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INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT BY HYDROPHILIC METAL CHELATORS

DETERMINATION OF DEHYDROGENASE TOPOGRAPHY

H. J. HARMON* and F. L. CRANE

Department of Biological Sciences, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

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SUMMARY

The topography of the inner mitochondrial membrane was investigated using inhibitors of electron transport on preparations of beef heart mitochondria and electron transport particles of opposite orientation. Reductions of juglone, ferricyanide, indophenol, coenzyme Q, duroquinone, and cytochrome *c* by NADH are inhibited to different extents on both sides of the membrane by the impermeant hydrophilic chelators bathophenanthroline sulfonate and orthophenanthroline. The extent of inhibition for each acceptor increased in the order given. At least two chelator-sensitive sites are present on each membrane face between the flavoprotein and coenzyme Q and a chelator-sensitive site is present on the matrix face between the sites of coenzyme Q and duroquinone interaction. Duroquinol oxidation in mitochondria only is stimulated by bathophenanthroline sulfonate. Juglone reduction is stimulated in electron transport particles (only) by *p*-hydroxymercuribenzenesulfonate, but after mercurial treatment, juglone reduction in both particles and mitochondria is more sensitive to bathophenanthroline sulfonate.

Succinate dehydrogenase components are inhibited by hydrophilic orthophenanthroline or bathophenanthroline sulfonate in mitochondria only. Electron flow between the dehydrogenases of succinate and NADH occurs via a chelator-sensitive site located on the matrix face of the membrane. Inter-complex electron flow is prevented by rotenone or thenoyltrifluoroacetone. The lack of succinate-indophenol reductase inhibition by bathophenanthroline sulfonate in the presence of rotenone or thenoyltrifluoroacetone indicates that the rotenone-sensitive site may be located on the matrix face and demonstrates that electrons flow between the NADH and succinate dehydrogenases via a hydrophilic chelator and rotenone-thenoyltrifluoroacetone-sensitive site on the matrix face of the membrane. Inhibition by hydrophilic chelators only in mitochondria indicates that succinate dehydrogenase as well as NADH dehydrogenase has a transmembranous orientation.

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* Present address: Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19174 (U.S.A.)

Abbreviations: Cl_2-Ind, 2,6 dichlorophenol indophenol; Q, coenzyme Q (Ubiquinone); pOH-Hg-$BzSO_3^-$, *p*-hydroxymercuribenzene sulfonate; pCl-Hg-BzO^-, *p*-chloromercuribenzoate.

INTRODUCTION

Impermeable electron acceptors and impermeable inhibitors have been employed in this study to determine the location of the components of complex I and II in the inner membrane. Chelator inhibitions of NADH and succinate oxidations in mitochondria and oppositely oriented electron transport particles have determined differences in the topography of acceptor sites in both types of particles. A transmembranous orientation of complex I has been suggested previously [1, 2].

Orthophenanthroline has been reported to inhibit coenzyme Q and duroquinone reduction by NADH in electron transport particles [3, 4]. Juglone and ferricyanide reduction is insensitive to 1, 10 phenanthroline.

Thenoyltrifluoroacetone inhibits succinate oxidation by all acceptors except phenazine methosulfate [5–8]. The chelators orthophenanthroline, $\alpha\alpha'$-dipyridyl, and Tiron do not inhibit isolated complex II although chelators are bound and form a colored complex with the protein [9–11]. Furyltrifluoroacetone [5, 12], bathocuproine, and bathophenanthroline [5, 12, 3] have been shown to inhibit succinate oxidations. Palmer [13] reported bathophenanthroline sulfonate inhibition of succinate oxidase in mitochondria that was uncoupler reversed, but attributed the inhibition to a site in cytochrome oxidase.

This communication presents evidence for a transmembranous orientation of the dehydrogenases of both NADH and succinate. Evidence is also presented to demonstrate a connection between the flavoproteins via a chelator-sensitive component common to both chains.

MATERIALS AND METHODS

Intact beef heart mitochondria were obtained from fresh heart as described previously [2]. The layer of heavy mitochondria was used. Electron transport particles were obtained from beef heart mitochondria by the alkaline treatment of Crane, et al. [14].

NADH oxidase and duroquinol oxidase were performed in a mixture containing 83 mM sodium phosphate and 83 μM EDTA (pH 7.4) with 0.7 mM NADH or 0.25 mg duroquinol as substrate. Succinate oxidase was performed in the same medium with 27.8 mM succinate as substrate. Cytochrome *c* oxidase was assayed polarigraphically under the conditions used to assay NADH oxidase. 0.4 mg cytochrome *c* (Sigma, Type VI), 5 μmol ascorbate, and 1.2 mg *N,N,N',N'*-tetramethylphenylenediamine were used as substrate. The reaction volume for all oxidase reactions was 1.8 ml.

NADH-juglone reductase was assayed polarigraphically in a mixture 83.4 mM sodium phosphate and 83.4 μM EDTA, pH 7.4. 0.35 mM NADH was used as substrate with 22 μM juglone and 5.6 μM rotenone present in 1.8 ml volume.

Duroquinone reduction was assayed spectrophotometrically at 37 °C by following the disappearance of NADH at 340 nm according to Ruzicka and Crane [4].

Reduction of 2,3-dimethoxy-5-methyl-6-heptyl-1,4-benzoquinone (Q_1) was followed spectrally at 550 nm in medium composed of 100 mM sodium phosphate, 10 μM EDTA, pH 7.4 with 0.1 μgm antimycin A and 0.3 mM KCN. 0.18 mM NADH or 50 mM succinate was used as substrate. 25 μgm CoQ_1 and 0.75 mg cytochrome *c* (Sigma, Type VI) were used as acceptor in 1 ml volume.

2,6-dichlorophenolindophenol (Cl_2-Ind) reduction was followed at 620 nm in the same reaction mixture employed for Q_1 reduction. 3.5 μgm Cl_2-Ind was used as acceptor with 0.19 mM NADH or 50 mM succinate in a 1 ml reaction mixture.

Succinate-phenazine methosulfate reductase was assayed in 100 mM sodium phosphate, 10 μM EDTA pH 7.4 with 225 μM phenazine methosulfate and 2 μgm Cl_2-Ind as acceptor in 1 ml reaction volume. 50 mM succinate was added as substrate. The reaction was assayed spectrophotometrically at 620 nm.

Ferricyanide reductase assays were performed by the method of Crane, et al. [14] with 0.19 mM NADH or 50 mM succinate.

NADH- and succinate-cytochrome *c* reductases were performed in 40 mM sodium phosphate, pH 7.4. 0.35 mM NADH or 50 mM succinate and 0.25 mg cytochrome *c* were used in 1 ml volume.

Mercurial treatments of membrane particles were performed by adding equal volumes of 1.6 mM *p*-hydroxy mercuribenzene sulfonate in 0.05 M sodium phosphate buffer (pH 7.4) and membrane (4 mg protein/ml) in 0.35 M sucrose 7.5 mM $MgCl_2$ and incubating for 10 min at room temperature.

Rhein was isolated from rhubarb extract by F. L. Crane. Rhein concentration was determined using $E_{mM} = 11$ at 435 nm.

Membrane particles loaded with $FeSO_4$ were made by resuspending mitochondria in 0.35 M sucrose and 0.01 M $FeSO_4$. The particles were sonicated on ice at full power with a Branson sonicator for 3 min and centrifuged at 27 000 × *g* for 15 min. They were then washed twice in 0.35 M sucrose at 27 000 × *g* to remove excess $FeSO_4$ and resuspended in 0.35 M sucrose. 10 μmol ascorbate were present in the determination of chelator complexes. Measurements were made after 5 min of incubation.

RESULTS

Orientation of membrane particles

That the electron transport particles and mitochondria used in this study are of opposite orientation is demonstrated in Table I. Electron transport particles show high rates of NADH oxidase activity and only slight stimulation of rates upon addition of cytochrome *c*. In contrast, mitochondria exhibit large stimulations of oxidase activity by exogenous cytochrome *c* because the cytochrome *c* site is exposed. Mitochondria also exhibit low specific activity of NADH oxidase. The membrane is poorly

TABLE I

PARAMETERS OF ORIENTATION OF MITOCHONDRIA AND ELECTRON TRANSPORT PARTICLES

	NADH → O_2		
	μatom O/mg protein/min		% inhibition by 11 μM rhein
	−cytochrome *c*	+cytochrome *c*	
Electron transport particles	6.9	7.2	70 % (−cytochrome *c*)
Mitochondria	0.02	0.22	0 (+cytochrome *c*)

permeable to NADH [15] and NADH must cross the membrane to react with the flavoprotein of NADH dehydrogenase on the matrix face of the cristae. Beef heart mitochondria characteristically have moderately low rates of antimycin-sensitive NADH oxidase that are higher than those of intact liver mitochondria. Topographical locations of the flavoproteins, cytochrome *c*, and other membrane components have recently been reviewed [16].

The orientation of the vesicles is substantiated by the inhibition of NADH oxidase by rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) in electron transport particles, but not in mitochondria. Rhein competes with NADH for the flavoprotein [17] and can be used as an indicator of flavoprotein exposure to exogenous NADH. At higher concentrations of rhein, mitochondria become inhibited slightly (20 % at 16 μM rhein), possibly indicating penetration of the membrane by rhein.

Chelator inhibitions of electron transport between NADH and cytochrome b

Data presented in Table II indicate that under the conditions used, bathophenanthroline sulfonate and 1, 10 phenanthroline are unable to cross the mitochondrial inner membrane although bathophenanthroline can. Using particles sonicated in the presence of $FeSO_4$ and washed, we observed that only bathophenanthroline, but not 1,10-phenanthroline or bathophenanthroline sulfonate would form a colored complex in the absence of detergent after 5 min incubation. Upon the addition of deoxycholate, a colored complex was formed by all three chelators, demonstrating that only bathophenanthroline could cross the membrane, although on the basis of its partition coefficient (15/1 = chloroform/arachis oil) [18] one would expect that 1-10 phenanthroline could penetrate partially into the membrane. Tetramethyl-1,10-phenanthroline is more lipophilic (26/12) = chloroform/arachis oil) as would be bathophenanthroline (diphenyl substituted phenanthroline). Aryl substitutions at the 4 and 7 positions of 1,10-phenanthroline will increase the hydrophobicity of 1,10-phenanthroline [19]. Since 1,10 phenanthroline and bathophenanthroline sulfonate cannot cross the membrane, they will react at sites on or near the membrane surface. Bathophenanthroline, however, can react with sites in more lipophilic regions of the membrane. Thus the apparent hydrophilicity of the chelators increases in the order bathophenanthroline < 1,10 phenanthroline < bathophenanthroline sulfonate.

Data in Table III indicates that inhibitions of NADH oxidation reactions by bathophenanthroline sulfonate and 1,10 phenanthroline are different in electron

TABLE II

FORMATION OF COLORED CHELATE COMPLEXES IN $FeSO_4$ – CONTAINING PARTICLES

ETP, Electron transport particle with enclosed $FeSO_4$ as described in Methods.

	Absorbance	Wavelength
ETP+bathophenanthroline sulfonate	0.00	535 nm
+deoxycholate	0.13	
ETP+1,10 phenanthroline	0.00	510 nm
+deoxycholate	0.04	
ETP+bathophenanthroline	0.06	540 nm
+deoxycholate	0.10	

TABLE III

CHELATOR INHIBITIONS OF ELECTRON TRANSPORT REACTIONS

	Percent inhibition						Typical control values	
	BPS		OP (0.55 mM)		BP (55 μM)		μmol acceptor mg protein/min	
	Electron transport particles*	Mitochondria	Electron transport particles*	Mitochondria	Electron transport particles*	Mitochondria	Electron transport particles*	Mitochondria
NADH → O_2	90	90	80	65	95	95	7.2	0.3–0.7
NADH → juglone	60	15	< 10	< 10	+20	+70	3.4	0.6
NADH → ferricyanide	80	40	< 10**	0**			7.8	2.5–3.0
NADH → Cl_2-Ind (+rotenone)	85	0	80	20			0.11	0.11
NADH → Cl_2-Ind (−rotenone)	100	20–40					0.18	0.15
NADH → Q_1	80	90	60**	50**			0.65	0.22
NADH → duroquinone	75	100	95**	70**			0.5	0.06
NADH → cytochrome *c* (AA sensitive)	–	80–100	–				–	0.4
TMPD-ascorbate → O_2	–	0	–	0 → +40	–	75	–	2.0–4.0
Duroquinonol → O_2	0	+90	20	25	55	55	5.2	3.0
Q_1H_2 → O_2	8	+ 4	+ 4	13			4.3	2.1

BPS, bathophenanthroline sulfonate; OP, 1,10 phenanthroline; BP, bathophenanthroline; TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

* Inverted vesicles

** 1 mM 1,10 phenanthroline used.

TABLE IV

ROTENONE INHIBITION OF COMPLEX I AND II REACTIONS

Data expressed in μmol acceptor/min/mg protein**

	Electron transport particles*		Mitochondria	
	−rotenone	+rotenone (10 μM)	−rotenone	+rotenone (10 μM)
NADH → Cl_2-Ind	0.12	0.08	0.15	0.11
NADH → Q	0.31	0.01	0.13	0.00
Succinate → Cl_2-Ind	0.21	0.16	0.17	0.12
Succinate → Q	0.24	0.20	0.20	0.22
Succinate → phenazine methosulfate	–	–	0.15	0.08

* Inverted vesicles.

** 0.1 mM thenoyltrifluoroacetone present.

transport particles than in mitochondria. Bathophenanthroline sulfonate inhibits the reactions in inverted vesicles more extensively than in mitochondria. Increase in bathophenanthroline sulfonate inhibition parallels the sequence of acceptor interaction with the NADH oxidation chain (juglone, ferricyanide, Q, duroquinone, cytochrome *c*) [3, 4, 20]. Also apparent is the inability, under the conditions used, of Cl_2-Ind, ferricyanide, and juglone to cross the membrane readily. The inability of ferricyanide to cross the membrane has been reported [21, 22]. That Cl_2-Ind and phenazine methosulfate are impermeant is seen in Table VI where thenoyltrifluoroacetone inhibits all succinate-Cl_2Ind or succinate-phenazine methosulfate reduction in mitochondria but is only partially inhibitory in electron transport particles. Were these compounds able to cross the membrane freely, they could interact with a non-inhibited site within the membrane vesicle and differences in inhibitions in the two particles would not be observed.

The inability of bathophenanthroline sulfonate to completely inhibit the reduction of some acceptors (juglone and ferricyanide) may be due to the interaction of the acceptors at multiple sites, all of which are not bathophenanthroline sulfonate-sensitive.

Inhibition of juglone reduction by bathophenanthroline sulfonate can be

TABLE V

BATHOPHENANTHROLINE SULFONATE INHIBITION OF NADH-JUGLONE REDUCTASE IN MERCURIAL TREATED MEMBRANES

	μatom O/mg protein/min	
	−pOH-Hg-$BzSO_3^-$	+pOH-Hg-$BzSO_3^-$
Electron transport particles	2.7	6.4
+1 mg bathophenanthroline sulfonate	1.5	1.8
Mitochondria	1.5	1.5
+1 mg bathophenanthroline sulfonate	1.1	0.56

increased by treatment of the membrane with mercurials as indicated in Table V. Juglone reductase is stimulated in electron transport particles in agreement with Ruzicka and Crane [3]. In both types of membranes, pOH-Hg-$BzSO_3^-$ treatment increased sensitivity to bathophenanthroline sulfonate. This indicates that attack on a sulfhydryl group may expose an iron site in the dehydrogenase. That the sulfonate group of bathophenanthroline sulfonate is not the reactive entity is shown by the lack of inhibitions by *p*-toluene sulfonate or pyrocatechol sulfonate.

While juglone reduction is inhibited by bathophenanthroline sulfonate on both sides of the membrane, 1,10 phenanthroline has only little effect. Bathophenanthroline stimulates juglone reduction in mitochondria more than in electron transport particles. Similarly, bathophenanthroline sulfonate prevents ferricyanide reduction while 1,10 phenanthroline does not inhibit significantly as observed previously [4].

To check if detergent action of bathophenanthroline sulfonate was responsible for the inhibition of ferricyanide and juglone reductions, 0.02 % final concentration Triton X-100 was added with 1,10 phenanthroline to electron transport particle. Although NADH-juglone reductase was stimulated 40 %, 1,10 phenanthroline did not inhibit activity, suggesting that bathophenanthroline sulfonate inhibits by a mechanism other than membrane disruption.

Reductions of Cl_2-Ind, Q_1, duroquinone and cytochrome *c* exhibit different degrees of inhibitions in electron transport particles and mitochondria by bathophenanthroline sulfonate and 1,10 phenanthroline. Only in duroquinone reductase is inhibition greater in mitochondria than in electron transport particles. The greater extent of bathophenanthroline sulfonate and 1,10 phenanthroline inhibitions of acceptors near or beyond the rotenone site in comparison to other acceptors (c.f. Table III) is probably a result of a decrease in the number of reactive sites for the acceptor and the combined effect of the chelator on other sensitive sites on the substrate side of the point of acceptor interaction. Sites inhibited by bathophenanthroline sulfonate that are involved in juglone or ferricyanide reduction would also inhibit reactions of all other acceptors reacting closer to oxygen. Similarly, sites of Cl_2-Ind reduction inhibited by 1,10 phenanthroline will also be inhibited in Q_1 or duroquinone reduction.

Differences in Cl_2-Ind and Q_1 reaction sites are seen by rotenone inhibition of the reduction of these acceptors (Table IV). Cl_2-Ind reduction is partially sensitive to rotenone, indicating that one or more Cl_2-Ind sites are located at or beyond the rotenone site. The presence of sites on the substrate side of rotenone is also indicated. A bathophenanthroline sulfonate-sensitive site affecting rotenone-sensitive Cl_2-Ind reduction is present on the C-side. In contrast, Q_1 and duroquinone reductions are almost entirely sensitive to rotenone, indicating their point of interaction on the oxygen side of the rotenone site.

The increase in chelator inhibition between Cl_2-Ind and the quinone sites indicates that at least one bathophenanthroline sulfonate- or 1,10 phenanthroline-sensitive site is involved in electron transport between Cl_2-Ind and Q_1 sites and between Q_1 and duroquinone sites in mitochondria. While bathophenanthroline sulfonate and 1,10 phenanthroline-inhibit Cl_2-Ind reduction more completely in electron transport particles, the effects of bathophenanthroline sulfonate on Q_1 and duroquinone reduction do not indicate additional sensitive sites on the M-side between Cl_2-Ind and the quinones, although at least one additional 1,10 phenanthroline-

sensitive component may exist between Q_1 and duroquinone. From these findings and the different inhibitions of ferricyanide and juglone reductions, we can conclude that bathophenanthroline sulfonate and 1,10 phenanthroline do not affect common sites.

Further differences in 1,10 phenanthroline and bathophenanthroline sulfonate inhibitions are seen in the quinol oxidases. 1,10 phenanthroline inhibits duroquinol oxidation, but bathophenanthroline sulfonate has no effect in electron transport particles and bathophenanthroline sulfonate stimulates activity in mitochondria. Neither bathophenanthroline sulfonate nor 1,10 phenanthroline significantly inhibit Q_1H_2 oxidase.

Connection between complexes I and II via a chelator sensitive site

Data in Tables IV and VI illustrate the effects of rotenone and thenoyltrifluoroacetone on complex I and II reactions. Rotenone inhibits succinate-Cl_2-Ind or Q_1 reduction in electron transport particles but does not inhibit Q_1 reduction in mitochondria (c.f. Table IV). Thenoyltrifluoroacetone inhibits NADH-Cl_2-Ind reduction in mitochondria and electron transport particles suggesting that reducing equivalents from one complex are reducing acceptors at sites in the other. Thenoyltrifluoroacetone inhibitions of NADH oxidations have been observed [6, 7] as have piericidin inhibitions of succinate oxidations [27].

Most significant is the inhibition of succinate-Cl_2-Ind reductase in electron transport particles by bathophenanthroline sulfonate in the absence of rotenone. However, when rotenone is present, bathophenanthroline sulfonate no longer inhibits, indicating that electrons from complex II are entering complex I via the rotenone site and reducing Cl_2-Ind at a site that is inhibited by bathophenanthroline sulfonate (this is seen only in electron transport particles). Were the connection via Q in the membrane, rotenone would have little effect on the bathophenanthroline sulfonate inhibition since a bathophenanthroline sulfonate-sensitive Cl_2-Ind site is seen on the oxygen side of the rotenone block.

Chelator inhibitions of electron transport between succinate and cytochrome b

Succinate oxidase activity is inhibited by bathophenanthroline sulfonate in mitochondria, but is not inhibited by bathophenanthroline sulfonate in inverted

TABLE VI

THENOYLTRIFLUOROACETONE INHIBITION OF ELECTRON TRANSPORT

Data expressed in μmol acceptor reduced/min/mg protein. 10 μM thenoyltrifluoroacetone (TFFA) added where indicated.

	Electron transport particles		Mitochondria	
	−TTFA	+TTFA	−TTFA	+TTFA
NADH → Cl_2–Ind	0.10	0.09	0.12	0.09
NADH → Q_1	0.27	0.26	–	–
Succinate → Cl_2Ind	0.25	0.04	0.14	0.00
Succinate → phenazine methosulfate	0.41	0.19	0.20	0.00
Succinate → Q_1	0.24	0.02	0.26	0.06

TABLE VII

EFFECT OF CHELATORS ON SUCCINATE OXIDATIONS

For bathophenanthroline sulfonate inhibition, concentration was 0.5–1 mM

	% Inhibition						Typical succinate oxidation rates*	
	BPS 10 mg		OP 1mM		BP 56 μM			
	mg protein							
	Electron transport particles	Mitochondria	Electron transport particles	Mitochondria	Electron transport particles	Mitochondria	Electron transport particles	Mitochondria
Succinate → O_2	0	40	15**	10**	30	40	3.5	1.1
→ PMS (−TTFA)	53	45		30			0.41	0.18
→ PMS (+TTFA)	0	–	0	0			0.18	0.00
→ Ferricyanide (−TTFA)	0–20	50	10	0–20			0.4	0.84
→ Ferricyanide (+TTFA)	0	5					0.3–0.4	0.60
→ Cl_2−Ind(−rotenone)	50	50					0.27	0.17
→ Cl_2−Ind(+rotenone)	13	50	0	25			0.6	0.17
→ Q_1 (−rotenone)	45	27				5	0.24	0.22
→ Q_1 (+rotenone)	6	27			21		0.20	0.20
→ Cytochrome *c*	–	30					–	0.30
→ PMS (−rotenone)	25	11					0.34	0.20
→ PMS (+rotenone)	0	21	0	30			0.26	0.18

BPS, bathophenanthroline sulfonate; OP, 1,10 phenanthroline; BP, bathophenanthroline; TTFA, thenoyltrifluoroacetone; PMS, phenazine methosulfate.

* μmol acceptor/mg protein/min

** 0.56 mM 1,10 phenanthroline used.

vesicles. Maximum inhibition of 40–50 % of mitochondrial activity occurs at concentrations of 10 μg bathophenanthroline sulfonate per μg mitochondrial protein.

A comparison of the effects of phenanthroline-type chelators on succinate oxidation by various acceptors is shown in Table VII. Succinate oxidation by various acceptors in mitochondria is inhibited by bathophenanthroline sulfonate under all conditions. Succinate oxidation by acceptors such as phenazine methosulfate and indophenol in electron transport particles is also inhibited by bathophenanthroline sulfonate in contrast to the lack of inhibition of the overall succinate oxidase activity. However, when thenoyltrifluoroacetone or rotenone are included in the assay mixture, bathophenanthroline sulfonate does not inhibit the oxidation of succinate by artificial acceptors in electron transport particles. As shown earlier, communication between complexes I and II is prevented by thenoyltrifluoroacetone or rotenone. Furthermore, the communication between the complexes involves a bathophenanthroline sulfonate-sensitive site on the M-side that reacts with indophenol. When thenoyltrifluoroacetone or rotenone are not present, Cl_2-Ind is reduced at sites in complex I as well as in complex II. The fact that bathophenanthroline sulfonate does not inhibit when rotenone or thenoyltrifluoroacetone are present indicates that Cl_2-Ind reduction in complex II does not involve a bathophenanthroline sulfonate sensitive site on the M-side. The observed inhibition by bathophenanthroline sulfonate in the absence of thenoyltrifluoroacetone or rotenone occurs at a site in complex I.

A similar explanation is possible for the apparent bathophenanthroline sulfonate-induced inhibition of phenazine methosulfate reduction in electron transport particles. Rotenone and thenoyltrifluoroacetone cause a partial inhibition of phenazine methosulfate reduction in electron transport particles. Thenoyltrifluoroacetone-insensitive phenazine methosulfate reduction occurs at the flavoprotein of complex II. However, Cl_2-Ind, which is used as the external indicator of the phenazine methosulfate reaction, can also be reduced at sites beyond the thenoyltrifluoroacetone site in complex II as well as at sites in complex I. If thenoyltrifluoroacetone is present to prevent the reduction of Cl_2-Ind at sites other than the flavoprotein, no inhibition of phenazine methosulfate reduction by bathophenanthroline sulfonate is seen. Thus, no sites sensitive to bathophenanthroline sulfonate are present on the M-side in complex II.

Several investigators have shown that chelator inhibitions of succinate oxidations may be due partially to inhibitions of succinate transport into the mitochondria [23–25]. Several investigators have also assumed that the inhibition of succinate oxidations by bathophenanthroline sulfonate is due solely to inhibition of the dicarboxylate carrier [26]. To show that bathophenanthroline sulfonate inhibits succinate electron transport, inhibitions under various conditions were studied. In the presence of 0.05 % Tween 80 to disrupt mitochondria, 40 % inhibition by bathophenanthroline sulfonate was observed while an equal amount of *p*-toluene sulfonate (0.56 mg/ml) inhibited only 13 %. When succinate oxidase activity was reconstituted by mixing red and green fractions derived by deoxycholate fractionation [28], bathophenanthroline sulfonate still inhibited 20 % of the activity while *p*-toluene sulfonate stimulated the activity 6 %. To test the effect of bathophenanthroline sulfonate on dicarboxylic acid transport, tannic acid and oxalate were used to inhibit succinate transport. In the presence of 28 mM oxalate or 140 μM tannic acid to inhibit succinate transport [23], 30 % inhibition was still observed and the response was not modified by increasing

the succinate concentration. The data indicates that bathophenanthroline sulfonate is not inhibiting only transport of succinate into the mitochondria. Furthermore, inhibition of succinate-Q_1 reductase by lipophilic bathophenanthroline in electron transport particles (in the presence of rotenone) indicates the presence of a chelator-sensitive site in complex II that does not involve the dicarboxylate carrier system.

Succinate oxidations by Cl_2-Ind and phenazine methosulfate+Cl_2-Ind in electron transport particles are only partially thenoyltrifluoroacetone-sensitive (Table VI) while these reactions are completely thenoyltrifluoroacetone-sensitive in mitochondria. The insensitive rate reflects the exposure of the flavoprotein of complex II on the M-side and shows that the thenoyltrifluoroacetone site is not involved in flavoprotein-acceptor activity (7).

Differences in chelator inhibitions are seen in Tables III and VII for the oxidation of NADH, succinate, duroquinol, and ferrocytochrome *c* to oxygen. Only for NADH oxidase reactions do electron transport particles and mitochondria differ in their inhibition by bathophenanthroline. The order of sensitivity to bathophenanthroline sulfonate is NADH oxidase > cytochrome *c* oxidase > duroquinol oxidase > succinate oxidase.

DISCUSSION

Topographical differences between the matrix and cytochrome *c* faces of the cristae are apparent and are summarized as follows: (a) juglone-reactive bathophenanthroline sulfonate-sensitive sites on each face with a pOH-Hg-$BzSO_3^-$ stimulated site on the M-side. Bathophenanthroline inhibition does not occur at this site. (b) ferricyanide-reactive bathophenanthroline sulfonate-sensitive sites on each face. (c) indophenol-reactive bathophenanthroline sulfonate- and 1,10 phenanthroline-sensitive sites on each membrane face. 1,10 phenanthroline and bathophenanthroline sulfonate inhibit strongly on M-side but may not act at the same site. Bathophenanthroline sulfonate inhibitions may be a result of inhibitions at site a) above. (d) 1,10 phenan-

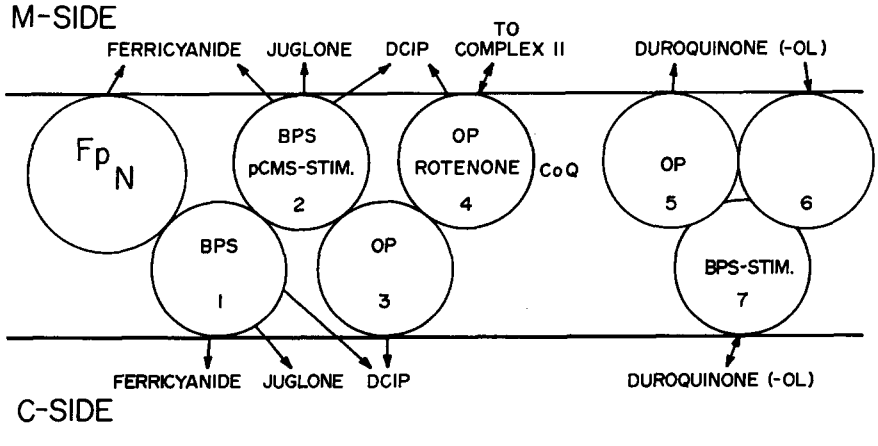

Fig. 1. Diagrammatic representation of the topography of the electron carriers between NADH and cytochrome *b*. F_{PN} denotes the flavoprotein of NADH dehydrogenase; OP, 1,10-phenanthroline; BPS, bathophenanthroline sulfonate.

throline-sensitive sites on both faces on the substrate side of Q that affect Q and duroquinone reduction. This latter site may be the same site as in (c). (e) rotenone- and bathophenanthroline sulfonate-sensitive component on M-side involved in Cl_2-Ind reduction of both complexes I and II and connects both complexes. (f) bathophenanthroline sulfonate-stimulated duroquinone site on the C-side. (g) Cl_2-Ind reduction site on C-side on oxygen side of rotenone. Bathophenanthroline sulfonate inhibits Cl_2-Ind reductase here.

On the basis of data presented, a topographical model of the inner mitochondrial membrane can be constructed. A possible orientation of components between NADH and cytochrome *b* is diagrammed in Fig. 1. Possible interactions of artificial electron acceptors with the components and inhibitors binding at these loci (numbered 1–7) are shown in the diagram. As is seen, NADH dehydrogenase exhibits transmembranous characteristics.

The chelator inhibitions which we report here are consistent with previous studies which showed chelator inhibition of NADH and succinate oxidase [30, 13]. The non-heme iron proteins in complex I and the coenzyme Q-cytochrome *b* region of complex III detected by electron paramagnetic resonance studies could account for the chelator effects we have observed. Orme-Johnson et al. [31] have reported the reduction sequence of iron centers in complex I to be 1, 3, 4, 2. Correlations between this data and the acceptor reductions we observe are possible. Center 1, or centers la or 1b of Ohnishi et al., ref. 32, could be the juglone and ferricyanide reductase sites on the C-side while centers 3 and 4, if surface oriented, could react with indophenol and be on the M-side. Centers 5 and 6 described by Ohnishi [33, 34] could possibly be the duroquinone-reactive protein on the M-side (center 5) or the bathophenanthroline sulfonate-stimulated duroquinol site on the C-side. Rotenone-sensitive Cl_2-Ind reduction in mitochondria may involve center 6 or a site on the C-side in complex II. Assignment of bathophenanthroline-sensitive sites cannot be made.

Multiple non-heme iron proteins have been observed in complex II [35–38]. One component is associated with the flavin of the 70 000 molecular weight flavoprotein [39], is insensitive to thenoyltrifluoroacetone [6, 8] and from this study is insensitive to bathophenanthroline sulfonate inhibition. A second component is of molecular weight 27 000 [36, 40]. Three Fe-S EPR signals in complex II have been detected [38]

TABLE VIII

EFFECT OF BATHOPHENANTHROLINE SULFONATE ON SUCCINATE OXIDASE ACTIVITY IN MITOCHONDRIA AND ELECTRON TRANSPORT PARTICLES

100 μg protein in each 1.8 ml assay mixture. Control rates: mitochondria, 0.54 μatom O/mg protein/min; Electron transport particles, 1.74 μatom O/mg protein/min.

BPS added (μg)	Percent inhibition	
	Mitochondria	Electron transport particles
250	13	0
500	23	3
1000	40	0
2000	51	–

BPS, bathophenanthroline sulfonate.

and high potential iron-sulfur proteins have been described [41]. We suggest that the chelator-sensitive sites we observe may be one or more non-heme iron proteins. Recently, Ohnishi, et al. [42] demonstrated the location of a high potential iron-sulfur protein of succinate dehydrogenase (center S-3) on the matrix face of the cristae by the use of bathophenanthroline sulfonate to quench the EPR signal. Our data, however, indicate that bathophenanthroline sulfonate does not inhibit the reduction of artificial acceptors by succinate in electron transport particles unless thenoyltrifluoroacetone is absent, suggesting that the thenoyltrifluoroacetone site and S-3 may be the same.

Albracht and Slater [29] have reported the partial reduction of non-heme iron center 2 of NADH dehydrogenase by succinate. Center 2 (N-2) is closely associated with the rotenone site in mammalian systems [33]. Based on the effects of bathophenanthroline sulfonate and rotenone on succinate-Cl_2-Ind reduction in electron transport particles only, we believe center 2 to be located on the M-side and be the last component reduced in complex I before coenzyme Q. Also, NADH has been shown to cause the appearance of an EPR signal of complex II in electron transport particles [43]. These data suggest inter-complex electron flow that could be mediated by a chelator-sensitive site.

Since the flavoprotein of complex II is insensitive to impermeant chelators and located on the M-side of the membrane [44] and one or more other components are inhibited by impermeant chelators on the C-side only, we conclude that the orientation of complex II is also transmembranous and allows vectorial electron flow across the membrane.

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