A CORRELATION OF THE NEAR-INFRARED ABSORPTION BAND OF CYTOCHROME OXIDASE WITH ENZYMIC ACTIVITY AND COPPER CONTENT

David C. Wharton and Alexander Tzagoloff[1]

Institute for Enzyme Research
University of Wisconsin
Madison 6, Wisconsin

Received August 12, 1963

Griffiths and Wharton (1961) described a broad spectral absorbance band with a maximum near 830 mμ in preparations of their purified oxidized cytochrome oxidase. The extinction coefficient reported was 1.4 $mM^{-1} cm^{-1}$ as compared to 9.0 $mM^{-1} cm^{-1}$ for the alpha peak of the oxidized cytochrome at 599 mμ. The absorbance near 830 mμ disappeared when the enzyme was fully reduced and reappeared on oxidation. The rate of this change was found to be grossly similar to the rate of changes observed in the alpha peak of the enzyme during oxidoreduction, although the absorbancy of the latter increased rather than decreased on reduction. Gibson (1963) demonstrated by rapid reaction techniques that the kinetics of the changes in the absorbance of cytochrome oxidase at 605 mμ and at 830 mμ were practically identical. Furthermore, absorbance of the oxidized chromophore near 830 mμ disappeared on reduction in the presence of cyanide but the reduced form thus obtained was not reoxidizable by oxygen (Griffiths and Wharton, 1961). In this communication we shall show that certain treatments which cause a loss of absorption at 830 mμ also result in a parallel loss of enzymic activity. We interpret these data as further evidence that this chromophore is an integral component of cytochrome oxidase. During the course of these experiments we have also observed a close correlation between the removal of copper and both the loss of absorbancy at 830 mμ and the loss in specific activity of the enzyme.

---

[1] Post-doctoral trainee of the Institute for Enzyme Research.

METHODS

Cytochrome oxidase was prepared from beef heart mitochondria according to the procedure of Griffiths and Wharton (1961) or that of Fowler et al. (1962). In both preparations the concentration of cytochrome a was approximately 9 mμmoles per mg of protein; the specific activities were essentially the same. Cytochrome a was determined according to the procedure of Yonetani (1959) and copper was assayed as the cuprous-2,2'-biquinoline complex by a slight modificatio (Wharton and Tzagoloff, 1963) of the method of Felsenfeld (1960). Iron was determined as the ferrous-bathophenanthroline complex (Smith et al., 1952) after wet ashing with sulfuric acid and hydrogen peroxide. Specific activity was assayed spectrophotometrically at 38° in a Beckman model DU spectrophotometer (Smith, 1955). Protein was determined by the biuret reaction (Gornall et al., 1949).

RESULTS

To explore further the association of the 830 mμ absorbing chromophore with cytochrome oxidase, the enzyme was subjected to treatments which resulted in a gradual and controlled loss of absorbancy in the 830 mμ band. The results of one type of treatment are illustrated in Fig. 1; the enzyme was first dialyzed against 1 M KCN, and then was dialyzed extensively against 0.05 M Tris chloride buffer, pH 8.0, to remove the cyanide. This treatment, as seen in the figure, results in a loss of specific activity which parallels the loss of the absorbancy at 830 mμ. The chromophore can also be destroyed quantitatively by dialyzing the oxidase against certain buffers, i.e. borate, glycine, or phosphate at concentrations higher than 0.4 M, above pH 9. This loss in absorbancy in the presence of the buffers is accelerated by adding KCN at concentrations of 0.01 M or more. Dialysis against 0.01 M KCN alone does not result in loss of the chromophore. Tris buffer, either alone or with 0.01 M KCN, is ineffective whereas cyanide itself loses its effectiveness at concentrations of 0.1 M and below. It appears that an alkaline pH itself is not responsible for the loss of the absorbancy at 830 mμ and that a minimum ionic strength is also necessary.

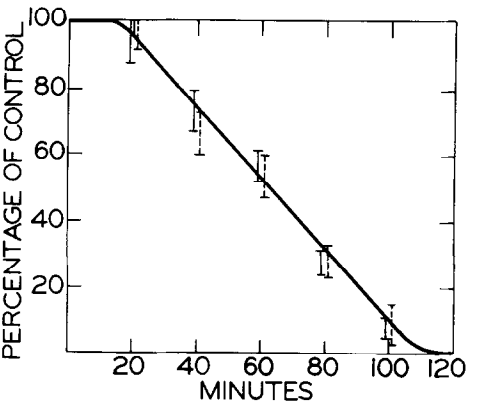

Fig. 1 Loss of enzymic activity and absorbancy at 830 mμ as a function of time of dialysis against 1 M KCN. Vertical unbroken line represents range of values of 830 mμ absorbancy and broken line, the range of values of specific activity.

In other experiments it was shown that the absorption band at 830 mμ could be eliminated by treatment of the oxidase with a combination of the water-soluble, copper-chelating agent bathocuproine sulfonate (BCS) and acetate buffer, at a pH below 5. This treatment produced a loss of specific activity proportional to the loss of the chromophore. Both the BCS and the buffer alone were without effect on the chromophore. The absorption at 830 mμ was not appreciably restored by adding oxidizing agents such as ferricyanide.

All treatments which were shown to lower absorbancy in the 830 mμ region also shifted the peaks of the α and Soret bands from 605 and 444 to 600 and 438 mμ, respectively; however, the bands characteristic of the pyridine hemochromogen (with peaks at 587 and 431 mμ) remained unchanged within the time of the treatments.

Since the loss of absorbancy at 830 mμ and the parallel loss of activity of the enzyme might result from the loss of certain metal ions due to complex formation, we decided to examine the treated enzyme protein for iron and copper content. Analyses showed no significant loss of iron. A loss of copper was found, however, which was proportional both to the decrease in absorbancy at 830 mμ and to the decline in specific activity under certain conditions. These conditions included dialysis of the enzyme against high concentrations of cyanide,

or against 0.01 M cyanide combined with the alkaline buffers mentioned above, or treatment with the BCS-acetate buffer. When cytochrome oxidase was dialyzed against the alkaline buffers alone, the loss of copper lagged behind the loss of specific activity and the loss of absorbancy at 830 mμ, presumably because no chelating agent was present to remove the displaced copper from the apoprotein.

We believe that the available evidence indicates that the absorption band with a maximum at 830 mμ is an integral part of the spectrum of enzymically active cytochrome oxidase and suggests that this band has its origin in the chemical binding of copper to the hemoprotein. The evidence which favors these proposals is: (a) the chromophore at 830 mμ has been found in all of our preparations of cytochrome oxidase; (b) the kinetics of changes in the 830 mμ chromophore on oxidoreduction are similar to those of one of the classical absorption bands (Gibson, 1963); (c) under specified conditions there is a parallel loss of absorbancy at 830 mμ, of specific activity, and of copper; (d) copper is reduced to the steady-state level as quickly as the alpha and Soret bands (Beinert and Palmer, 1963); (e) there is no significant loss of iron during the treatments described; and (f) several copper-containing proteins in the oxidized state have an absorption maximum near 800 mμ (Blumberg et al., 1963; Omura, 1961; Katoh, et al., 1962). However, at present we cannot eliminate entirely the possibility that at least part of the absorption band at 830 mμ results from a specific linkage of heme a especially in view of the fact that Caughey (1963) has observed absorption bands in the near infrared region of some Fe (III) porphyrins. A test of our proposals will come with the reintroduction of copper into the copper-free cytochrome oxidase. Work is currently being directed to that end.

ACKNOWLEDGMENTS

The authors appreciate the interest and encouragement of Dr. D. E. Green during the course of these studies and thank Drs. Helmut Beinert and Mary Buell for their helpful criticism of the manuscript. This work was supported in part by National Institutes of General Medical Sciences graduate training grant 5-T1-GM-88 (USPHS) and by U. S. Atomic Energy Commission Contract AT(11-1)-1151.

Cytochrome oxidase was expertly prepared by Mrs. Margret Wellmann. Meat by-products were generously supplied by Oscar Mayer and Co., Madison, Wisconsin.

REFERENCES

1. Beinert, H., and Palmer, G., personal communication.
2. Blumberg, W. E., Eisinger, J., Aisen, P., Morell, A. G., and Scheinberg, I. H., J. Biol. Chem., 238, 1675 (1963).
3. Caughey, W. S., personal communication.
4. Felsenfeld, G., Arch. Biochem. Biophys. 87, 247 (1960).
5. Fowler, L. R., Richardson, S. H., and Hatefi, Y., Biochim. et Biophys. Acta, 64, 170 (1962).
6. Gibson, Q. H., personal communication.
7. Gornall, A. G., Bardawill, C. J., and David, M. M., J. Biol. Chem., 177, 751 (1949).
8. Griffiths, D. E., and Wharton, D. C., J. Biol. Chem., 236, 1850 (1961).
9. Katoh, S., Shiratori, I., and Takemiya, A., J. Biochem. (Tokyo), 51, 32 (1962).
10. Omura, T., J. Biochem. (Tokyo), 50, 394 (1961).
11. Smith, G. F., McCurdy, W. H., and Diehl, H., Analyst, 77, 418 (1952).
12. Smith, L., in D. Glick (Editor), Methods of Biochemical Analysis, Vol. II, Interscience Publishers, Inc., New York, 1955, p. 427.
13. Wharton, D. C., and Tzagoloff, A., in preparation.
14. Yonetani, T., J. Biochem. (Tokyo), 46, 917 (1959).