LIPOPHILIC CHELATOR INHIBITION OF MITOCHONDRIAL MEMBRANE-BOUND ATPase ACTIVITY AND PREVENTION OF INHIBITION BY UNCOUPLERS

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SUMMARY

Oligomycin sensitive, membrane bound ATPase of beef heart mitochondria is strongly inhibited by the lipophilic chelator bathophenanthroline. The inhibition is reversed by uncouplers such as carbonylcyanide-3-chlorophenyl hydrazone but not by ionophores such as gramacidin. Oligomycin-insensitive soluble ATPase is not inhibited by bathophenanthroline. Since the inhibition effects parallel bathophenanthroline inhibition of electron transport associated with coupling sites and uncoupler reversal is similar we propose metalloproteins function at the juncture of the electron transport and energy coupling systems.

INTRODUCTION

All phosphorylating sites in the mitochondrial chain share the following characteristic: all are uncoupled by the same uncouplers (1); the energy transfer from the electron transport chain to ATP formation at all three sites is inhibited by the same energy transfer inhibitors such as oligomycin (1-3) [the guanidines as an exception show some site specificity (3)] and the energy conserved from electron flow through one site can be used to drive reverse electron flow through the other phosphorylating sites (4). Until recently no inhibitor of electron transport could be found which 1) directly inhibited electron flow associated with all coupling sites, 2) the inhibition of which could be reversed by uncouplers and 3) which gave a clue as to possible mode of inhibition. We have recently reported that the metal chelator bathophenanthroline inhibits in a manner consistent with all of the above criteria (5). In this paper we show that at concentrations which inhibit electron transport bathophenanthroline also inhibits

membrane bound ATPase activity as completely as oligomycin but does not inhibit oligomycin insensitive ATPase. Unlike oligomycin, the uncoupler carbonylcyanide-3-chlorophenyl hydrazone (CCCP) reverses chelator inhibition and oligomycin can prevent the reversal.

METHODS

Heavy and light mitochondria were isolated according to Hatefi and Lester (6). ETP_H were made by a Lee and Ernster procedure (7). Oligomycin-insensitive ATPase was made according to Senior and Brooks through the ammonium sulfate fractionation (8). All 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 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 vol. 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. Pi was measured according to Lindberg and Ernster (9). NADH-ubiquinone reductase activity was measured as previously described (5).

RESULTS

Bathophenanthroline inhibits membrane bound ATPase in ETP_H at concentrations which inhibit electron transport. The inhibition is reversed by the uncoupler CCCP as shown in Table I. Table II shows that not only does oligomycin inhibit ATPase activity in the presence of CCCP as previously reported (1), but that oligomycin inhibits the the ATPase activity restored by CCCP in the presence of bathophenanthroline. Other uncouplers also reverse the bathophenanthroline inhibition of ATPase. As shown in Table III the ionophore gramicidin is ineffective in reversing the bathophenanthroline inhibition of either electron transport (5) or ATPase activities. Table IV shows that the

Table I

Comparison of CCCP Preventable Inhibition by Bathophenanthroline of Membrane Bound ATPase Activity with Inhibition of Electron Transport Through Coupling Site I

µg Bathophenanthroline/assay	µg CCCP/assay	ATPase Activity[a] µmoles Pi/min/mg prot.	NADH-ubiquinone[b] Reductase nmoles NADH/min/mg prot.
Experiment 1			
0	0	1.0	32
0	3	1.0	33
30	0	0.2	17
30	1	0.5	19
30	3	0.8	37
50	0	0.1	17
50	1	0.2	17
50	3	0.8	24
Experiment 2			
no additions		1.2	48
1 µg oligomycin/mg prot.		0.2	48

[a]protein was 0.29 mg for experiments 1 and 2

[b]For electron transport studies 60 ng oligomycin per mg protein was added to induce maximum coupling. 0.18 mg protein in experiment 1 and 0.09 mg protein in experiment 2.

Table II

Inhibition by Oligomycin of Membrane Bound ATPase Activity in the Presence of Both CCCP and Bathophenanthroline

	Additions		
µg Bathophenanthroline	µg CCCP	µg Oligomycin mg prot.	ATPase activity µmole Pi/min/mg prot.
0	0	0	1.4
0	3	0	1.7
0	0	1.0	0.2
0	3	1.0	0.4
50	0	0	0.0
50	0	1.0	0.0
50	3	0	1.5
50	3	1.0	0.8

protein was 0.27 mg

Table III

Prevention of Bathophenanthroline Inhibition of Membrane Bound ATPase Activity by Various Uncoupling Agents

Uncoupling agent	ATPase Activity μmoles Pi/min/mg prot.	
Experiment 1[a]	no Bathophenanthroline	+30 μg Bathophenanthroline
No uncoupling agent	1.0	0.1
3 μg CCCP	1.1	0.8
3 μg S_6[c]	0.4	0.7
30 mM sodium arsenate	0.9	0.2
20 nmoles Gramicidin + 100 μmoles potassium acetate	1.4	0.1
10 nmoles Valinomycin + 14 nmoles Nigericin + 100 moles potassium acetate	1.2	0.1
Experiment 2[b]	no Bathophenanthroline	+50 μg Bathophenanthroline
No uncoupling agent	1.4	0.0
0.3 μmoles 1799[c]	1.5	0.2
0.5 μmoles DNP[c]	1.7	0.4

[a,b] protein was 0.27 mg

[c] S_6, 5-chloro-3-(p-chlorophenol)-4'-chlorosalicylanilide

1779, α,α-bis (hexafluoroacetonyl) acetone

DNP, 2,4-dinitrophenol

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soluble oligomycin-insensitive ATPase is also insensitive to bathophenanthroline.

Studies with preformed ferrous-bathophenanthroline (55 μg per assay) show that although the chelate can give up to 60 to 70 percent inhibition of ATPase activity the reversal of inhibition by CCCP is less (45 percent reversal as compared to 80 to 90 percent reversal of inhibition by free

Table IV

Effect of Bathophenanthroline and Oligomycin on Soluble ATPase Activity

additions	ATPase activity μmoles Pi/min/mg
none	0.30
2 μg Oligomycin	0.33
50 μg Bathophenanthroline	0.33

protein was 0.37 mg

chelator). The ferrous bathophenanthroline chelate has essentially no effect on electron transport (5). Ferrous bathophenanthroline chelate inhibits ATPase activity in mode different from bathophenanthroline.

DISCUSSION

The metal chelator, bathophenanthroline shares some properties with the energy transfer inhibitor, oligomycin in that any inhibition of electron transport through the coupling sites is prevented by uncoupling agents (1-4), and both strongly inhibit membrane bound but not soluble ATPase activity (1,3,8). Bathophenanthroline differs from oligomycin in that it strongly inhibits electron transport associated with coupling sites in particles which are no longer stimulated by ADP plus Pi and in that uncouplers can reverse bathophenanthroline inhibition of ATPase activity. Partial inhibition of ATPase in mitochondria by bathophenanthroline and bathophenanthroline sulfonate has previously been reported (10). Because an uncoupling agent must be used in intact mitochondria in order to see high ATPase activity, maximum inhibition could not be shown. The uncoupler used in the previous studies with bathophenanthroline (10) was dinitrophenol which we find to cause incomplete reversal of the ATPase inhibition. Our observations are consistent with the previous observations. (10)

We hypothesize that bathophenanthroline interfers with an energy transfer component or complex which may either react with or contain an electron transport component. This hypothesis is based on the fact that bathophenanthroline causes CCCP preventable inhibitions of both membrane-bound ATPase and electron transport at similar concentrations of bathophenanthroline and CCCP. This hypothesis is consistent with both the failure of bathophenanthroline to inhibit soluble oligomycin-insensitive ATPase and the oligomycin inhibition of the activity of membrane bound ATPase which had been restored by CCCP in the presence of bathophenanthroline. The lack of reversal of bathophenanthroline inhibition of either ATPase activity or electron transport activities (5) by an ionophore implies that ion gradients are secondary rather than primary reactions in energy transfer.

The fact that bathophenanthroline is a metal chelator suggests that the inhibition of both electron transport and ATPase might be caused by chelation of bathophenanthroline with a non-heme iron at coupling sites I and II and copper at site III.

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