RECONSTITUTION OF RESPIRATORY CHAIN ENZYME SYSTEMS. IX. CYTOCHROME c-CYTOCHROME OXIDASE COMPLEX OF HEART MUSCLE

Morio Kuboyama, Shigeki Takemori and Tsoo E. King

Department of Chemistry and Laboratory for Respiratory Enzymology,
Oregon State University, Corvallis, Oregon

Received November 14, 1962

The existence of a cytochrome c-cytochrome oxidase complex is deducible from the concept of the respiratory chain. The deduction is also in accord with the kinetic results which have been reported from time to time (see, for example, Yonetani, 1960; Smith and Conrad, 1961; Nicholls, 1961; Orii, et al., 1962). However, the isolation of the complex, from natural material, has previously been unsuccessful. The failure has been due mainly to the fact that cytochrome c is easily leached from respiratory particles in the presence of dilute salt solutions (Tsou, 1951) especially of bile salts (Ball and Cooper, 1957). This unfavorable property is further abetted by the extreme insolubility of cytochrome oxidase*. These characteristics make the isolation and purification of this segment of the respiratory chain containing cytochrome c and cytochrome oxidase almost impossible.

On the other hand, the successful reconstitution of succinate oxidase (Keilin and King, 1958, 1960) and succinate cytochrome c reductase (King and Takemori, 1962) has inspired us to attack the problem of the complex from the reconstitutive approach. This idea has been further encouraged by the recent findings that cytochrome oxidase is an acidic protein (Takemori, et al., 1961), whereas cytochrome c is strongly basic and comparatively small in size. The complex formed by electrostatic attraction, perhaps also reinforced by other bindings, might be isolable under suitable conditions by means of "molecular sieve" chromatography.

*In this paper, cytochrome oxidase refers to cytochromes a + a_3. The question of whether the latter are functionally or structurally different entities does not affect the conclusion.

This communication reports the actual separation of the cytochrome c-cytochrome oxidase complex of 1 to 1 molar ratio from the mixture of the two reactants. Other forms of the complex are also found which are evidently dependent on the polymerization state of the cytochrome oxidase employed.

Materials and Methods

Purified cytochrome oxidase was prepared from beef heart muscle according to the method of Okunuki, et al. (1958), with minor modifications. The ratio of copper to heme or to heme iron of the oxidase was 1.0. Crystalline cytochrome c was also isolated from beef heart muscle by the method of Hagihara, et al. (1958), or of Margoliash (1962). The concentrations of cytochrome oxidase and cytochrome c were determined spectrophotometrically according to the difference (reduced - oxidized) absorbancy indexes of 11.0 x mM^{-1} x cm^{-1} at 605 mμ and 19.0 x mM^{-1} x cm^{-1} at 550 mμ, respectively.

Emasol No. 1130 (polyoxyethylene-sorbitan-monolaurate), a non-ionic detergent, was kindly supplied by Kao Soap Co., Tokyo. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden. Sonic oscillation was conducted in a Raytheon 250 W, 10 KC Sonic Oscillator, Model DF101; and the temperature of the chamber was kept at about 4° by circulating ice water.

Results and Discussion

One and four-tenths μmoles (based on the heme content) of a lyophilized cytochrome oxidase preparation were dissolved in 10 ml of cold 0.05 M phosphate buffer, pH 7.4, containing 0.1% Emasol. Subsequent operations were performed at 4° unless otherwise indicated. The solution was treated in the Raytheon Oscillator for 20 minutes at the maximal power output. To the solution were then added 5.5 μmoles of cytochrome c. The mixture was further treated for 25 minutes. One-half of one ml of the mixture was carefully layered on the top of a Sephadex column (1.5 x 12cm) which had been equilibrated with 0.05 M phosphate buffer, pH 7.4, containing 0.1% Emasol. The column was subsequently developed with the same buffer. Sixty fractions, 0.6 ml per fraction, were collected at a flow rate of 7.2 ml per hour. During the development, the reddish-brown colored zone was rapidly separated into

two fractions. The first band, consisting of cytochrome c and cytochrome oxidase, appeared at a R_f value of approximately 0.6. The second, slow-moving band containing cytochrome c, showed a R_f value of 0.1. Clean separations of these two fractions were always obtained; indeed, more than 15 experiments employing different batches of oxidase and cytochrome c in widely varied proportions gave identical results. The elution pattern is shown in Figure 1.

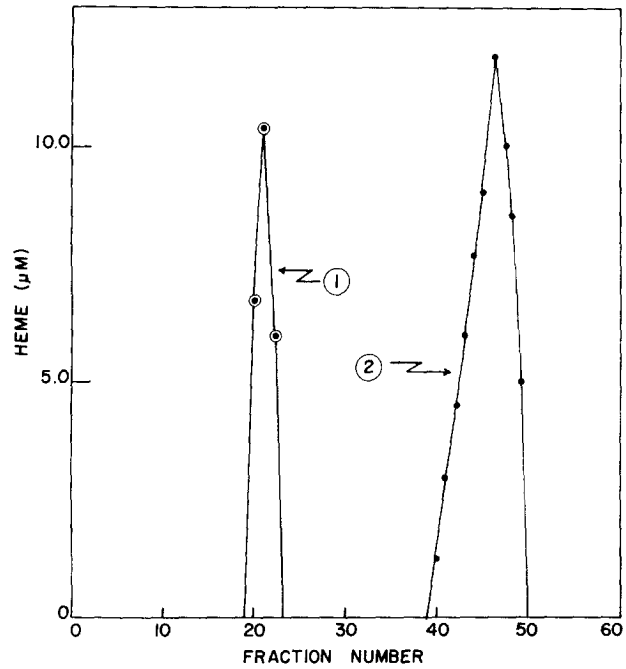

Figure 1. Elution pattern of the cytochrome c-cytochrome oxidase complex of molar ratio 1 from a Sephadex column ——●—— ●—— cytochrome c; ——⊙—— ⊙—— cytochrome oxidase. (1), The complex; (2), free cytochrome c.

That the fast-moving fraction was not merely a mixture of cytochrome oxidase and cytochrome c, but a new entity in the form of a complex in which the two components were firmly combined with each other, was concluded from three different lines of evidence. (1) The catalytic activities of the complex were higher than those of the components, as described in the accompanying

paper. (2) The components, cytochrome c and cytochrome oxidase, of the complex could not be separated by differential centrifugation in spite of their difference in molecular size. But weak cation exchange resin, such as Amberlite IRC-50, easily split the complex into cytochrome c and cytochrome oxidase, quantitatively. (3) Re-chromatography of the complex gave only one band. Samples from the initial chromatography were concentrated with the aid of polyvinyl pyrrolidone and subsequently chromatographed on a smaller column (0.7 x 5 cm). Only one single homogeneous band with the R_f value of 0.6 was observed by the aforementioned method of elution, and the isolated complex also showed the cytochrome c to cytochrome oxidase ratio of 1.

The absorption spectra of the complex are given in Figure 2. Its molar ratio of cytochrome oxidase to cytochrome c was found to be 1.0.

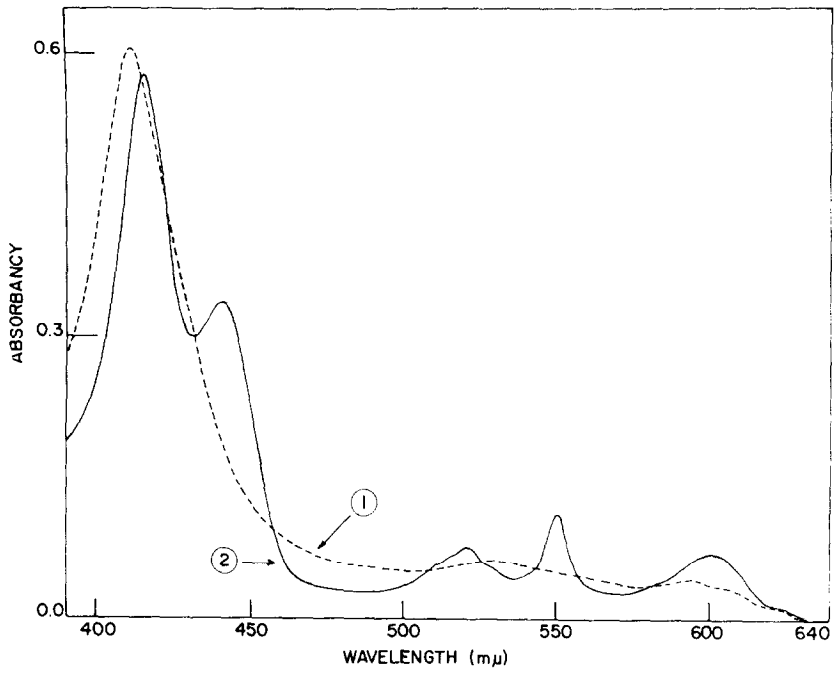

Figure 2. Absorption spectra of the cytochrome c-cytochrome oxidase complex.

The complex was 1 to 1 (cytochrome c to cytochrome oxidase) and was dissolved in 50 mM phosphate buffer, pH 7.4, containing 0.1% Emasol at about 25°. Curve 1 is the oxidized form; curve 2 is the reduced form. The reduced form was obtained by the addition of solid $Na_2S_2O_4$ in the presence of 1 mM NaN_3. The latter was added to inhibit re-oxidation of the reduced complex.

It has been previously reported (Takemori, et al., 1961) that cytochrome oxidase exists in Emasol solution as a monodispersed pentamer; the polymerization perhaps takes place in the course of the isolation. The sonic treatment used in the present experiment evidently depolymerized the pentamer into subunits, and the complex of 1:1 ratio of cytochrome c to cytochrome oxidase was thus obtained. The argument for depolymerization was borne out from the following experiment. Mixtures of cytochrome oxidase and excess amounts of cytochrome c were subjected to sonic oscillation for different lengths of time. Aliquots of the treated solution were then chromatographed on Sephadex columns. The molar ratio of cytochrome c to cytochrome oxidase in the complex thus separated, as shown in Table I, increased with the time of sonic treatment. In spite of the great excess of cytochrome c, the resulting complex always gave a ratio between 0.2 and 1.0. Further experiments with reaction mixtures of widely varied proportions showed that the composition of the complex formed did not depend on the composition of the initial mixture but on the time of sonic treatment. However, no complex of a ratio higher than 1.0 was observed even after prolonged sonic treatment. These facts are very significant from the point of view of the relationship between the protein conformation of cytochrome oxidase and its binding with cytochrome c.

Table 1. Effect of Sonic Oscillation of the Reaction Mixture on the Composition of the Cytochrome c-Cytochrome Oxidase Complex Formed*

Net time of sonic oscillation	Composition of the complex formed
(Minutes)	(Molar ratio of cytochrome c:cytochrome oxidase)
0	0.2
30	0.5
45	1.0

*The reaction mixture contained 1 μmole of cytochrome oxidase and 5 μmoles of cytochrome c in 10 ml of 50 mM phosphate buffer, pH 7.4, and 0.1% Emasol. The mixture was treated in the Raytheon Sonic Oscillator for the net time indicated. (A one-minute interlude followed each two minutes of sonic exposure.) Other conditions and chromatography are detailed in the text.

Acknowledgment

We are greatly indebted to Dr. E. Margoliash who has kindly made his unpublished, improved method for the isolation of cytochrome c to us and to Karen Nickel for the copper and iron analyses. This work was supported by the National Science Foundation, the U. S. Public Health Service, the Oregon Section of the American Cancer Society and the American Heart Association.

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