Chelator Inhibition as a New Approach to the Mechanism of Energy Coupling in Biological Membranes

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Abstract

There has been very little evidence for a common intermediate coupling electron transport to energy linked functions in biological membranes such as mitochondria or microbial plasma membranes. Such an intermediate would be expected in the chemical intermediate hypothesis. We now find that the chelator, bathophenanthroline, inhibits electron transport at three sites associated with energy coupling in mitochondria and two sites in *E. coli* membranes when the membranes are coupled but not when uncouplers are present. We also find that the membrane-bound ATPase activity is inhibited by bathophenanthroline and that the inhibition is reversed by uncouplers in both mitochondria and *E. coli* membrane. It thus appears that a chelator sensitive site such as non heme iron proteins (or copper protein in mitochondrial cytochrome oxidase) may be the long sought chemical link between electron transport and energy coupling. Since reversal of bathophenanthroline inhibition is not brought about by ionophores which prevent a proton gradient across the membrane, it appears that the chelator sensitive step occurs before the establishment of a proton gradient and that the gradient cannot be the primary step in energy coupling.

Introduction

Two theories have been proposed for the mechanism for transduction of energy from the redox system of biological electron transport to the chemical potential of ATP. The chemical intermediate theory proposes that one of the carriers of the electron transport chain forms a high energy intermediate which can be used to form ATP (19). The chemiosmotic theory calls for the formation of a proton or ion gradient across an anisotropic membrane as a result of electron flow. This gradient would then be used to energize the formation of ATP through an ATPase which would be completely independent of the electron transport system (8) (9) (10).

There has been much support for the chemiosmotic theory since it can be shown that electron transport causes formation of ion gradients (17); preinduced ion gradients can cause ATP formation (10); and most agents which prevent energy coupling make the membrane permeable to ions (14).

There has been little experimental support for the chemical intermediate theory except for evidence that some redox elements of the electron transport chain change their redox potential under coupled or uncoupled conditions (2) (12) (13) (21).

In this paper we will present evidence that the electron transport chain and the ATP forming system have a chelator sensitive site in common, that this site responds in the coupled state and that ionophores do not influence the chelator sensitivity. These effects would support the chemical intermediate hypothesis by showing that the ATP forming system is linked to the electron transport system independent of an ionic gradient. The fact that the inhibition is caused by a chelator

suggests that certain of the many non heme iron and copper proteins in electron transport chains act as the chemical intermediate.

Materials and Methods

Chemicals used were from commercial sources except the ferrous bathophenanthroline complex which was made by the reaction of bathophenanthroline with ferrous sulfate in water and extraction of the complex into isobutanol.

The medium used for the growth of *E. coli* strain K12 wild type was that described by Monod *et al.* as medium 56 (11). The strain AN236 (ilv⁻, arg⁻, purE, thi⁻) was grown in a manner described by Cox *et al.* (1). Cells were harvested by centrifugation at 27,000 g for 10 minutes, washed in cold 0.25M sucrose—0.1 M phosphate buffer pH 7.0 then spun down and resuspended in the same buffer. Membrane fraction was prepared by disruption of cells which was accomplished by sonication with a Branson sonifier at 7-9 amps for 3 minutes in ice. The cell extract was centrifuged at 27,000 g for 20 minutes and the supernatant fraction separated which was then centrifuged at 100,000 g for 2 hours. The resulting gelatinous yellow pellet was resuspended in a mineral volume of 0.1 M phosphate buffer pH 7.0 in the presence of 20% (v/v) glycerol and designed as the membrane fraction. Soluble ATPase was made according to Cox *et al.* (1) by low-ionic-strength treatment.

Heavy beef heart mitochondria were isolated according to Hatefi and Lester (5). ETPH were made by Lee and Ernster procedure (6). Oligomycin-insensitive ATPase was made according to Senior and Brooks through the ammonium sulfate fractionation (18). M.O.P. were isolated by Hansen and Smith procedure (3) except that 0.5 M sucrose and 15 mM $MgCl_2$ were used.

Protein concentration was determined by biuret method (17) with bovin serum albumin as a standard.

All ATPase assays were run in a medium which gives maximal coupling as measured by ATP dependent reverse electron transport (.45 M sucrose, 50 mM glycylglycine, 12 mM MgO, pH 8.0 referred to as SGM8 medium). The ATPase activity was measured at 30°C as follows: enzyme was incubated with inhibitor and uncoupler 5 minutes in 1.9 ml total volume, 10 μmoles ATP in 0.1 ml was added and incubation continued five minutes. The reaction was stopped by the addition of 3.0 ml cold 10% TCA. P_i was measured according to Wadkins and Lehninger (20).

NADH ubiquinone reductase, all cytochrome c reductases and cytochrome oxidase were assayed in SGM8 medium at 30°C (16). Succinate ubiquinone reductase was measured in 0.08 M phosphate buffer pH 7.4 in presence of 8×10^{-5} M EDTA.

E. coli NADH and other oxidase systems were assayed as follows: enzyme (0.5 ~ 1.0 mg protein/assay) was incubated in 1.4 ml of buffer (0.1 M phosphate pH 7.0 for the strain K 12 wild type SGM8 for the strain AN236) for 3 minutes in the presence or absence of the indicated amounts of inhibitors and uncoupler, 50 μl of NADH (0.1 M), 50 μl

of lactate (0.1 M), 50 μl of succinate (0.1 M) or 10 μl of duroquinone (20 mg/ml) and 20 μl of dithiothreitol (30 mg/ml) were added as substrates to start the reaction for assay of NADH oxidase, lactate oxidase, succinate oxidase and duroquinol oxidase respectively.

E. coli NADH- menadione rductase activity was measured by following oxygen uptake from the non-enzymatic autooxidation of menadiol. Enzyme and 50 μl KCN (0.1 M) were incubated in 1.4 ml buffer in the presence or absence of inhibitors and uncoupler for 3 minutes. The reaction was started by addition of 25 μl menadione (75 mM) and 50 μl NADH (0.1 M). To determine the hydroxyquinoline-N-oxide (HOQNO) insensitive rate, enzyme, 30 μl KCN (0.1 M) and 10 μl HOQNO (4.0 mg/ml) were incubated with the appropriate inhibitors and uncoupler, and the reaction was started as described above.

Results

Bathophenanthroline (BP) inhibits electron transport of beef heart mitochondria in all three complexes, NADH- ubiquinone reductase, ubiquinolcytochrome C reductase and cytochrome oxidase. Table I shows the maximum percent of inhibition observed and the inhibition of these three systems is partially reversed by uncoupling agents in preparations which are coupled. Uncoupler has no effect on the BP inhibition of succinate-ubiquinone reductase which does not contain a coupling site. Dithizone, a Cu chelator, inhibits cytochrome oxidase but the inhibition of this complex is not reversed by the uncoupler as shown in Table I. NADH and succinate ubiquinol reductase can be inhibited by hydrophilic bathophenanthroline sulfonate or orthophenanthroline at surface sites depending on the orientation of the membrane (4). Inhibition at these sites is not reversed by uncouplers under our assay condition. Any slight inhibition of the three coupling complexes

TABLE 1. *Effect of Uncouplers on Chelator Inhibition of Mitochondrial Electron Transport.*

partial reactions	coupling site	maximum percent of inhibition by chelators	maximum percent of reversal by uncouplers
		BP	CCCP
NADH➤ubiquinone	I	70	20
ubiquinone➤cytochrome c	II	50	86.7
cytochrome c➤oxidase	III	56.5	26.1
succinate➤ubiquinone	none	15.4	
		BP	gramacidin
succinate➤cytochrome c	II	85	2.5
cytochrome c➤oxidase	III	65.9	34.1
NADH➤ubiquinone	I	75	8.3
		dithizone	CCCP
cytochrome c➤oxidase	III	73.9	—43.5

Concentrations: BP, 0.05 μmoles/ml; CCCP, 5.5 μmoles/ml for cytochrome oxidase and 1.7 μmoles/ml for all other reactions. Gramacidin, 11 nmoles/ml for cytochrome oxidase and 7 nmoles/ml for all others. Dithizone, 4.5 x 10^{-5}M. It should be noted that the effect of gramacidin on cytochrome oxidase is not dependent on the presence of monovalent cations.

by the preformed chelate, ferrous bathophenanthroline complex are not reversed by uncoupling agents either. The reversal is effected by various uncoupling agents such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), α,α bis (hexa-fluoro acetonyl)-acetone (1799) and 5-chloro-3-tert-butyl-2-chloro-4'-nitro-salicylanilide (S13). The uncoupler, pentachlorophenol, causes an inhibition of electron transport activities by itself, which is increased with chelators. No reversal by the ionophore, gramacidin, in partial reactions such as succinate → cytochrome c and NADH → ubiquinone has been obseerved as also shown in Table I. Although gramacidin does show a significant reversal of inhibition of cytochrome oxidase, its effects are at 100 times the concentration required for uncoupling.

Table II shows the inhibition of *E. coli* electron transport at two sites associated with energy coupling by the lipophilic chelator, bathophenanthroline. It gives good inhibition in both systems; NADH oxidase and duroquinone oxidase in both strains K 12 wild type and AN236 (ilv⁻, arg⁻, purE, thi⁻). Less inhibition has been observed in the system of succinate oxidase by this chelator. Hydrophilic chelating agent such as a,a'-dipyridyl and bathophenanthroline sulfonate show only 11.8% and 0% inhibition respectively of the NADH oxidase system. This indicates that the chelator sensitive protein which affects electron transport activity is buried deep in the lipid layer of the membrane.

The comparison of BP concentrations which cause 50% inhibition of electron transport activities and ATPase activity is listed in the Table III. Since at a similar level of BP, the same extent of inhibition is observed in both the ATP synthesis system and electron transport chain, we propose that a common functional intermediate, which participates in both systems, is the bathophenanthroline sensitive component.

TABLE 2. *Effect of Uncoupler on Chelator Inhibition of* E. coli *Plasma Membrane Electron Transport.*

partial reactions	coupling site	maximum percent of inhibition by chelator-BP	maximum percent reversal by uncoupler CCCP
NADH→oxidase (K-12)	I and II	81	13.2
NADH→menadione	I	51.5	19.4
lactate→oxidase	II	50	17
succinate→oxidase	II	34.3	7.3
duroquinone→oxidase	II	65.4	24.4
NADH→oxidase (AN236)	I and II	61.8	17
NADH→menadione	I	86.8	20
succinate→oxidase	II	34	12
duroquinone→oxidase	II	82.4	27.4

Concentrations: BP, 3.2 x 10^{-4}M for both K12 W.T. NADH oxidase and lactate oxidase; 6.3 x 10^{-6}M for K12 W.T. NADH—menadione reductase, 1.0 x 10^{-4}M for K12 W.T. succinate oxidase, duroquinone oxidase and AN236 NADH oxidase, duroquinone oxidase; 5 x 10^{-4}M for AN236 succinate oxidase CCCP, 3 x 10^{-5}M for both K12 W.T. succinate oxidase and AN236 duroquinone oxidase; 7 x 10^{-5}M for K12 W.T. NADH-menadione reductase; 8 x 10^{-5}M for the other two K12 W.T. reactions; 2 x 10^{-5}M for AN236 NADH-menadione reductase and 4 x 10^{-5}M for the other AN236 reactions.

Both mitochondria and *E. coli* membrane bound ATPase activities are inhibited by BP and can be reversed by various uncoupling agents as shown in Table IV and Table V respectively. The lack of effect with pentachlorophenol in mitochondria contrasts with *E. coli* where this uncoupler causes 40% reversal of BP inhibited ATPase activity. Study with other chelators on *E. coli* membrane bound ATPase, such as 200 μg of orthophenanthroline and 40 μg of a,a'-dipyridyl show less than 10% and 15% inhibition respectively. Hydrophilic chelators such as bathophenanthroline sulfonate show essentially no inhibition. The copper chelator diphenylthiocarbazone (dithizone) also shows no inhibitory effect. These results suggest that a BP sensitive component participates in energy linked reactions and is hidden in a lipophilic region within the membrane. The preformed ferrous-bathophenanthroline shows no inhibition of *E. coli* ATPase activities in both membrane bound and solubilized form; but this preformed chelate can give up to 70 percent inhibition of mitochondrial ATPase activity and the reversal of inhibition by CCCP is only 45 percent as compared to 90 percent reversal of inhibition by free chelator. This implies that ferrous-bathophenanthroline has a different mode on inhibition than the free chelator. Table

TABLE 3. *The Comparison of Bathophenanthroline Concentration at 50% Inhibition of Both ATPase Activity and Electron Transport Activities.*

enzyme systems	beef-heart mitochondria	E. coli K12 wild type
NADH→ubiquinone	20 μM	--
ubiquinone→cytochrome c	13 μM	--
cytochrome c→oxidase	6 μM	--
membrane bound ATPase	8 μM	72 μM
NADH→oxidase	--	50 μM
NADH→menadione	--	63 μM
lactate→oxidase	--	70 μM
duroquinone→oxidase	--	60 μM

TABLE 4. *Prevention of Bathophenanthroline Inhibition of Mitochondria Membrane Bound ATPase Activity by Various Uncoupling Agents.*

Uncoupling agent	Maximum percent of inhibition by chelator 30 μg BP	Maximum percent of reversal by uncouplers
3 μg CCCP	90	80
3 μg S_6	90	150
20 nmoles gramicidin + 100 μmoles potassium acetate	90	0
10 nmoles valinomycin + 14 nmoles nigericin + 100 μmoles potassium acetate	90	0
	50 μg BP	
0.33 μmoles 1799	100	13.3
0.5 μmoles DNP	100	23.5

S_6, 5-chloro-3-(p-chlorophenol)-4'-chlorosalicylanilide
1799, α, α-bis (hexa-fluoro-acetonyl) acetone
DNP 2.4-dinitrophenol

IV and Table V also show that ionophores are essentially ineffective in reversing ATPase activities from both *E. coli* and mitochondria. The soluble ATPase extracted from either mitochondria or *E. coli* is insensitive to BP. This indicates the chelator sensitive site is in the membrane.

TABLE 5. *Prevention of Bathophenanthroline Inhibition of* E. coli *K 12 Wild Type Membrane Bound ATPase Activity by Various Uncoupling Agents.*

Uncoupling agent	Maximum percent of inhibition by chelator 50 μg BP	Maximum percent of reversal by uncoupler
3 μg CCCP	70	60
1 μg TTFB	70	60
1 μg S_6	70	50
6 μg pentachlorophenol	70	40
4 μg dicumarol	70	60
20 nmoles gramicidin + 100 μmoles potassium acetate	70	0
10 nmoles valinomycin + 14 nmoles nigericin + 100 μmoles potassium acetate	70	0

TTFB, 4, 5, 6, 7 Tetrachloro-2-trifluoromethylbenzimidazole.

Table VI shows that not only does oligomycin inhibit mitochondrial ATPase activity very nicely in the presence of CCCP but also oligomycin inhibits this enzyme activity after restoration by CCCP in the presence of bathophenanthroline. The fact that oligomycin does not reverse the bathophenanthroline effect, together with the fact that CCCP does not reverse oligomycin inhibition indicates that the BP binding site and the oligomycin sensitive site are not identical.

The inhibition by N,N'-dicyclohexyl carbodiimide (DCCD) of *E. coli* ATPase activity is shown in Table VII. Evidence is also shown in this Table that CCCP does not reverse the DCCD effect on ATPase activity, bathophenanthroline inhibition is not reversed by DCCD and DCCD inhibits ATPase activity reversed by CCCP in the presence of BP. We conclude here that bathophenanthroline binding component is different from the DCCD binding protein.

TABLE 6. *Inhibition by Oligomycin of Mitochondria Membrane Bound ATPase Activity in the Presence of both CCCP and Bathophenanthroline.*

μg Bathophenanthroline	μg CCCP	μg oligomycin mg protein	Percent of inhibition in ATPase activity
0	0	1.0	85.8
0	3	1.0	71.5
50	0	0	100
50	0	1.0	100
50	3	0	0
50	3	1.0	42.9

TABLE 7. *Effect of DCCD on ATPase Activity of* E. coli *AN236 in the Presence of BP or CCCP or Both.*

μg of Bp	μg of CCCP	nMoles of DCCD	Percent of inhibition in ATPase activity
0	0	5	50
0	0	10	40
0	5	5	50
0	5	10	40
10	0	0	30
30	0	0	40
10	0	5	30
30	0	5	40
10	5	0	0
30	5	0	0
10	5	5	20
30	5	5	20

DCCD, N,N'-dicyclohexyl carbodiimide.

Discussion

These studies using a metal chelator, bathophenanthroline, as an inhibitor of both electron transport and energy coupling systems show effects that must be related to the location of non-heme iron and copper proteins in the membrane. Since both systems are inhibited by bathophenanthroline at similar concentrations, it strongly indicates that either a common functional component which is bathophenanthroline sensitive, may participate in both systems, or an energy transport component, which is sensitive to bathophenanthroline, may be required to react with an electron transport component.

The fact that uncouplers reverse chelator inhibition suggests a competition between uncoupler and chelator either for the same site on closely adjacent sites will be the basis for the prevention of chelator inhibition by the uncoupler.

Since ionophores, such a gramacidin, valinomycin and nigericin are essentially ineffective in reversing the bathophenanthroline inhibition of either ATPase activity or electron transport activities; it is therefore suggested that ion gradients can not be the primary reactions in the energy transfer.

The preformed ferrous-bathophenanthroline shows no inhibition of *E. coli* ATPase activity. This is in contrast to that of mitochondria in which the chelate gives a good inhibition, but CCCP causes only up to 50% reversal compared to the reversal of bathophenanthroline inhibition. A different mechanism of inhibition by ferrous-bathophenanthroline is indicated in the eukaryotic organells which is probably missing in the prokaryotic *E. coli* cells.

The lack of inhibition of both energy conserving system and electron transport system by hydrophilic chelators indicates that the common functional intermediate is protected by a hydrophobic environment of the membrane. The fact that bathophenanthroline fails to inhibit soluble ATPase from both *E. coli* and mitochondria also implies that the bathophenanthroline responsive site is probably hidden deep in the membrane but not in the enzyme itself.

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