PURIFICATION AND PROPERTIES OF CYTOCHROME OXIDASE

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The cytochrome oxidase system catalyzes the oxidation of reduced cytochrome c by molecular oxygen and has been isolated in a soluble form as a protein-bile salt complex by many investigators; Smith and Stotz (1954), Okunuki et al. (1958), Yonetani (1960). The present communication describes the isolation and general properties of a cytochrome oxidase preparation from beef heart mitochondria. This preparation, containing equimolar amounts of cytochrome a and copper, is of high spectral purity and of high activity (Q_{O_2} = 55,000 - 60,000 at 38°).

The isolation procedure is a modification of a procedure described previously by Hatefi (1958). All operations were performed at 0-5° and all centrifugations were carried out in a Spinco Model L ultracentrifuge at 30-40,000 rpm.

(a) Beef heart mitochondria were treated with t-amyl alcohol (10% v/v) for 5 min at 20° as described by Basford et al. (1957). After centrifugation, the hard packed green-brown residue was collected, washed with a mixture of 0.9% KCl and mM succinate, and resuspended in 0.25 M sucrose containing mM succinate at a concentration of 40-45 mg protein/ml.

(b) Cytochrome oxidase was extracted by adding 2 mg cholate/mg protein and solid KCl to 3 M. After stirring for 30 min the mixture was centrifuged for 5 min at 30,000 rpm. The green-red turbid supernatant was collected and allowed to stand at 4° overnight.

(c) After standing overnight, the cholate-KCl extract was centrifuged for 10 min and the residue discarded. The supernatant was dialyzed and the precipitate formed after dialysis (green residue) was fractionated with $(NH_4)_2 SO_4$

in presence of deoxycholate (1 mg/mg protein). Cytochrome oxidase precipitates between 20-27% saturation as a red oil which is dissolved in 0.25 M sucrose and refractionated in the absence of added deoxycholate. The final precipitate, a dark red oil, is taken up in 0.25 M sucrose to give a clear reddish green solution of cytochrome oxidase. The preparation is readily soluble in sucrose, water, Tris and phosphate buffers but is more stable in sucrose solution. The results of a typical fractionation procedure are summarized in Table 1.

Table 1

Fractionation of Cytochrome Oxidase

Fraction	Total Heme a μmoles	Heme a mμmoles/mg protein	Activity* 38° Q_{O_2} (μl/mg prot./hr)
1. Mitochondria	19.8	1.4	11,800
2. Cholate-KCl-extract	12.5	3.28	15,500
3. Green Residue	7.4	4.75	15,100
4. $(NH_4)_2 SO_4$ fraction 15-27% saturation	5.85	6.6	41,000
5. $(NH_4)_2 SO_4$ fraction 20-27% saturation	5.15	8.15	62,100

* Assayed manometrically as described by Slater (1949). Values extrapolated to infinite cytochrome c concentration.

A 5-8 fold increase in specific activity over the initial mitochondrial activity has been attained with a concomitant increase in cytochrome a concentration, i.e. the specific activity per unit heme a in the final preparation approximates that of the initial mitochondria. The composition of the purified cytochrome oxidase preparation is summarized in Table 2. The copper to heme a ratio approximates 1:1 (analysis of many preparations showed that this ratio ranged from 1.05 - 1.25). No acid extractable flavin was detected before or after tryptic digestion. No enzymic activities were detected with succinate and DPNH as substrates and ferricyanide, cytochrome

Table 2

Composition of Cytochrome Oxidase Preparations

Component	1/mg protein
Heme a	8.1 - 9.2 mμmoles
Iron	8.2 - 9.4 mμmoles
Copper	9.2 - 10.6 mμmoles
Lipid	0.20 - 0.28 mg*
Deoxycholate	0.8 - 1.4 mg

* All preparations contained variable amounts of Coenzyme Q (1.8 - 3 mμmoles/mg protein).

c and oxygen as electron acceptors.

The spectral properties of the enzyme are similar to those reported by other workers [α band - 599 mμ (oxidized), 605 mμ (reduced); β band - 517 mμ (reduced); γ band - 423 mμ (oxidized), 444 mμ (reduced); 444 mμ (reduced)/423 mμ (oxidized) = 1.37]. A previously undescribed absorption band has been noted at 830 mμ which disappears on reduction and reappears on reoxidation. As the reoxidation of this band is inhibited by cyanide it is assumed to be an integral part of the cytochrome oxidase system.

Copper in cytochrome oxidase is tightly bound to the enzyme and is not removed by dialysis against cyanide and a number of chelating agents. An increase in copper concomitant with an increase in cytochrome a concentration and activity has been demonstrated (Table 3). A 1:1 relation between copper and cytochrome a can be demonstrated at all stages of purification. These results suggest that cytochrome oxidase is a cytochrome a - copper protein complex and that copper is involved in cytochrome oxidase activity.

Using the method of Felsenfeld (1960), we have demonstrated that copper in cytochrome oxidase is in the cupric state in oxidized cytochrome oxidase and in the cuprous state in reduced cytochrome oxidase. Copper has been shown to undergo reversible oxidation and reduction and the oxido-reduction

Table 3

Distribution of Copper and Cytochrome a During Fractionation

Fraction	Cytochrome a mμmoles/mg protein	Copper* mμmoles/mg protein	Cu/cyt. a
Mitochondria /	1.52	1.47	0.97
Cholate KCl supernatant	3.5	4.1	1.17
Green Residue	4.65	5.4	1.17
1st $(NH_4)_2 SO_4$ fractionation 20-27% fraction	7.4	7.9	1.07
2nd $(NH_4)_2 SO_4$ fractionation // 20-27% fraction	7.9	8.2	1.04

* Copper was estimated after dialysis for 16 hrs against 0.01 M KCN, in 0.02 M phosphate buffer pH 8.
/ The Q_{O_2} of this fraction was 12,000.
// The Q_{O_2} of this fraction was 57,500.

state of copper is similar to that of cytochrome a under oxidized, reduced and steady state conditions. The reoxidation of reduced copper and cytochrome a is inhibited by cyanide and azide. Copper chelating agents such as Bathocuproine sulfonate and EDTA have no effect on the oxidation and reduction of copper in our preparation. These results have been confirmed by EPR spectroscopic studies (Griffiths, D.E., Wharton, D.C. and Beinert, H., to be published) and support the previous observations of Sands and Beinert (1959), who showed that copper in cytochrome oxidase was reduced by ferrocytochrome c. We have been able to demonstrate a cytochrome c dependent reduction of copper and cytochrome a by ascorbate in some cytochrome oxidase preparations. This behavior is not typical as most preparations show slow reduction of cytochrome a and copper by ascorbate and this rate is greatly increased by addition of small amounts of cytochrome c. In general, we have found that only reagents capable of reducing cytochrome a cause reduction of copper in cytochrome oxidase.

The results presented in this communication, together with the recent work of Takemori (1960), provide strong evidence that cytochrome oxidase is a copper hemoprotein complex containing equimolar amounts of copper and cytochrome a and that copper acts as an electron carrier during terminal electron transport.

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