TOPOGRAPHICAL DEFINITION OF NEW SITES ON THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

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Received March 7,1974

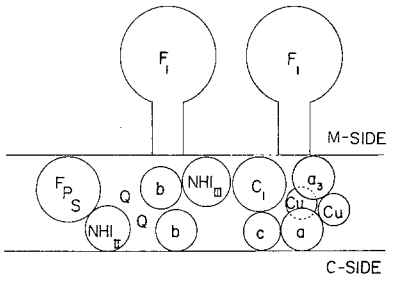
SUMMARY

The use of water soluble bathophenanthroline sulfonate as an inhibitor of electron transport in mitochondria and electron transport particles exhibits effects that may be related to the location of the multiple non-heme iron and copper proteins in the membrane. In contrast to lipophilic bathophenanthroline, hydrophilic bathophenanthroline sulfonate gives inhibition dependent upon membrane orientation. While NADH oxidation is sensitive in both intact mitochondria (C-side exposed) and electron transport particles (M-side exposed), succinate oxidation is inhibited only in mitochondria. Duroquinol oxidase is stimulated in mitochondria but unaffected in electron transport particles. Inhibitions of NADH and succinate by electron acceptors such as juglone, ferricyanide, duroquinone, and cytochrome c in the presence of appropriate inhibitors indicate at least one chelator-sensitive site on each side of the membrane for the NADH dehydrogenase complex and at least one site on the C-side in the succinate dehydrogenase complex. In contrast, lipophilic bathophenanthroline shows substantial inhibition at these and additional sites regardless of membrane orientation. A transmembranous orientation of dehydrogenase components is proposed having both surface exposed and buried chelator sensitive sites.

INTRODUCTION

Presently, six non-heme iron centers have been reported in the NADH-coenzyme Q reductase segment (Complex I) of the mitochondrial electron transport chain (1-5). Two non-heme iron centers are associated with the succinate-coenzyme Q portion (Complex II), but only one is reducible by succinate (6). The second center is believed not to be located on the succinate electron transport chain.

Metal chelators have been used to inhibit mitochondrial electron transport. Thenoyltrifluoroacetone (TTFA) has been used extensively to inhibit succinate oxidation (7). Furyltrifluoroacetane (FTFA) has been shown to inhibit electron transport in complexes I, II, and III (ubiquinol-cytochrome c reductase) (8,9). Bathophenanthroline has been shown to inhibit NADH and



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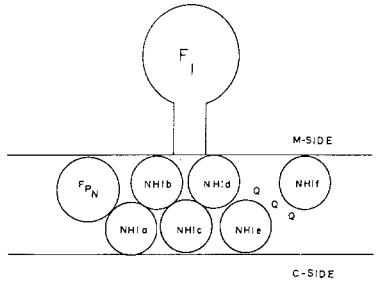

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Figure 1. Diagramatic representation of the location of electron carriers of succinate oxidation to oxygen. Fp_S=succinate dehydrogenase, NHI= non-heme iron proteins, Cu=copper protein, Q=coenzyme Q.

Figure 2. Possible arrangement of Complex I electron carriers. Coenzyme Q→ O_2 segment is the same as in Fig. 1. Juglone, duroquinone, and ferricyanide may interact with more than one site on the same side of the membrane. Other arrangements compatible with the data are possible. The non-heme iron sites indicated may correspond to those described by Ohnishi (5). Fp_N=NADH dehydrogenase, NHI_{a-f}=non-heme iron sites. Q= coenzyme Q.

duroquinol oxidation (10,11). Because of their permeability, these inhibitors are of only limited value in topographical determinations of non-heme iron centers.

Palmer employed water soluble bathophenanthroline sulfonate (BPS) to show inhibition of succinate oxidase and phosphorylation at site III in rat liver mitochondria (12). Hare obtained inhibition of NADH oxidase and slight stimulation of duroquinol oxidase in sonicated beef heart mitochondria with BPS (13). The topographical significance of these results, however, was confused by the fact that these particles could reduce exogenous cytochrome c, indicating the presence of broken vesicles or intact mitochondria and interactions of reagents with both the M- and C-surfaces.

TABLE 1

Particle Homogeneity as Determined by Respiratory Activity

		NADH Oxidase+	Succinate Oxidase+	Ferrocyanide Oxidase*
Electron Transport Particle	-cytochrome c	8.36	3.46	3.3 mM
	+cytochrome c	8.36	3.46	
Mitochondria	-cytochrome c	0.043	0.126	0.67 mM
	+cytochrome c	0.43	1.26	

+Data expressed as μatom O/mg protein-min. Activity is antimycin sensitive

*Data expressed as concentration of ferrocyanide necessary for half maximal antimycin insensitive activity.

In this paper we shall present evidence locating at least two sites on the C-side of the membrane and at least one chelator sensitive site on the M-side based on oriented inhibition of dehydrogenase function by hydrophilic chelators. In addition selected hydrophobic chelators show strong inhibition of buried sites related to oxidase function.

MATERIALS AND METHODS

Electron transport particles (ETP) were isolated from beef heart mitochondria by the alkaline treatment of Crane, et al. (14).

Intact beef heart mitochondria were obtained from fresh heart trimmed of excess connective tissue, minced, and homogenized in 0.35 M sucrose, 7.5 mM $MgCl_2$, 1 mM succinate, 0.01 M Na_2HPO_4. pH was adjusted with concentrated Na_2HPO_4 to achieve 7.0-7.2 after homogenization. The homogenate was then treated as previously described (8) with the inclusion of two washes in 0.35 M sucrose, 7.5 mM $MgCl_2$, 0.15 M KCl.

NADH oxidase, succinate oxidase, and ferrocyanide oxidase were performed oxygraphically at 37°C as described previously (8). Cytochrome c oxidase was performed under conditions similar to NADH oxidase except that 5 μmole sodium ascorbate and 1.2 mg N-N-N'-N' tetramethyl phenylenediamine were

used as substrate with cytochrome c. Antimycin-sensitive duroquinol oxidase was measured by oxygen electrode at 37°C in a mixture 83.4 mM in sodium phosphate (pH 7.4) and 83.1 mM EDTA. 0.25 mg duroquinol or 0.25-0.50 mg duroquinone and 5 μmoles dithiothreitol were used as substrate.

NADH-duroquinone or juglone reductases were performed by the method of Ruzicka and Crane (15). Duroquinone and juglone (Sigma) were added as a methanol solution to final concentrations of 0.03 and 0.057 M respectively. The assay was followed spectrophotometrically at 340 nm.

NADH- and succinate ferricyanide reductases were performed as described by Crane, et al. (14) using 0.23 mM NADH and 0.033 M succinate. The reaction was monitored at 410 nm.

NADH- and succinate cytochrome c reductase assays were performed in 4 mM sodium phosphate buffer, pH 7.4, at 37°C with 0.3 mM KCN. 0.25 mg cytochrome c (Sigma, horse heart, type VI) and either 0.23 mM NADH or 0.033 M succinate were added to the enzyme. The reaction was recorded at 550 nm. Permeability studies with ferrous iron trapped inside vesicles show that bathophenanthroline penetrates the membrane but that BPS does not.

DATA AND DISCUSSION

That the ETP and mitochondria employed in this study are of opposite orientation is indicated in Table 1. ETP show little, if any, reaction with exogenous cytochrome c while mitochondria show a ten-fold stimulation in respiratory activity. Thus, the ETP are oriented with the cytochrome c site sequestered and the M-side (matrix face) exposed, while 90% of the mitochondria have the C-side (cytochrome c side) exposed. The difference in ferrocyanide concentrations necessary for half maximal activity of ferrocyanide oxidation substantiates the finding that different sites are exposed in these preparations.

Markedly different effects of BPS on respiration in oppositely oriented particles are seen in Table 2. Succinate oxidations in ETP are unaffected but are partially inhibited in mitochondria while NADH oxidations are equally sensitive in both particles. Since duroquinol oxidation is unaffected by

TABLE 2

Effect of Bathophenanthroline Sulfonate on Electron Transport in Mitochondria and Electron Transport Particles

	% Inhibition	
	Mitochondria	Electron Transport Particles
NADH→O_2	88	88
Succinate→O_2	37	0
Duroquinol→O_2	190% stimulation	0
Cytochrome oxidase	0	-
Succinate→cytochrome c	22-30	-
Succinate→$Fe(CN)_6^{3-}$ (antimycin insensitive)	56-85	0
Succinate→$Fe(CN)_6^{3-}$ (TTFA insensitive)	0-5	0
NADH→cytochrome c	60-80	-
NADH→Juglone	40-66	40-50
NADH→duroquinone	100	76
NADH→$Fe(CN)_6^{3-}$ (rotenone insensitive)+	33-48	66-80

+Data not corrected for possible non-inner membrane activity.

Where indicated, 0.1 μgm antimycin, 4 μgm rotenone, or 0.1 nmole TTFA (thenoyltrifluoroacetone) were added.

All data obtained at w/w ratio BPS/protein = 10/1 for essentially maximum inhibition

TABLE 3

Inhibition of Mitochondrial Respiration by Bathophenanthroline

	% Inhibition	
	Electron Transport Particles	Mitochondria
NADH→O_2	>95	>95
Succinate→O_2	40	30
Duroquinol→O_2	50-60	50-60
Cytochrome→oxidase	--	50

Bathophenanthroline added as methanol solution to 55 μM final concentration.

BPS in ETP and stimulated in mitochondria, a BPS sensitive site is not present on the oxygen side of the duroquinol reactive site in the electron transport chain. Cytochrome oxidase is insensitive to BPS. Since Fe^{+3} and Cu^{+2} have been observed to inhibit electron transport in the NADH-coenzyme Q segment (16) we can suggest that BPS is chelating endogenous Fe^{+3} or Cu^{+2} on the C-side of the membrane, relieving inhibitions imposed by these cations.

Since succinate oxidation is not BPS inhibited in ETP, all sensitive sites are on the C-side. TTFA insensitive ferricyanide reduction is not inhibited in either particle but antimycin insensitive ferricyanide reduction is inhibited in mitochondria. Thus the sensitive site, at the TTFA sensitive site or between the sites of TTFA or antimycin action, is located on the C-side. Since only one Fe-S center has been shown to be reduced by succinate (6), this is presumably the BPS and TTFA site.

Bathophenanthroline sulfonate inhibition of succinate oxidase is also observed in both detergent-treated mitochondria and the red fraction obtained by deoxycholate fractionation of the membrane (17). This indicates that BPS inhibits electron transfer from succinate and not succinate transport across the membrane (18,19).

The topography of Complex I sites is difficult to elucidate since all Fe-S centers are on the substrate side of the rotenone site. As shown in Table 2, BPS sensitive sites are present on both the M- and C-sides of the membrane. Juglone reduction is inhibited to the same extent in both particles indicating the presence of a BPS sensitive site before the point of juglone interaction on each side of the membrane. Duroquinone reduction is sensitive to BPS from the C-side. The results observed could be due to either multiple BPS sites and/or multiple quinone reactive sites. If only one BPS sensitive site were present on the substrate side of the juglone site, then we would expect the inhibitions of juglone, duroquinone, and ferricyanide to be similar and occurring in only one type of particle (in the case of ferricyanide). This is not the case, and thus multiple sites of inhibition on opposite sites of the membrane are present.

Antimycin insensitive NADH-cytochrome c reductase in mitochondria is not inhibited by BPS at the level indicated in Table II, indicating the lack of a sensitive site in non-inner membrane catalyzed reactions.

The difference in action between hydrophilic BPS and lipophilic bathophenanthroline is illustrated in Table 3. The extent of succinic oxidase inhibition is similar to that obtained with BPS. Inhibition of duroquinol oxidase is observed in both types of vesicles. Cytochrome oxidase inhibition is observed with bathophenanthroline but not with BPS (Table 2). Lipophilic chelator inhibition sites are therefore present in the cytochrome region of the electron transport chain.

Possible transmembranous arrangements of components of the NADH dehydrogenase and succinate dehydrogenase portions of the electron transport chain compatible with the data are diagrammed in Figures 1 and 2.

ACKNOWLEDGEMENT

H.J.H. is supported by training grant TO1-GM1195. F.L.C. is supported by Career Award KG-218339 from the National Institute for General Medical Science.

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