH→$Fe(CN)_6^{3-}$
Antimycin A 1.01×10^{-5} M	80	8
Rotenone 5.55×10^{-5} M	--	6
TTFA 5.55×10^{-5} M	100	-
FTFA 4.2×10^{-2} M	100	48
Typical Control Values	2.2→2.5	1.3 μmole reduced/mg/min

TTFA: thenoyltrifluoroacetone

Mg^{++} -dependent ATPase activity was measured according to Ernster et al. (12). ATPase inhibitor was produced by the method of Horstman and Racker (13).

DATA AND DISCUSSION

Using the criterion of lack of respiratory stimulation upon addition of exogenous cytochrome c as evidence of ETP preparation homogeneity, at least 95% of the particles in the ETP preparation used were intact inverted vesicles and showed no stimulation with cytochrome c. In contrast, mitochondria exhibited at least a 2.5 fold stimulation in electron transport activity upon cytochrome c addition. Because of the possibility that broken vesicles could react with cytochrome c, this data does not indicate per se that 60% of this preparation was intact mitochondria. Rather, it shows that either ETP and/or endogenous cytochrome c was responsible for 40% of the respiration. The use of the natural ATPase inhibitor, to which the membrane should be impermeable and which inhibits non-sequestered, external ATPase, inhibited only 30-40% of the ATPase activity, suggesting that 60-70% of the membrane was indeed intact mitochondria.

Data shown in Table I indicates that ferricyanide reduction by succinate is antimycin sensitive while reduction by NADH is not, indicating that most of the NADH-ferricyanide activity is associated with Complex I, as shown by Ruzicka et al. (14,15), However, a ferricyanide reduction site between the antimycin site and

TABLE II

Effect of Inhibitors on Electron Transport in ETP

	% Inhibition			
Inhibitor	NADH Oxidase	Succinoxidase	Ferrocyanide Oxidase	Duroquinol Oxidase
FTFA 4.2×10^{-2} M	99	100	15-21	94
TTFA 5.55×10^{-5} M	9	74-82	0	--
Hydroxyquinoline-N-Oxide 10 µgm	91	100	0	--
Antimycin A, 1.01×10^{-5}M	100	100	0	93
KCN, 5×10^{-4} M	100	100	100	100
Typical Control Rates in µmoles O/mg protein/min	7.0	2.1	0.37	1.25

cytochrome c (but including neither) must be exposed in ETP and therefore be on the M-side. These sites are presumably NHI_{III} and/or cytochrome c_1. Cytochrome c cannot be involved in ETP since it is sequestered inside the vesicle.

As shown in Table II ferrocyanide oxidase in ETP is antimycin insensitive, but partially sensitive to trifluorofurylbutanedione (FTFA). As in the case of ferricyanide reduction, ferrocyanide must be reacting with either the NHI_{III} or cytochrome c_1. Ferrocyanide added to ETP causes reduction of cytochromes c_1 and aa_3. A cytochrome oxidase preparation oxidized ferrocyanide only in presence of cytochrome c at one-fifteenth the rate expected based on the heme a content, explaining the low rate of oxidation in mitochondria. FTFA is seen (Tables I and II) to inhibit electron transport reactions in complexes I, II, and III, which is consistent with the postulate that this inhibitor interferes with non-heme iron sites. Therefore the partial inhibition of ferrocyanide oxidation can best be related to non-heme iron III.

That cytochrome c_1 is not exposed on the C-side of the cristae is shown by study of ferricyanide reduction in mitochondrial membranes that are cytochrome c-depleted. Addition of exogenous c inhibits 55-70% of ferricyanide reduction by succinate, suggesting interaction between c_1 and ferricyanide through the cytochrome

c site. In a cytochrome c-depleted membrane this site would be vacant. Cytochrome c appears to sterically block this interaction.

FTFA, an analog of TTFA, completely inhibits succinate driven ferricyanide reduction, as does TTFA, indicating a site of action at or on the substrate side of the TTFA site. That FTFA inhibits 48% of the rotenone-insensitive NADH-ferricyanide reductase indicates that two ferricyanide reactive sites are present before the rotenone site, one of them before and one after non-heme iron protein(s) inhibited by FTFA.

ACKNOWLEDGMENT

Research supported under Grant AM04663 from the National Institute for Arthritis and Metabolic Diseases and Grant GB27501 from the National Science Foundation. Support for H.J.H. is under training grant T01-GM1195 and F.L.C. under Career Award KG-21839 from the National Institute for General Medical Science.

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