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THE CRYSTALLIZATION OF MITOCHONDRIAL CYTOCHROME OXIDASE-CYTOCHROME *c* COMPLEX

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Summary

Conditions are described under which crystals are formed with equimolar complex of mitochondrial cytochrome oxidase and cytochrome *c*. Characteristic absorption bands of the solubilized crystals could be attributed to the cytochrome oxidase-cytochrome *c* complex with heme *a*:*c* ratio of 2:1. Activity of crystals shows more close heme-heme interaction between two cytochromes than that of the mixture.

Since the discovery of Atmungsferment by Warburg [1], mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) has been recognized to be very tightly bound to the other components of mitochondrial inner membrane. High hydrophobicity of the oxidase together with its structural integrity in the membrane has made isolation and purification of the enzyme difficult. In the previous paper [2], we have reported an affinity chromatography by which the oxidase could be highly purified by interacting with Sepharose-bound cytochrome *c*. As for a large scale preparation of the oxidase, we have presented [3] a hydrophobic affinity chromatography using phenyl-Sepharose. By these affinity chromatographic procedures, it becomes possible to obtain the oxidase sample which is highly depleted of phospholipids. By forming a complex with hydrophilic cytochrome *c*, the phospholipids-depleted oxidase changed its conformation to be more hydrophilic and more suitable for the crystallization, as an enzyme-product complex similar to the case of D-amino-acid oxidase [4]. In this paper, we first describe the crystallization of mitochondrial cytochrome oxidase as its enzyme-product complex.

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Cytochrome oxidase was prepared from beef heart mitochondria using phenyl-Sepharose CL-4B affinity chromatography as reported previously [3]. It was dissolved in 0.1 M $NaHCO_3$, pH 8.0, containing 0.1 M NaCl and 0.1% deoxycholate. Phospholipids were estimated to be 0.7%, w/w, from an analysis of total phosphorus by the method of Chen [5]. The protein concentration was 38.5 mg/ml determined by the biuret method [6] using crystalline bovine serum albumin as the standard. The values were corrected for overestimate of 30% by heme. The heme *a* content in the purified oxidase was 14.5 nmol of heme *a* per mg of protein, determined by the difference in absorbance at 604 and 630 nm after the reduction with a few grains of sodium dithionite, using a millimolar extinction coefficient of 16.5 $mM^{-1} \cdot cm^{-1}$ [7]. It was diluted in 10 mM sodium phosphate, pH 7.4, containing 1.5% cholate to a final concentration of 36 μM in terms of heme *a* content. Horse heart cytochrome *c* (Type III) was purchased from Sigma. The heme *c* content was determined by the difference in absorbance at 550 and 535 nm after the reduction with dithionite, using a millimolar extinction coefficient of 20.5 $mM^{-1} \cdot cm^{-1}$ [8]. Cytochrome *c* was added to this solution to be its final concentration of 64 μM. After setting the mixture for 20 min in an ice bath, 5 ml of the mixture was put into a cellophane tube and dialyzed against 500 ml of 10 mM sodium phosphate, pH 7.4, without stirring on an ice bath, with two changes of the buffer during the first 24 h, and the dialysis was continued for 48 h. At 24 h after the beginning of dialysis, no

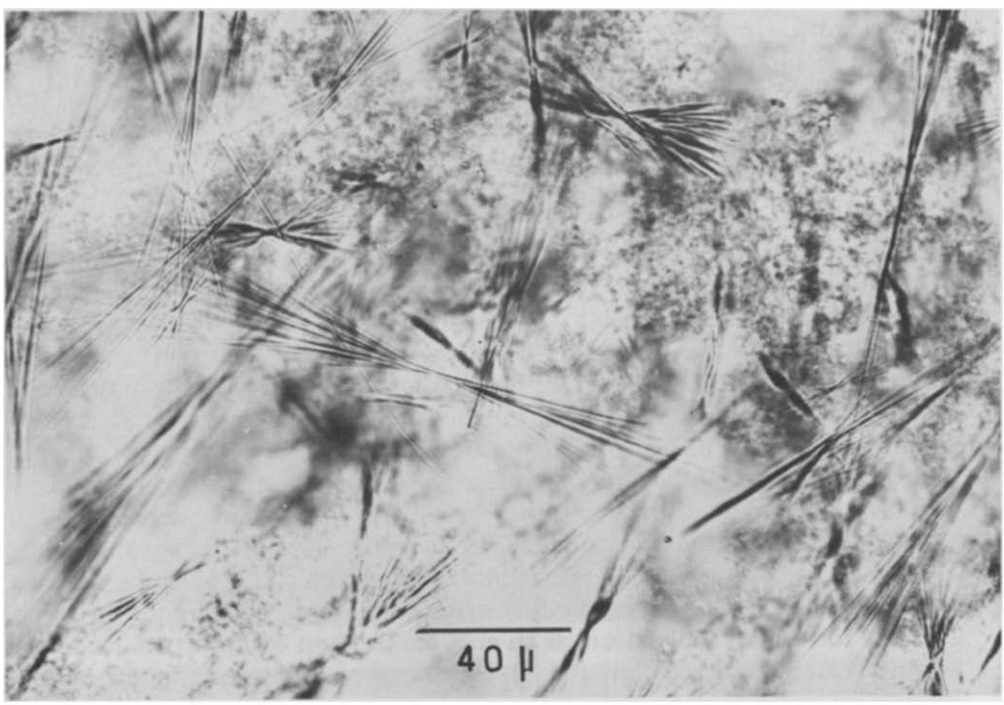

Fig. 1. Crystals of the cytochrome oxidase-cytochrome *c* complex (X 300). The background is a pile of numerous crystals which are out of focus.

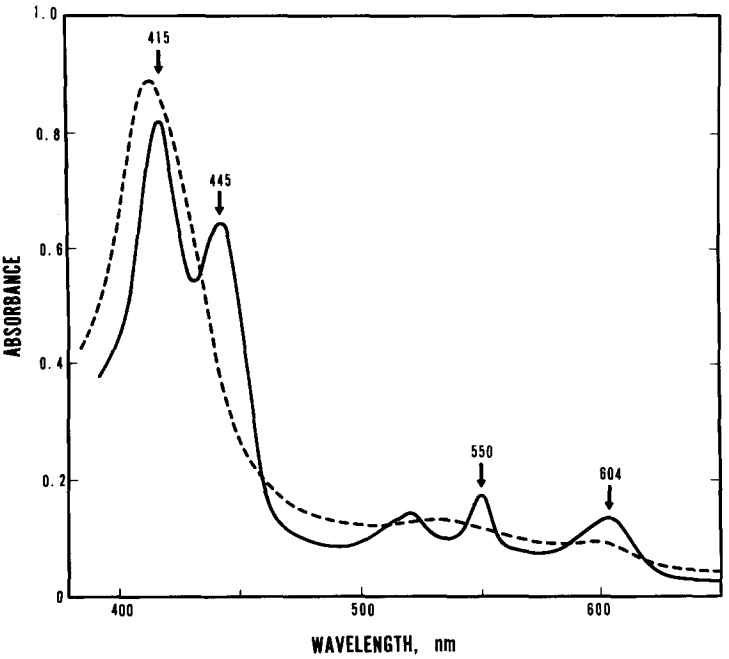

Fig. 2. Spectra of the cytochrome oxidase-cytochrome *c* complex. Dashed curve: spectrum of the solution of the complex (oxidized form); the complex was dissolved by sonication in 10 mM sodium phosphate, pH 7.4, containing 0.5% Tween 80. Solid curve: spectrum of the same solution after the addition of a few grains of sodium dithionite (reduced form).

precipitate was seen, the solution remained transparent and slight birefringence of flow was observed. At 48 h, the solution showed strong birefringence with a little turbidity, showing fine needle-shaped crystals under a microscope. After the dialysis, the dialysate was centrifuged at 500 × *g* for 5 min. The precipitated crystals were washed three times with the buffer by centrifugation at 1 000 × *g* for 10 min until no cytochrome *c* was detected in the supernatant. Finally the reddish-green colored crystals were suspended in the same buffer, as shown in Fig. 1.

Washed crystals were practically free from bile acids, cholate or deoxycholate, which were determined by the method of Kier [9]. Crystals were also free from anions such as SO_4^{2-}, except phosphate. Nitrogen analyses [10] on the crystal suspension showed that the crystals are indeed composed largely of protein free from salt.

The absorption spectra of the crystallized complex showed little difference compared with those of the mixture of cytochrome oxidase and cytochrome *c* with molar ratio of 1:1, but not of 1:2 (Fig. 2). The molar ratio of cytochrome oxidase to cytochrome *c* in the crystallized complex determined by the method of Williams [11] was found to be unity, i.e., heme *a*:*c* ratio of 2:1. King and co-workers [12] reported that a complex of cytochrome oxidase and cytochrome *c* (heme ratio of 1:1) was obtained in a particulate form after extensive dialysis of the mixture of cytochrome oxidase and cytochrome *c* for 24 h. Their conditions request more extensive dialysis

than ours for the crystallization of the equimolar complex of cytochrome oxidase and cytochrome *c*.

The enzymatic activity of the complex was also examined. The oxygen consumption was monitored polarographically using 7 mM ascorbate as reductant in 10 mM sodium phosphate, pH 7.4, containing 0.5% Tween 80. The crystallized sample was dispersed in the same buffer by sonication in the presence of 0.5% Tween 80. At 25°C, 1 μM of this complex in terms of oxidase reduced oxygen at a rate of 17.4 μM O_2 per min, whereas the equimolar mixture (1 μM) of cytochrome oxidase and cytochrome *c* reduced 1.3 μM O_2 per min. The catalytic activity per mol of the complex of cytochrome oxidase and cytochrome *c* was 13 times higher than that of the mixture of the two cytochromes. When cytochrome *c* was added stepwise into the same reaction mixture, the activity was increased linearly with increasing cytochrome *c* and leveled off at a saturating concentration of cytochrome *c*, as described by Orii et al. [13]. At the saturating concentration of cytochrome *c*, 10 μM, the same rate of oxygen uptake, 39 μM O_2 per min, was observed in both cases of the complex and the free cytochrome oxidase. These results indicate that the cytochrome oxidase and the cytochrome *c* in this complex are arranged in an intrinsic way by which electron transfer can readily occur by close heme-heme interaction between the two cytochromes.

Crystallization of the cytochrome oxidase-cytochrome *c* complex will open new era in the study of the electron-transfer chain. For example, heme-heme interaction or the exact distance between hemes will be elucidated by X-ray diffraction analysis of the crystals of the complex.

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