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INHIBITION OF CYTOCHROME *c* OXIDASE BY HYDROPHOBIC METAL CHELATORS

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Summary

Cytochrome oxidase, either purified form or in the intact mitochondria, is inhibited by lipophilic bathophenanthroline and bathocuproine, but unaffected by the hydrophilic sulfonated form of these chelators. Orthophenanthroline and neocuproine exhibit no inhibitory effects. Difference spectra of bathophenanthroline and bathocuproine-treated oxidase show decreases in the 830 nm absorption peak and differences in the 535—540 nm region when CN^- is present. Inhibition by lipophilic bathophenanthroline involves intrinsic copper and is not affected by exogenous copper levels. This evidence suggests that oxidase inhibition occurs at the copper protein and that the chelator reactive site is not accessible to hydrophilic chelators.

The participation of copper-containing protein in cytochrome *c* oxidase has been determined by several experimental approaches. The 830 nm peak of the copper protein cytochrome oxidase disappears upon reduction [1,2], with CN^- sensitive copper contributing approximately half of the peak amplitude [3]. In addition, electron paramagnetic resonance studies have shown both endogenous and exogenous copper to undergo spectral changes upon reduction [4], while only extraneous copper can be chelated by bathocuproine sulfonate [5]. Chelation of intrinsic copper by bathocuproine sulfonate has been reported only in the presence of detergents. Sodium dodecylsulfate has been employed to permit copper removal by CN , complete reactivity with sulfhydryl reagent, and chelation and inhibition of cytochrome oxidase activity [6,7]. Mason and Ganapthy also reported a decrease in turnover number by bathocuproine sulfonate only when Tween 80 was present [8]. Deoxychelate, however, would not allow bathocuproine sulfonate to inhibit oxidase activity [2].

In the present study, the effects of several hydrophilic and lipophilic chelators on cytochrome oxidase activity and its visible spectrum were investigated. The results indicate that only the diphenyl derivatives of the phenanthroline and cuproine chelators are effective in both inhibition of activity and decrease in 830 nm peak. Other less lipophilic chelators had no inhibitory effect on activity or effect on the copper protein absorption peak.

Cytochrome oxidase was obtained by deoxycholate fractionation according to Hall and Crane [9] using mitochondria prepared by the method of Crane et al. except that 1 M Tris was used to adjust pH. 0.32 mg deoxycholate/mg protein was used in the fractionation. Intact mitochondria were isolated as described previously [10] with the inclusion of 2 washes with 0.15 M KCl. Cytochrome oxidase activity was measured polarigraphically in a mixture 83.4 mM sodium phosphate (pH 7.4) and 83.4 μM EDTA. 5 μmoles sodium ascorbate and 1.2 mg *N,N,N',N'*-tetramethyl phenylenediamine were used as substrate with 0.4 mg cytochrome *c* (Sigma, Type VI).

TABLE I

EFFECT OF CHELATORS ON CYTOCHROME OXIDASE ACTIVITY

Chelator	Concentration at maximum effect (μM)	Effect	
Bathophenanthroline	55	55—60%	inhibition
Bathocuproine	14	62—75%	inhibition
Diphenylthiocarbazone (dithizone)	44	40%	inhibition
1,10-Phenanthroline	550	40%	stimulation
Neocuproine	55	0—3%	stimulation
α,α'-Dipyridyl	110	9%	stimulation
Bathophenanthroline sulfonate	519	no effect	
Bathocuproine sulfonate	138	no effect	

The inhibition of cytochrome oxidase is shown in Table I. Studies using washed mitochondrial particles sonicated in the presence of $FeSO_4$ indicate that the membrane is permeable to bathophenanthroline but not to orthophenanthroline or bathophenanthroline sulfonate. However, addition of detergent allows the latter two chelators to bind the trapped iron. The data for phenanthroline-type chelators is consistent with their observed permeabilities. The variable stimulation by orthophenanthroline is not observed in all membrane preparations and cannot be explained at this time.

One experiment involving the effect of varying bathophenanthroline concentration on oxidase activity is seen in Fig. 1. At no time in our experiments was inhibition greater than 75% observed. This indicates that either all copper is not bound or a non-copper-requiring pathway is operative. The data in Table I and Fig. 1 are the same for oxidase activity in intact mitochondria or the fractionated enzyme.

The difference spectra of cytochrome oxidase incubated with bathophenanthroline and bathocuproine in the presence of KCN are shown in Fig. 2. Both chelators allow the decrease of the 830 nm peak, and do not alter the amplitude of the 605 nm peak upon reduction of the oxidase. However,

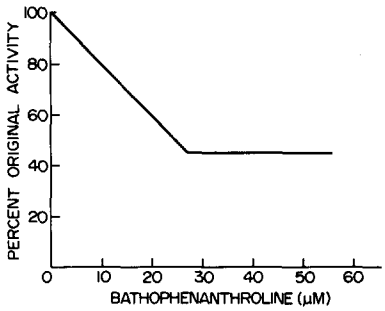

Fig.1. Effect of increasing bathophenanthroline on cytochrome oxidase activity. All determinations were made after 5-min incubation with bathophenanthroline. The control rate was 5.8 μatoms O/min per mg protein.

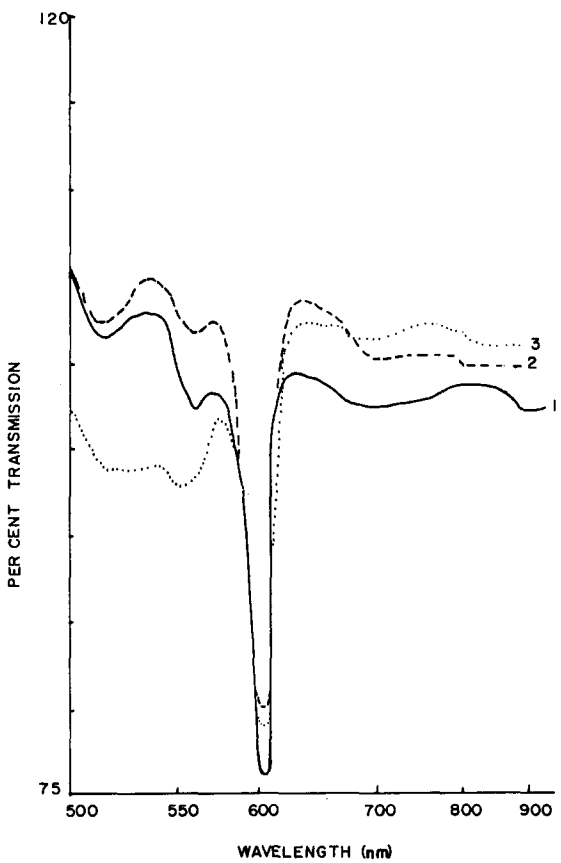

Fig. 2. Difference spectra of deoxycholate green fractions treated with chelators. Measurements of dithionite reduced vs ferricyanide oxidized samples containing 2.5 mg protein and KCN were made on a Beckman DK-2 after 5 min incubation. Spectra were recorded using the 75—125% T scale. Deoxycholate was used to clarify the protein samples. Curve 1, control; Curve 2, bathocuproine; Curve 3, bathophenanthroline.

bathophenanthroline but not bathocuproine caused an increase in absorption at 540 nm upon reduction. This corresponds with the absorption peak for an Fe^{2+}—bathophenanthroline complex and the reduced sample attains a red color. The Cu^{+}—bathophenanthroline complex has an absorption maximum at 492 nm. However, neither orthophenanthroline nor bathophenanthroline sulfonate had an effect on the difference spectra. Diphenylthiocarbazone, a copper specific chelator, also causes a decrease in 830 nm amplitude, confirming the participation of copper in the 830 band and the chelation of copper by bathophenanthroline and bathocuproine.

That bathophenanthroline is interacting with intrinsic copper is shown in the following experiments. In our studies, the addition of dodecylsulfate or deoxycholate did not allow either bathophenanthroline sulfonate or bathocuproine sulfonate to inhibit activity. Furthermore, in preparations of cytochrome oxidase clarified with deoxycholate, bathophenanthroline sulfonate did not alter the 830nm absorption band. This also indicates that the intrinsic copper is responsible for the 830 nm spectrum. No difference in extent of bathophenanthroline inhibition was observed in mitochondria washed with

TABLE II

CHELATOR INHIBITIONS ON BATHOCUPROINE-WASHED MITOCHONDRIA

	% Inhibition		
	Before wash	After wash	After wash + Cu^{2+}(*)
Bathophenanthroline	60	55	50
Cu^{2+}—Bathophenanthroline	50	20	45

*0.2 nmoles $CuSO_4$/0.1 mg protein added.
Control value = 1.5 μmole 0/min/mg protein

bathocuproine sulfonate. It has been shown previously that bathocuproine sulfonate removes adventitious copper [12]. However, as indicated in Table II, a marked difference in inhibition by a performed Cu^{2+}—bathophenanthroline chelate was seen. Restoration of inhibition upon addition of Cu^{2+} indicates that the bathocuproine sulfonate treatment has removed copper from the enzyme. This copper however does not relate to inhibition with lipophilic phenanthroline chelators.

The effect on copper protein absorption spectra and inhibition of cytochrome oxidase activity by lipophilic chelators exclusively (in the absence of detergents) indicates that the copper site of cytochrome oxidase is buried within the enzyme and inaccessible to impermeable reagents.

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