COPPER IN CYTOCHROME OXIDASE

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The presence of significant amounts of copper in respiratory chain particles was first noted by Keilin and Hartree (1938) which led them to propose that the terminal oxidase might be a copper enzyme. However, their demonstration that cytochrome a_3 satisfied all the criteria of the terminal oxidase cast doubt on the role of copper in the oxidase reaction (Keilin and Hartree, 1939). The demonstration of significant amounts of copper in purified cytochrome oxidase preparations (Eichel et al., 1950; Mackler and Penn, 1957; Okunuki et al., 1958) has revived speculation as to the role of copper in cytochrome oxidase as evidenced by the recent papers of Wainio et al. (1959) and Takemori (1960). Also EPR studies (Sands and Beinert, 1959) have shown that copper in cytochrome oxidase undergoes valence change during the cytochrome oxidase reaction.

Recently, we have isolated a cytochrome oxidase preparation from beef heart mitochondria of high specific activity (Q_{O_2} = 60,000 at 38°) and high spectral purity (Griffiths and Wharton, 1961 a,b). During this purification an increase in copper proportional to the increase in cytochrome a and the specific activity of the preparation has been demonstrated. We have also demonstrated (i) that an equimolar relationship between copper and cytochrome a is maintained at all levels of purification and that this copper moiety is not removed by dialysis against cyanide (cf. Takemori, 1960); (ii) that copper in cytochrome oxidase is reduced by the substrates of the oxidase in a cytochrome c - dependent reaction and that the reoxidation of copper and cytochrome a is inhibited by cyanide and azide (Griffiths and Wharton, 1961 a,c). These results have also been confirmed

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by EPR spectroscopy (Griffiths, Wharton and Beinert, 1961); (iii) that copper chelating agents (EDTA, 8-hydroxyquinoline, potassium ethyl xanthate, neocuproine, bathocuproine sulfonate, sodium diethyldithiocarbamate) have little or no effect on cytochrome oxidase activity. The results presented (Griffiths and Wharton, 1961 a,b,c) provide strong evidence for the contention that copper is involved in cytochrome oxidase activity and that cytochrome oxidase is a copper hemoprotein complex containing equimolar amounts of copper and cytochrome a.

In a recent communication, Yonetani (1960 b) has concluded that copper is not involved in cytochrome oxidase activity as the result of experiments with a cuprous-specific chelating agent, bathocuproine sulfonate (BCS). Using this reagent he was able to demonstrate that the major portion of the copper in his cytochrome oxidase preparation could be trapped in the cuprous state as Cu-BCS and thus constrained from further valency change. He also concluded that copper does not contribute to the spectrum of cytochrome oxidase at 605 mμ and 445 mμ and that the reduction of copper in cytochrome oxidase is a non-specific reaction unrelated to the sequence of reactions which underlie cytochrome oxidase activity.

As these observations and conclusions are contrary to our own we deemed it necessary to examine the effect of BCS on our cytochrome oxidase preparation. On treatment of cytochrome oxidase (15 μM cytochrome a, 17 μM copper in 0.02% (w/v) DOC) with 100 μM BCS at pH 7.2 there was no change in the absorption spectrum in the oxidized state. On reduction with dithionite or ascorbate-cytochrome c there was no increase in absorption at 479 mμ (Cu-BCS formation) as observed by Yonetani (1960 b). No formation of Cu-BCS was observed after several cycles of oxido-reduction by ascorbate-cytochrome c and incubation with BCS for 30 min at 25°. No formation of Cu-BCS was observed after addition of potassium borohydride. Thus, it is apparent that none of the copper in our cytochrome oxidase preparation reacts with BCS. No formation of Cu-BCS was observed when these experiments were carried out in the presence of a non-ionic detergent (0.5% (w/v) Tween 80) and with a cytochrome oxidase preparation which

had been refractionated in the presence of Tween 80. We have been unable, therefore, to confirm any of the observations on which Yonetani bases his conclusion that copper is not involved in cytochrome oxidase activity. Our only point of agreement is that the chelating agent BCS does not inhibit cytochrome oxidase activity, a property we have previously demonstrated for other copper chelating agents. We have been able to demonstrate all of the spectral changes at 479 mμ in the presence of BCS reported by Yonetani after addition of 15 μM $CuSO_4$ to our cytochrome oxidase preparation. Under these conditions added ionic copper is reduced by dithionite, borohydride, and ascorbate-cytochrome c and is trapped in the cuprous state by BCS.

Experiments with Cu^{64} have provided further evidence to support the contention that Yonetani's results are due to contamination by ionic copper. After fractionation of mitochondria to which Cu^{64} was added (Table I) the cytochrome oxidase preparation which was isolated contained appreciable amounts of radioactivity and had a Cu/cytochrome a ratio of 1.76. (Normal ratio = 1.1-1.2).

Table I

Distribution of Added Cu^{64} During Cytochrome Oxidase Fractionation

Fraction	Cu/cyt. a	Total Counts/min (corrected)	Counts/min/mg protein (corrected)	Counts/min/mμmole cyt. a (corrected)
Mitochondria	3.66*	128.6×10^6	1.07×10^4	9×10^3
Green Residue	---	20×10^6	1.0×10^4	2.3×10^3
Cyt. Oxidase Before Dialysis	1.76	1.94×10^6	1.39×10^4	1.8×10^3
Cyt. Oxidase After CN Dialysis	1.03	2×10^3	17.2	2.2

* Includes 16.85 μmoles of added Cu^{64} acetate.

After dialysis against 0.01 M cyanide (pH 8) for 12 hr and a further 3 hr dialysis against Tris buffer (0.01 M pH 8) to remove cyanide, only negligible amounts of radioactivity remained in the oxidase preparation, and the Cu/cyto-

chrome a ratio had dropped to 1.03. It is apparent that there has been no exchange with added copper and that added ionic copper, although tightly bound to the oxidase, can be readily removed by dialysis against cyanide. This experiment provides additional evidence for our contention that cytochrome oxidase is a copper hemoprotein complex containing equimolar amounts of copper and cytochrome a.

On treatment of the radioactive cytochrome oxidase preparation (Cu/cytochrome a ratio of 1.76) with BCS, as described by Yonetani, formation of Cu-BCS was observed on reduction with ascorbate-cytochrome c, dithionite, and potassium borohydride. On addition of cyanide, the Cu-BCS absorption at 479 mμ disappears as reported by Yonetani. On the other hand the non-radioactive, cyanide-dialyzed cytochrome oxidase preparation (Cu/cytochrome a = 1.03) showed no formation of Cu-BCS on reduction with ascorbate-cytochrome c, dithionite, and borohydride. (This negative result is not due to non-removal of cyanide as no Cu-BCS formation was observed with an enzyme which had been subjected to prolonged dialysis. Moreover, small amounts of added ionic copper reacted readily with the BCS reagent).

It is our contention that copper is involved in cytochrome c oxidase activity and that copper is firmly bound in a specific configuration so that it is not chelated by many copper chelating agents. If this configuration is modified so that copper is able to react with chelating agents there is a loss of a specific catalytic function exhibited by copper in the cytochrome oxidase reaction (Griffiths and Wharton, 1961 c). The observations reported by Yonetani (1960 b) are probably accounted for in terms of the contamination of his cytochrome oxidase preparation by ionic copper and/or a modification of the binding of copper such that it is able to react with BCS. It should be noted that a significant amount of copper in Yonetani's preparation (30-40% of the total copper; equivalent to a Cu/cytochrome a ratio of 0.5 - 0.65) is not enzymically reducible so that it reacts with BCS. We contend that it is this copper moiety which undergoes oxido-reduction during cytochrome oxidase activity and that a

major portion of the catalytically active copper has been modified in the isolation procedure used by Yonetani. In this respect it should be pointed out that, whereas the activity per unit cytochrome a of our purified cytochrome oxidase preparation approximates that of mitochondria (Griffiths and Wharton, 1961 a,b), the activity per unit cytochrome a of Yonetani's preparation is less than 20% of that of the original activity in mitochondria (Yonetani, 1960 a).

Finally, EPR spectroscopic studies have shown (1) that BCS has no effect on the reduction and reoxidation of copper in cytochrome oxidase and (2) that copper in cytochrome oxidase is not reduced by borohydride (Griffiths, Wharton and Beinert, 1961). It is thus apparent that the conclusions of Yonetani (1960 b) regarding the role of copper in cytochrome oxidase are not valid. The participation of copper in the absorption peaks of cytochrome oxidase at 605 mμ and 830 mμ still remains an attractive hypothesis.

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