EFFECT OF BATHOPHENANTHROLINE AND CARBONYLCYANIDE-m-CHLOROPHENYL HYDRAZONE ON CYTOCHROME C REDUCTASE ACTIVITY OF RESOLVED SUCCINATE CYTOCHROME C REDUCTASE COMPLEX

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Summary Electron transport activity of resolved succinate-cytochrome c reductase complex is inhibited by bathophenanthroline, a hydrophobic chelator of ferrous ions. This inhibition is partially reversed, and more effectively prevented, by carbonylcyanide-m-chlorophenyl hydrazone, an uncoupler of mitochondrial oxidative phosphorylation. This effect of the uncoupler can not be explained by a mechanism in which the uncoupler dissipates a transmembrane proton-motive force as envisioned in the chemiosmotic hypothesis.

Introduction

Multiple iron sulfur proteins have been described as components of the electron transport chain since the discovery of non-heme iron associated with mitochondria (1). Separate iron sulfur proteins have been found in the NADH-ubiquinone reductase (2), succinate-ubiquinone reductase (3), and ubiquinone-cytochrome c reductase (4) segments of the electron transport chain.

Recent interest in iron sulfur proteins has focused on the possibility that these redox components may participate in the primary energy conserving steps of oxidative phosphorylation. Specific chelators of iron frequently have been employed in attempts to implicate iron sulfur proteins in energy conservation. Butow and Racker (5) described conditions under which orthophenanthroline inhibited the ATP-Pi exchange reaction in submitochondrial particles, and Palmer (6) has reported that bathophenanthroline inhibits state 3 respiration and uncoupler stimulated ATPase in rat liver mitochondria.

Recently Phelps and coworkers (7) reported that bathophenanthroline inhibits electron transport at regions of the electron transport chain in the vicinity

of the coupling sites and that this inhibition is reversed by uncouplers such as carbonylcyanide-m-chlorophenyl hydrazone (CCCP). It was concluded that iron sulfur proteins, in a hydrophobic environment, are associated with the first 2 coupling sites on the electron transport chain.

Resolved segments of the electron transport chain offer several advantages for further elucidating the possible role of iron sulfur proteins in energy coupling. One can thus examine discrete sections of the electron transport chain, including a small number of redox components, in the absence of auxillary proteins such as the ATPase which are required for oxidative phosphorylation.

In the experiments reported below we have employed succinate-cytochrome c reductase complex to examine the interaction of bathophenanthroline with this portion of the electron transport chain and to test whether the uncoupler CCCP can relieve chelator inhibition in a redox system which is incapable of the terminal steps of energy coupling.

Materials and Methods

Bovine heart mitochondria and succinate-cytochrome c reductase complex were prepared according to Yamashita and Racker (8). The reductase complex, obtained in a cholate containing buffer, was dialyzed 18 hrs against 20 volumes of .25 M sucrose-10 mM sodium phosphate-0.5 mM EDTA, pH 7.4, with 3 changes of dialysis buffer. Thereafter the insoluble reductase complex was recovered by centrifugation for 90 min at 78,500g, suspended in 0.1 M sodium phosphate, pH 7.4 and stored at -70°C prior to use. Freshly prepared mitochondria were stored overnight at 4°C as a pellet, suspended in .25 M sucrose, and thus used without freezing.

Mitochondria and reductase complex were incubated with bathophenanthroline in "SGM8" medium (7). Further details, such as the additions of CCCP, are described in the figure legends. Succinate-cytochrome c reductase activity also was measured as described (7), except the concentration of cyanide was lowered to 0.5 mM. Succinate dehydrogenase activity was measured according to King (8).

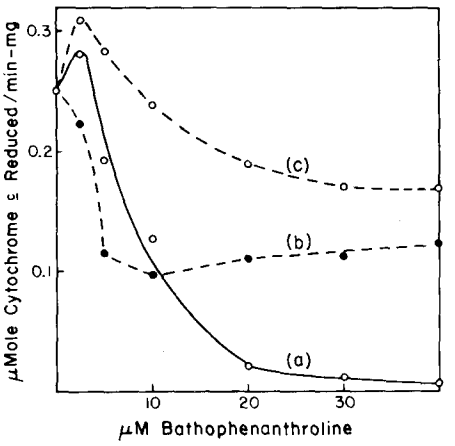

Figure 1 Effect of bathophenanthroline and CCCP on succinate-cytochrome c reductase activity of beef heart mitochondria. Mitochondria were incubated 4 min at 30°C with bathophenanthroline as described by Phelps et al (7). Curve (a) was obtained after incubation with bathophenanthroline and no CCCP. Curve (b) was obtained by addition of 5 μM CCCP after incubation with bathophenanthroline. Curve (c) was obtained by incubating mitochondria 30 seconds with 5 μM CCCP prior to incubation with bathophenanthroline.

Results and Discussion

Experiments with electron transport particles (ETPH) established that bathophenanthroline inhibits electron transport activities, including succinate-cytochrome c reductase, and such inhibition was partially prevented by uncouplers such as CCCP (7). Similar results are obtained with fresh heart mitochondria as shown in Figure 1. Succinate-cytochrome c reductase is essentially completely inhibited with 40 μM bathophenanthroline, and subsequent addition of CCCP restores activity to approximately 50 percent of the original. At all concentrations of bathophenanthroline the reductase activity was greater when CCCP was added prior to, rather than after, the chelator. Figure 1 also illustrates an anomalous effect of CCCP which complicates interpretations of its mode of action. After incubation with low concentrations of bathophenanthroline, which partially inhibit electron transport, subsequent addition of CCCP results in further inhibiton. This effect of CCCP is not evident when uncoupler is added prior to bathophenanthroline.

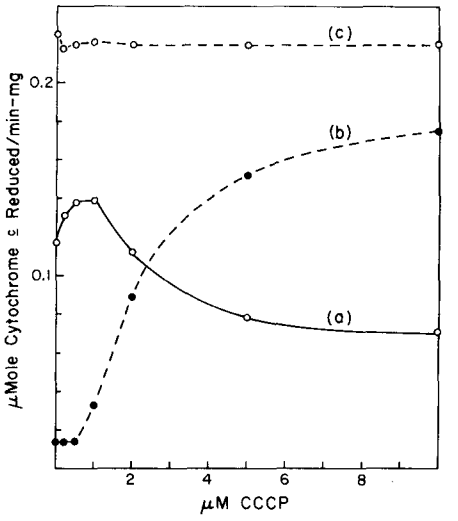

Figure 2 Effect of CCCP on succinate-cytochrome c reductase activity of mitochondria after incubation with bathophenanthroline. Mitochondria were incubated with bathophenanthroline as in Figure 1 and CCCP added subsequent to the bathophenanthroline. Curve (a) was obtained by adding various concentrations of CCCP after incubation with 5 μM bathophenanthroline. Curve (b) was obtained by adding CCCP after incubation with 30 μM bathophenanthroline. Curve (c) is a control which was obtained by adding various concentrations of CCCP after incubating mitochondria 4 min with no bathophenanthroline.

Figure 2 shows the effect of various amounts of CCCP when added subsequent to 5 μM and 30 μM bathophenanthroline. When added subsequent to 5 μM bathophenanthroline, there is a slight stimulation of cytochrome c reductase activity by concentrations of CCCP of 1 μM or less; as the uncoupler is increased to 5 and 10 μM there is a slight, but significant, inhibition in addition to that caused by bathophenanthroline alone. When CCCP is added subsequent to 30 μM bathophenanthroline there is no effect with up to 0.5 μM CCCP, above which concentration CCCP progressively stimulates activity. It is evident that the effects of chelator and uncoupler are complex and appear to be manifested through more than a single site. One possibility is that bathophenanthroline may sensitize a component of the electron transport chain to inhibition by CCCP, which is capable of reacting with cysteine residues (10). In experiments not described here, we have tested for, and been unable to detect, a chemical reaction between CCCP and bathophenanthroline.

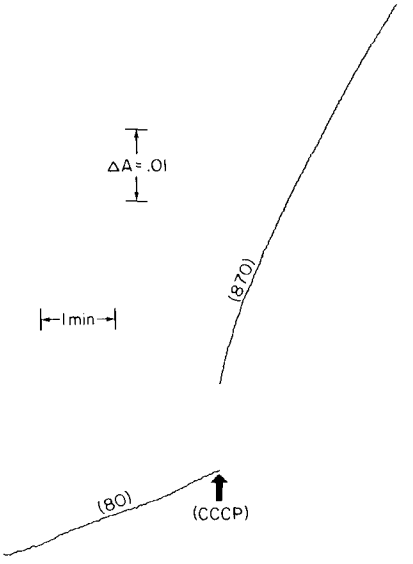

Figure 3 Cytochrome c reduction by succinate-cytochrome c reductase complex after inhibition by 30 μM bathophenanthroline, showing the effect of CCCP. Resolved reductase complex was incubated with bathophenanthroline for 4 min. CCCP (5 μM) was added during the course of the reductase assay as indicated by the heavy arrow. Numbers in parenthesis are rates of cytochrome c reduction, in nmole/min-mg. Particles not treated with bathophenanthroline had activity of 2800 nmole/min-mg.

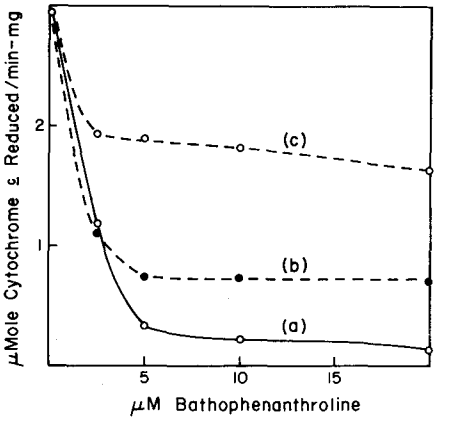

Figure 4 Effect of bathophenanthroline and CCCP on succinate-cytochrome c reductase activity of resolved succinate-cytochrome c reductase complex. Curve (a) was obtained after incubation with bathophenanthroline and no CCCP. Curve (b) was obtained by addition of 5 μM CCCP after incubation with bathophenanthroline. Curve (c) was obtained by incubating reductase complex 30 seconds with 5 μM CCCP prior to incubation with bathophenanthroline.

TABLE I

Effects of Ethanol, Antimycin, Bathophenanthroline, and CCCP on Succinate-Cytochrome c Reductase Activity of Resolved Reductase Complex

Additions	μMole cytochrome c reduced/min-mg
None	1.30
Ethanol	1.40
CCCP	1.23
Antimycin	0.08
Bathophenanthroline	0.17
Bathophenanthroline + CCCP	0.59
Bathophenanthroline + CCCP + antimycin	0.09
Bathophenanthroline + ethanol	0.19

Where indicated, components were added to obtain the following concentrations: ethanol, 5 μl per ml; CCCP, 5 μM; antimycin, 1 μg per mg of protein; and bathophenanthroline, 20 μM.

It was of particular interest to test if bathophenanthroline inhibits electron transport in resolved succinate-cytochrome c reductase complex in a manner which is reversible by uncoupler. Figure 3 shows the effect of CCCP on the resolved complex when electron transport was inhibited from a control value of 2.8 μmole/min-mg to 0.08 μmole/min-mg by 30 μM bathophenanthroline. Addition of uncoupler caused an immediate 10 fold increase in the rate of cytochrome c reduction.

The inhibitory effect of various concentrations of bathophenanthroline on resolved succinate-cytochrome c reductase complex is shown in Figure 4. The inhibition by bathophenanthroline is partially reversed, and more effectively prevented, by the uncoupler. This result is qualitatively the same as was obtained with mitochondria. Succinate dehydrogenase activity remained constant, at 8 μeq/min-mg, with concentrations of bathophenanthroline as high as 100 μM. Thus the site of bathophenanthroline inhibition in the resolved

complex is probably the non-heme iron of complex III (4) as was found with ETPH (7).

Table I summarizes results of control experiments which established that the effects of chelator and uncoupler are not attributable to the small amounts of ethanol employed in their addition. The electron transport which is restored by uncoupler is sensitive to antimycin and thus represents electron transfer through the cytochrome b-c_1 complex.

Resolved succinate-cytochrome c reductase complex has no ATP-Pi exchange or ATPase activity. It seems highly unlikely that the resolved complex is capable of generating an asymmetric proton-motive force. At the same time, the above results indicate that bathophenanthroline and CCCP interact with the resolved complex in the same manner as with intact mitochondria. We conclude that inhibition of electron transport by bathophenanthroline, and its release by CCCP, do not involve interaction with the ATPase (cf 6). Also, the stimulation of bathophenanthroline controlled electron transport by CCCP cannot be explained by a mechanism in which the uncoupler dissipates a transmembrane proton-motive force. Instead, it appears that CCCP interacts directly with a component of the electron transport chain and it remains to be established whether this effect of CCCP is synonymous with its ability to uncouple oxidative phosphorylation.

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