Topography of the Inner Mitochondrial Membrane

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Abstract

Using ferri/ferrocyanide as an artificial electron acceptor and donor the topographical organization of the proteins of the quinol-cytochrome c reductase segment of mitochondrial electron transport was studied. Kinetic differences in antimycin-insensitive ferrocyanide oxidase activity and antimycin-sensitive ferricyanide reduction in isolated beef heart mitochondria and electron transport particles indicate a site of interaction on both the outside and inside (M-side) of the inner membrane. Polylysine inhibits ferrocyanide oxidation in mitochondria, but not in electron transport particles. Spectral studies of intact electron transport particles and deoxycholate fractions indicate ferrocyanide reduces cytochrome c_1 but not b-type cytochromes. Thus, ferri/ferrocyanide interacts with cytochrome c in mitochondria but with cytochrome c_1 and possibly another component of electron transport in electron transport particles.

Introduction

An anisotropic organization of proteins in mitochondrial inner membranes has been postulated to provide vectorial proton translocation accompanying electron transport (15, 16, 18). Vectorial proton translocation across a proton-impermeable membrane can result in the formation of a proton gradient of sufficient magnitude to energize the membrane to carry on oxidative phosphorylation or other energy-linked reactions.

Asymetrical membrane structure has been observed in the inner mitochondrial membrane. Following fractionation of the cristae by detergent and salt, two membranous fractions differing in width, composition, and enzymatic activity are recovered (1, 4, 6). Recombination of the two fractions yields a structurally and functionally intact membrane. Evidence of asymmetric protein localization in the intact inner membrane would support both the chemiosmotic theory and the binary membrane model (3).

The study of membrane topography requires membrane preparations that are homogeneous in particles wholly of one orientation. Intact mitochondria can be isolated that show the physiological orientation, with the 90 Å knobs on the M-side (matrix side) facing the matrix (27). The surface exposed in these right-side out particles is called the C-side. Preparations of electron transport particles (ETP) can be prepared that have an inverted orientation to mitochondria (2), *i.e.* the M-side with 90 Å knobs is exposed while the C-side is on the inside of the membrane vesicle (Figs. 1 and 2).

The topography of the membrane can be elucidated in particles of homogeneous orientation by use of the following techniques: 1) observation of protein location by electron microscopy; 2) covalent bonding of surface proteins with impermeable radioactive surfactants; 3) protein extraction with loss of structure or enzymatic activity and reconstitu-

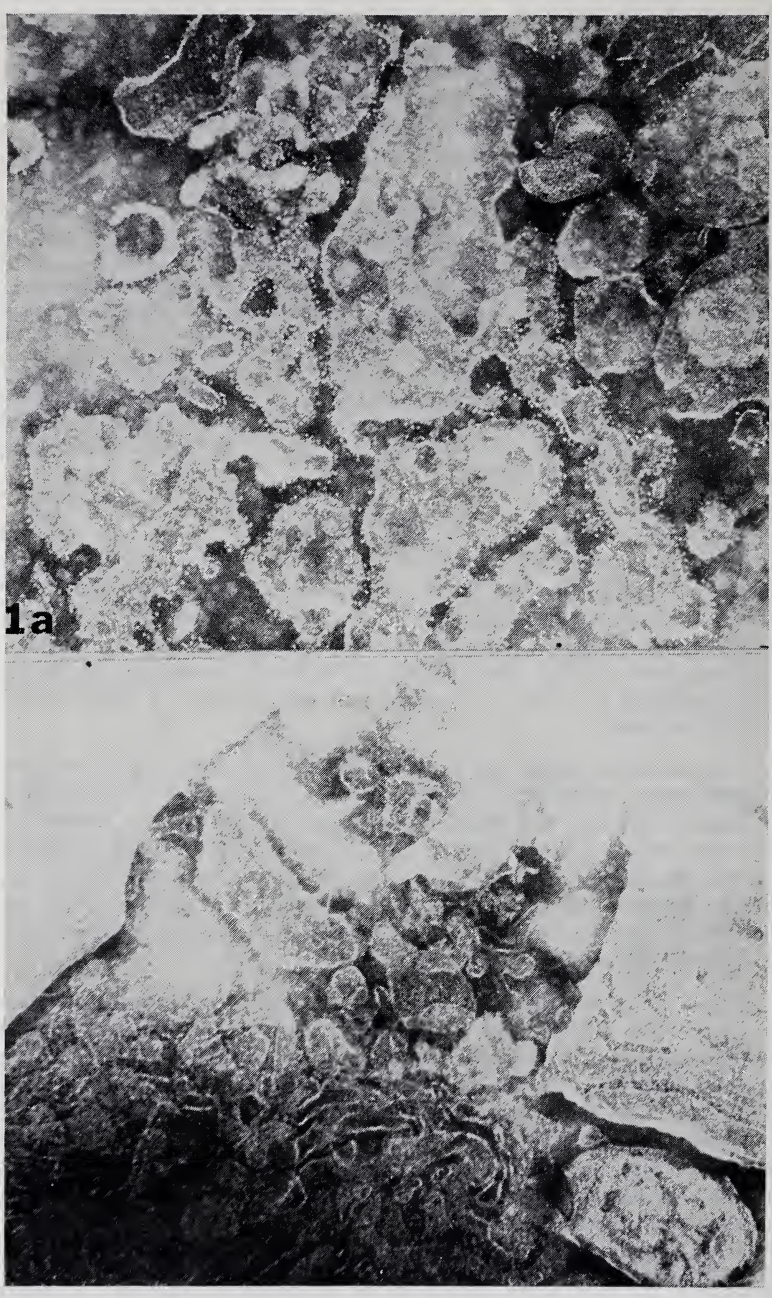

FIGURE 1. *A. Negative stained preparation of electron transport particles. B. Negative stained preparation of intact inner mitochondrial membrane vesicles with C-side exposed and 90 Å spheres not visible on the surface. 82,000 X magnification. Unfixed material was stained with 1% phosphotungstate pH 7.0.*

tion of structure or activity; 4) inhibition of electron transport or oxidative phosphorylation by impermeable agents, including specific antibody; 5) the use of impermeant donors and acceptors of electron transport. These techniques were used to locate some membrane proteins on either the M-side or the C-side. These data are summarized in Table 1. A third location of a protein, in the middle of the membrane but not necessarily exposed to both M- and C-sides, must also be considered.

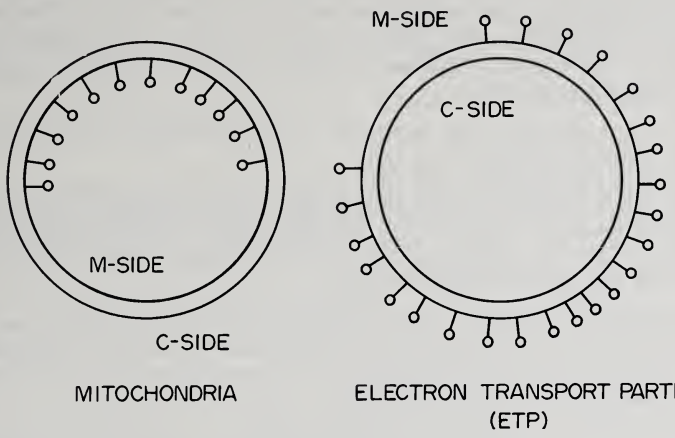

FIGURE 2. *Diagramatic representation of opposite orientations of beef heart mitochondria and electron transport particles.*

TABLE 1. *Location of inner mitochondrial membrane proteins.*

		Method of Determination					
Component	Side	E.M.	Extraction/Reconstitution	Impermeant Donor/Acceptor	Radioactive Surfactant	Impermeant Inhibitor	Reference
Cytochrome c	C		X	X	X	X	13, 19, 21, 22, 24
ATPase (F_1)	M	X	X		X	X	8, 14, 19, 20
Succinate Dehydrogenase	M		X	X			11, 25
NADH Dehydrogenase	M			X			10, 12, 25
Cytochrome a	C			X	Inconclus.	Inconclus.	19, 21, 22
Cytochrome a_3	?				Inconclus.	Inconclus.	19, 21, 22
Cytochrome c_1	M			X			7

Using the impermeant electron acceptor/donor ferri-/ferrocyanide (17), cytochrome c_1 was localized on the M-side of the inner membrane. The binding of antibodies against c_1 to mitochondria and the lack of antibody binding to ETP defined c_1 on the C-side of the membrane. Ferricyanide reductions in mitochondria have also suggested c_1 to be on the C-side (5, 9). Recently, cytochrome c_1 was shown to be located on the M-side on the basis of antimycin sensitive ferricyanide reduction

in ETP (7). The present study substantiates the M-side location of c_1 utilizing differences in ferrocyanide oxidation between ETP and mitochondria.

Materials and Methods

Beef heart mitochondria were obtained from fresh heart trimmed of excess connective tissue, minced, and homogenized in 0.25 M sucrose -0.075 M $MgCl_2$ -0.001 M succinate - 0.01 M Na_2HPO_4. The pH was adjusted with 0.5 M Na_2HPO_4 to achieve pH 7.0-7.2 after homogenization. The homogenate was then centrifuged at 300 x g for 20 min. Mitochondria were isolated from the filtered supernatant by centrifugation at 27,000 X g for 15 min. Mitochondria were washed in 0.25 M sucrose -0.075 M $MgCl_2$-0.15 M KCl.

ETP were isolated from beef heart mitochondria by the alkaline treatment method of Crane *et al.* (2).

Succinate—$Fe(CN)_6^{3-}$ reductase assays were performed according to Crane *et al.* (2) at 37°C, except that 0.2 μmole ferricyanide was used with the mixture 50 μM in succinate. The reaction was monitored at 410 nm on a Gilford spectrophotometer.

Ferrocyanide oxidation was measured polarigraphically in a reaction mixture 83.4 mM PO_4, 83.4 μM EDTA and 7.5 mM ferrocyanide. When indicated, 0.2 mg cytochrome c (Sigma, Type III) or 0.2 mg poly-L-lysine (M.W. 70,000) was added.

NADH oxidase or succinic oxidase assays were performed as were ferrocyanide oxidase assays except that either NADH or succinate was added to concentrations of 3.89 mM and 5.56 mM, respectively.

Fractionation of beef heart mitochondria was performed by the method of Wharton and Tzagaloff (26). Difference spectra of the resulting fractions were recorded on a Cary 15 spectrophotometer. Difference spectra of intact ETP were measured on an Aminco-Chance spectrophotometer. Ferricyanide was used to oxidize the reference in all measurements.

Results and Discussion

Homogeneous intact ETP should show no stimulation of NADH or succinate oxidation upon addition of exogenous cytochrome c since the location of cytochrome c is inside the vesicles and the membrane is impermeable to proteins of this size. In contrast, mitochondria should exhibit substantial respiratory stimulation in the presence of external cytochrome c. The ETP used in these experiments show a 6-8% increase in respiration (94% ETP oriented) while the mitochondria exhibit up to a 7-fold increase in respiration after two washes in dilute KCl (14% ETP).

Succinate reduction of ferricyanide in ETP and mitochondria is inhibited approximately 80% by antimycin A, which inhibits electron transport between cytochromes b and c_1 (23). Estabrook (5) has reported the interaction of ferricyanide with cytochrome c in rat liver mitochondria, as has Jacobs and Sanadi (9). However, in intact ETP,

where cytochrome c is sequestered within the vesicle, ferricyanide must be interacting with a component between the antimycin-sensitive site and cytochrome c. Thus the two sites of interaction are different.

TABLE 2. *Ferrocyanide oxidation rates in mitochondria, ETP and purified cytochrome oxidase.*

	uatom 0/mg protein/min		
	—cyt c[1]	+cyt c	pH
Mitochondria	0.06	0.23	7.4
ETP	0.56	0.56	7.4
Cytochrome Oxidase	0	0.07	8.0

[1] cyt c = cytochrome c

A similar result was observed in the antimycin-insensitive oxidation of ferrocyanide by the cytochrome chain in ETP and mitochondria. As seen in Table 2, ETP catalyze ferrocyanide oxidation at a faster rate than mitochondria. Ferrocyanide oxidation is stimulated by the addition of exogenous cytochrome c in mitochondria but not in ETP. Since cytochrome c is not accessible in ETP, ferrocyanide must be accessible to sites between cytochrome b and c. The sites exposed in ETP could be a non-heme iron component (NHI_{III}) and/or cytochrome c_1. Cytochrome aa_3 does not react directly with ferrocyanide because purified cytochrome c oxidase does not catalyze a significant rate of ferrocyanide oxidation in the absence of cytochrome c.

TABLE 3. *Effective of poly-l-lysine (M.W. 70,000) on ferrocyanide oxidation.*[1]

		Polylysine		
		None	0.2 mg	Inhibition
ETP	—cyt c[2]	0.66	0.74	None
	+cyt c	0.66	0.74	None
Mitochondria	—cyt c	0.035	0.02	57%
	+cyt c	0.27	0.04	86%

[1] Data expressed in uatoms 0/mg protein/min
[2] cyt c = cytochrome c

Further evidence that the site of ferrocyanide interaction is different in ETP and mitochondria is shown in Table 3. The addition of poly-L-lysine to ETP does not inhibit ferrocyanide oxidation. However, polylysine inhibits virtually all cytochrome c stimulated oxidation in mitochondria. These data are consistent with cytochrome c being exposed in mitochondria and thus inhibited by polycationic proteins, as is succinate oxidation (13), but sequestered in ETP. Lack of polylysine inhibition indicates cytochrome c is not directly involved in ferrocyanide interaction with ETP.

Ferrocyanide reduces cytochrome c_1 (reduction peak at 554 nm) in the red fraction following deoxycholate fractionation (spectrum not

shown). The red fraction has been shown to contain most of the cytochromes b, c, and c_1 of the inner membrane (1, 6). That ferrocyanide reacts with cytochrome c_1 and not with b is illustrated in Figure 3, where the difference spectrum of intact ETP displays cytochrome c_1 reduction by ferrocyanide but no reduction of b-type cytochromes (reduction peaks at 563 nm).

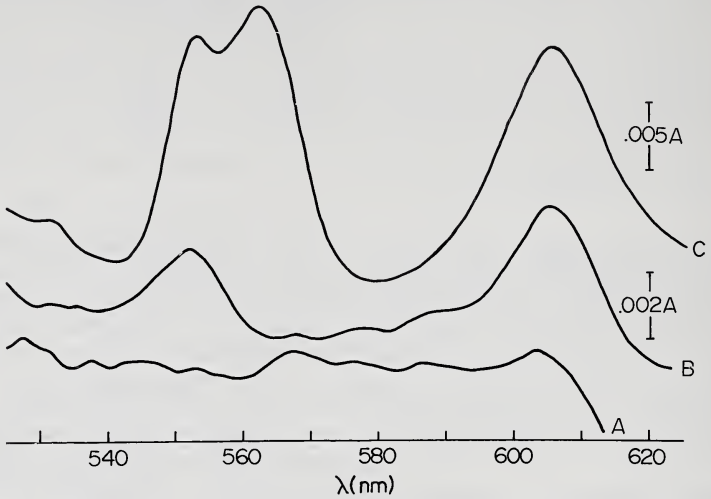

FIGURE 3. *Difference spectrum of intact ETP. Ferrocyanide was used to reduce the sample. A. ETP with no ferrocyanide added. B. ETP with 5 umole ferrocyanide added. C. ETP sample reduced with* $Na_2S_2O_4$. *Absorbancy indicated on figure.*

Conclusion

The evidence clearly indicates that cytochrome c_1 (and possibly nonheme iron III) is located on the matrix side of the mitochondrial cristae membrane.

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