COPPER IN CYTOCHROME OXIDASE

Takashi Yonetani

Johnson Research Foundation,
University of Pennsylvania,
Philadelphia, Pennsylvania.

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While presenting strong evidence in favor of the participation of the iron enzymes in the oxidase system, Keilin and Hartree also carefully considered the possible role of copper in their paper on cytochromes a and a_3 (1939). It has been recently confirmed that various purified preparations of cytochrome oxidase contain a significant amount of copper as well as heme iron (Eichel et al, 1950; Mackler and Penn, 1957; Okunuki et al, 1958). In addition, it has been found by electron spin resonance spectrometry (Sands and Beinert, 1959) and through the use of copper chelating agents (Takemori, 1960) that the copper in oxidized cytochrome oxidase is in the cupric state and is partially reduced by various reducing systems.

Through the use of a new cuprous specific chelating agent, Bathocuproine sulfonate sodium salt [2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline sodium disulfonate (BCS)(Zak, 1958)] we have succeeded in quantitatively trapping the copper in cytochrome oxidase under physiological conditions (pH 6.0 to 8.0 and without organic solvent) without affecting the absorption spectra of the heme prosthetic groups and enzyme activity, and in obtaining evidence to support the following conclusions: (1) the absorption peaks at 444 and 605 mμ of reduced cytochrome oxidase are entirely the contribution of the heme prosthetic groups; (2) the copper

in oxidized cytochrome oxidase is in the cupric state and can be reduced by various reducing-systems; and (3) at least the enzymatically reducible copper, which corresponds to 60 to 70 % of the total copper, is not required for the enzyme activity of cytochrome oxidase.

Materials and Methods. Spectrophotometry was carried out with a split beam recording spectrophotometer (Yang and Legallais, 1954). As the purified preparation of cytochrome oxidase, we used Direct Dialyzed Preparation (Yonetani, 1960 a), which was our previously described cytochrome oxidase (Yonetani, 1960 b) further purified by differential centrifugation and dialysis. The molar ratio of total copper to total iron of the preparation was found to be 1.7 by a quantitative metal analysis. The content of the total iron was equal to that of the heme iron calculated from the millimolar extinction coefficient, 21 ($mM\ Fe^{-1} \times cm^{-1}$ at 605 mμ)(Yonetani, 1960 a). All the measurements were carried out in 0.5 % Emasol-4130 - 0.05 M phosphate buffer, pH 7.3, at 25°. BCS is highly specific for the cuprous ion under physiological conditions, forming a deep yellow complex (Cu-BCS) having an absorption maximum at 479 mμ.

Experimental Results. As shown in Fig. 1, (B)-(A), upon the addition of 70 μM BCS to an aerobic solution of oxidized cytochrome oxidase (A), no significant change in its absorption spectrum is observed. Upon the addition of BCS to the dithionite-reduced preparation (C), extra absorption between 400 to 500 mμ peaking at 475 to 485 mμ is observed [Fig. 1, (D)-(A)], in addition to the typical absorption spectrum of reduced cytochrome oxidase [Fig. 1, (C)-(A)]. The difference spectrum between the BCS-reduced- and reduced-preparations [Fig. 1, (D)-(C)], subtracting the absorption due to the heme prosthetic groups, is identical to that of Cu-BCS made from inorganic cuprous ions. Titration of the dithionite-reduced preparation (C) with BCS showed that the protein bound copper can react stoichiometrically with the added BCS and all the copper in the preparation is converted to Cu-BCS.

Effects of inhibitors on the BCS-reduced preparation (D) were as

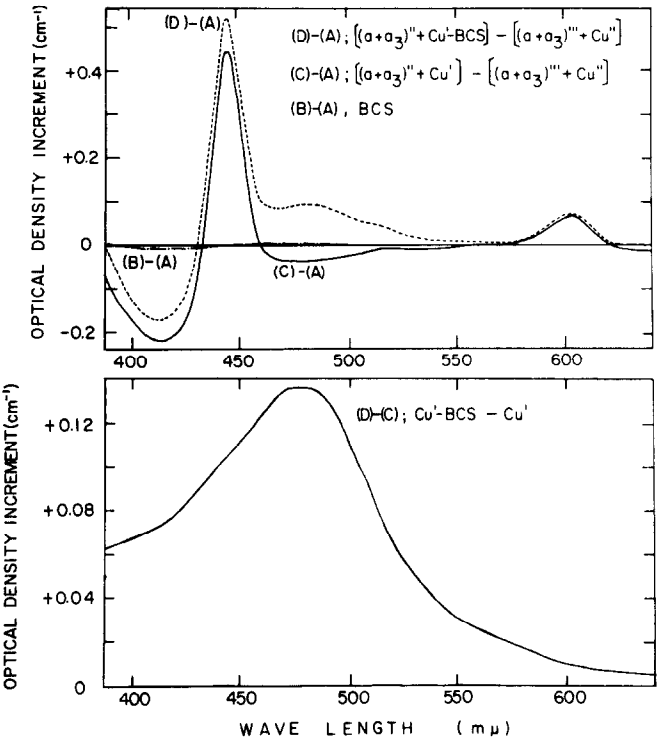

Fig. 1 Effect of BCS (70 μM) on difference spectra of cytochrome oxidase (7 μM heme iron and 12 μM copper equivalents). The baseline represents the oxidized preparation (A). (B), oxidized preparation treated with BCS: (C), dithionite-reduced preparation; and (D), reduced preparation treated with BCS.

follows: (1) upon the addition of 1 mM cyanide, the Cu-BCS absorption at 430 mμ disappears completely, indicating transformation of Cu-BCS to Cu-CN in addition to the effect of cyanide on the heme absorption (shift of the α peak from 605 to 600 mμ and a decrease in absorption at 444 mμ), (2) upon the CO treatment, the Cu-BCS absorption shows no change, while the heme absorption shows the typical change due to formation of the CO compound of reduced cytochrome a3 (shift of the α peak from 605 to 600 mμ, a decrease in 444 mμ absorption and an increase in 430 mμ absorption), and (3) azide does not change either the Cu-BCS- or the heme-absorptions.

Since the valency state of the copper can be easily detected by its

reactivity with BCS, effects of various reducing agents on the components of cytochrome oxidase were examined: (1) dimercaptopropanol, BAL, can reduce both the heme prosthetic groups and the copper as does dithionite. (2) borohydride can not reduce the heme groups, but can reduce 70 to 90 % of the total copper, and (3) other reducing-systems such as ascorbate-p-phenylenediamine, ascorbate-cytochrome c, and succinate-heart muscle preparation can reduce the heme groups and 60 to 70 % of the total copper after the dissolved oxygen is exhausted.

Kinetic observations of the effect of BCS on anaerobic oxidation-reduction cycles by adding oxygen were carried out with the preparation (C) reduced by the reducing-systems of category (3). By suddenly adding a small amount of oxygen (~ 3 μM) to an anaerobic solution of the reduced preparation the traces at 445 mμ and 605 mμ deflect suddenly to their steady state levels and then return to their fully reduced levels as observed by Chance (1955). No response of the trace at 461.5 mμ (the isosbestic point of the spectra of the heme groups) was observed. On adding 66 μM BCS, the trace at 461.5 mμ deflects significantly, indicating rapid formation of Cu-BCS. In few minutes, 60 to 70 % of the total copper is converted to Cu-BCS. Even after the BCS treatment the traces at 445 and 605 mμ respond to the added oxygen in the same way as observed before the BCS treatment, while the absorption at 461.5 mμ due to newly formed Cu-BCS does not change at all on adding the oxygen.

This amount of BCS shows no inhibitory effect on the enzyme activity of cytochrome oxidase measured by the polarographic method, although most of the copper (60 to 70 % of the total copper) is trapped so firmly that it is unable to change its valency.

Discussion and Conclusion. The participation of the copper in the absorption peaks at 444 and 605 mμ of reduced cytochrome oxidase as proposed by Wainio et al (1959) is now completely denied. This has been suggested in the oxidase/oxygen stoichiometry (Chance and Yonetani, 1959; Yonetani, 1960 c). Although the cupric copper in oxidized cytochrome

oxidase is certainly reduced to the cuprous state with various reducing-systems in agreement with the results of others (Sands and Beinert, 1959; Takemori, 1960), it should be concluded that most of the copper, at least enzymatically reducible copper (60 to 70 % of the total copper) does not participate in electron transfer of cytochrome oxidase. In other words, the reduction of the copper by various reducing-systems is a non-specific reaction independent of the enzme reaction of cytochrome oxidase.

Cyanide reacts with both cytochrome a_3 and copper, while CO reacts only with cytochrome a_3 (Yonetani, 1960 b).

The details of this study will be published elsewhere.

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