ON THE FUNCTION OF COPPER IN CYTOCHROME OXIDASE*

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Several investigators have reported that cytochrome oxidase preparations contain copper (Keilin and Hartree, 1938; Eichel, et al., 1950; Mackler and Penn, 1957; Okunuki, et al., 1958; Wainio, et al., 1959). Purified preparations, in which hemes other than those of the a-type could not be detected showed a ratio of heme:iron:copper of 1:1:1 (Hatefi, 1959)**. We have investigated such preparations*** by means of paramagnetic resonance (EPR) spectrometry. In the frozen state a pronounced copper signal was observed (Fig. 1). This signal diminished in intensity when the oxidase was reduced. The rate and total extent of decline in intensity was different with preparations of different age and deoxycholate concentration and the rate depended on the concentration of reductant and oxygen present, the half-time varying from <30 seconds to about 3 minutes at 0° and the total decrease between 50 and 80%.

The oxidase was generally reduced by ascorbate in the presence or absence of cytochrome c. The following observations are consistent with the idea that copper is reduced by substrates of the oxidase and that this reduction is specific and possibly connected with the function of the enzyme: (1) In every case in which the rate of disappearance of the copper signal could be measured by rapid freezing between short incubations, the rate in the presence

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of cytochrome c was found to be distinctly faster (Fig. 2) than that in its absence. Although spectrophotometric measurements of the appearance of the 605 mμ band of the oxidase could not be carried out exactly under the conditions of the EPR experiments, the difference in rate in the presence or absence of cytochrome c was similar in both types of measurement. (2) On reoxidation of the oxidase by oxygen the copper signal reappeared in its original shape and size. (3) When Cu^{++} ions were added to the reduced or oxidized oxidase the signal due to the added Cu^{++} remained unchanged when ascorbate was added with or without cytochrome c under conditions when the signal of the copper of the oxidase faded rapidly. (4) The very fact that a portion of the copper signal observed with the oxidase did not disappear, likewise supports the idea of a specific function of that portion which underwent the described reversible changes. (5) When 0.003 M cyanide was added to a 0.001 M aqueous $CuSO_4$ solution, the copper signal disappeared, whereas the signal of the enzyme-bound copper remained under these conditions. However, a change in shape was observed (Fig. 1) which shows a definite interaction of oxidase-copper and cyanide.

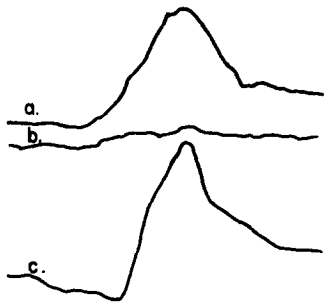

Figure 1. EPR signals (g=2.05 (1); peak to peak width for (a) 160 gauss) of 42 mg of cytochrome oxidase (10 μmoles of Cu per g of protein) in 0.3 ml of 0.1 M phosphate (pH 7.5) aerobically at -100°: (a) untreated; (b) 5 minutes after addition of 25 μl of 1 M ascorbate (pH 6.5); (c) as (a) in the presence of 0.003 M cyanide.

Since it is difficult to obtain preparations of the oxidase completely free of cytochrome c, it is not clear whether ascorbate is able to reduce the oxidase in the complete absence of cytochrome c. It appears, however,

from spectrophotometric measurements that ascorbate will reduce the oxidase slowly even in the absence of cytochrome c (Smith, 1955; Cooperstein, 1959; Hurwitz and Cooperstein, 1955), and it is therefore not surprising that the copper would be affected at a similar rate, as shown by EPR. In an experiment (Fig. 2) in which 0.33 μmole of reduced cytochrome c was added to the oxidase (0.24 μmole of bound copper) in the absence of reducing agents the copper signal decreased by 25% within <30 seconds, and further addition of 0.20 μmoles of ascorbate led to a total decrease of 50%, the maximal decrease observed with the preparation of oxidase used.

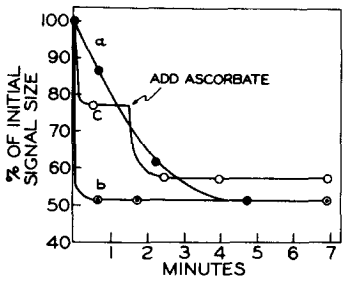

Figure 2. Rate of disappearance of EPR signal of 24 mg of cytochrome oxidase in tris-buffer (pH 7.5) anaerobically at 0°, when (a) 1 μmole of ascorbate, (b) 0.45 μmole of cytochrome c + 1 μmole of ascorbate were added, and (c) when 0.33 μmole of reduced cytochrome c was added at 0 time and 0.2 μmole of ascorbate at 90 seconds.

Our observations permit no conclusions as to the sequence of components--a hemes and copper--in the oxidase. In the presence of 0.003 M cyanide no pronounced effect on the reduction of the copper by ascorbate or on reoxidation by oxygen was observed. In view of the high protein concentration in these experiments, however, this level of cyanide may have been too low. If copper is a functional component of the oxidase, the question may be raised whether some of the spectral peculiarities ascribed to the a and a_3 hemes are not possibly due to the copper component.

References:

Cooperstein, S. J., J. Biol. Chem., 234, 392 (1959).

Eichel, B., W. W. Wainio, P. Person and S. J. Cooperstein, J. Biol. Chem., 183, 89 (1950).

Hatefi, Y., Biochim. Biophys. Acta, 30, 648 (1959).

Hurwitz, J. and S. J. Cooperstein, J. Biol. Chem., 212, 771 (1955).

Keilin, D. and E. F. Hartree, Nature, 141, 870 (1938).

Mackler, B. and N. Penn, Biochim. Biophys. Acta, 24, 294 (1957).

Okunuki, K., I. Sekuzu, Y. Yonetani and S. Takemori, J. Biochem., 45, 847 (1958).

Smith, L., J. Biol. Chem. 215, 833 (1955).

Wainio, W. W., C. Vander Wende and N. F. Shimp, J. Biol. Chem., 234, 2433 (1959).