Reactivity of Cytochrome Oxidase with Ascorbate

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SUMMARY

When cytochrome oxidase is solubilized in bile acid, ascorbate alone is capable of reducing the oxidase and induces oxygen uptake in the absence of cytochrome *c*. Cytochrome oxidase is organized into vesicular structures in the absence of detergent in phosphate buffer. In this, spectral changes are brought about by ascorbate, but there is negligible oxygen uptake in the absence of cytochrome *c*.

INTRODUCTION

It was observed by Orii and Okunuki (1) and Orii, Sekuzu and Okunuki (2) that the spectrum of cytochrome oxidase could be affected by ascorbate in the absence of cytochrome *c*, but significant oxygen uptake could not be provoked. Smith (3) and Jacobs *et al.* (4) reported that ascorbate alone can reduce cytochrome oxidase but oxygen uptake by the oxidase was not induced by ascorbate in the absence of cytochrome *c*. Wainio *et al.* (5) agreed with these observations. However, we have observed the reduction of cytochrome oxidase with ascorbate in the absence of cytochrome *c* accompanied by oxygen uptake when the enzyme is solubilized in bile acid. The present paper, therefore, deals with the difference in reactivity of cytochrome oxidase and its topological characteristic in the different media.

MATERIALS AND METHODS

Cytochrome oxidase was prepared from beef heart mitochondria by the method of Flower *et al.* (6), and was washed extensively according to Tzagoloff and MacLennan (lipid-deficient cytochrome oxidase, Preparation B in Ref. 7). The sample

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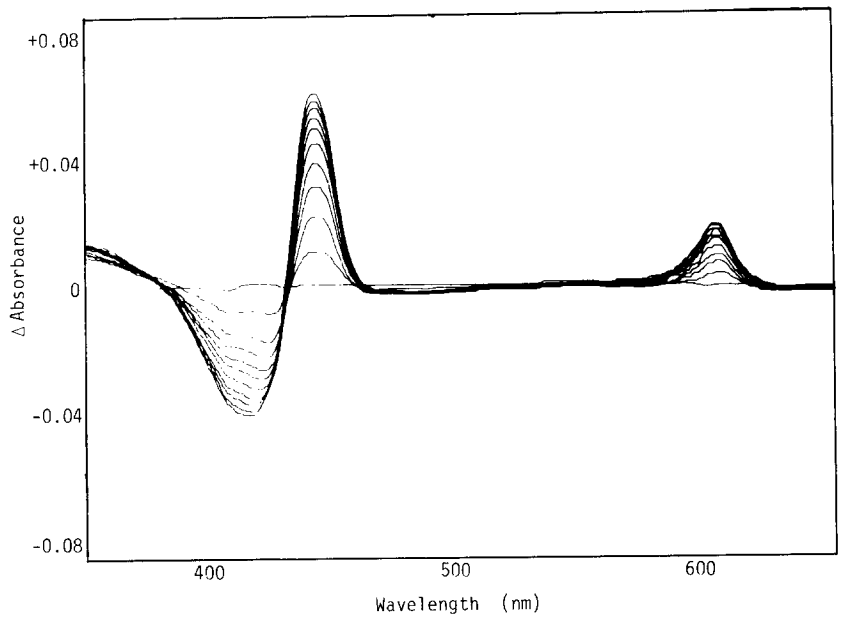

Fig. 1. Change in difference spectrum of cytochrome oxidase by ascorbate. Cytochrome oxidase was dissolved to give final concentration, 7.5 μM in terms of heme *a* in the solution containing 0.15 M KCl, 0.05 M Tris-HCl (pH 8.0) and 0.5% deoxycholate. Ascorbate (1 mM) was added to the solution in the cuvette. Temperature in the cuvettes was regulated at 30°C. Difference spectra were recorded, in every 1 minute intervals, against the solution without ascorbate.

contained 9.1 nmoles heme *a* per mg protein. Spectrophotometry was carried out using a Shimadzu Multipurpose Spectrophotometer, MSP-501. SDS-disc gel electrophoresis of the enzyme was performed according to the method of Dunker and Rueckert (8) with a modification of Capaldi and Hayashi (9). Ascorbic acid was purchased from Hayashi Pure Chemicals Ind. Ltd., and neutralized with KOH. The specimens for electron microscopy were prepared by negative staining with phosphotungstic acid (10). All specimens were examined in a Hitachi HU-11 electron microscope operated at 75 KV.

RESULTS AND DISCUSSION

When cytochrome oxidase was dissolved in a solution containing 0.15 M KCl, 0.05 M Tris-HCl (pH 8.0) and 0.5% deoxycholate, an optically clear solution was obtained. Fig. 1 shows the time course of the change in the difference spectrum of the oxidase solution by ascorbate. The peaks at 605 nm and 444 nm and the

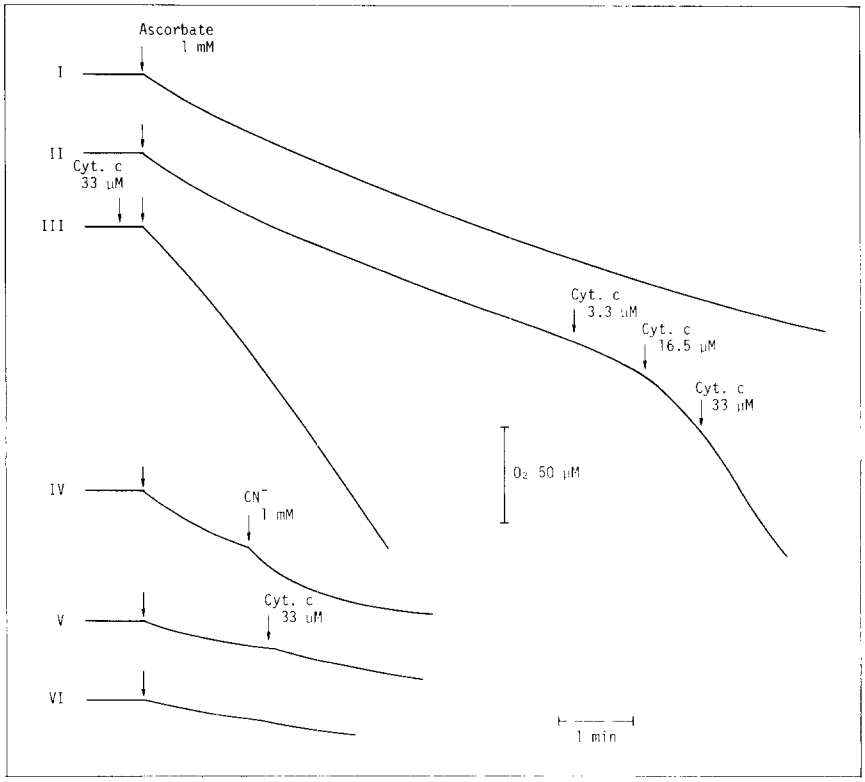

Fig. 2. Oxygen uptake by cytochrome oxidase with ascorbate. Conditions were the same as in Fig. 1, except the additions indicated. Trace V: The reaction mixture without the oxidase. Trace VI: The oxidase was suspended in the solution containing 0.1 M phosphate, pH 7.4, and 1 mM EDTA.

trough at 415 nm are characteristic of the reduced form of the enzyme. The rate of increase in intensity was equal at 605 nm and 444 nm, in contrast to reduction of the enzyme with dithionite, where a gradual increase in intensity at 444 nm after an instantaneous increase in intensity at 605 nm was observed.

Fig. 2, trace I represents the oxygen uptake by the solubilized oxidase with ascorbate, indicating that ascorbate alone is capable of inducing oxygen uptake by the enzyme. The rate of oxygen uptake gradually decreased, but continued until anaerobiosis was reached. The effect of cytochrome *c* under the same conditions is represented by trace II, which shows that the oxygen uptake is enhanced with increasing concentration of cytochrome *c*. Trace III shows the oxygen uptake by the oxidase in the presence of excess cytochrome *c*. Trace IV shows that the oxygen

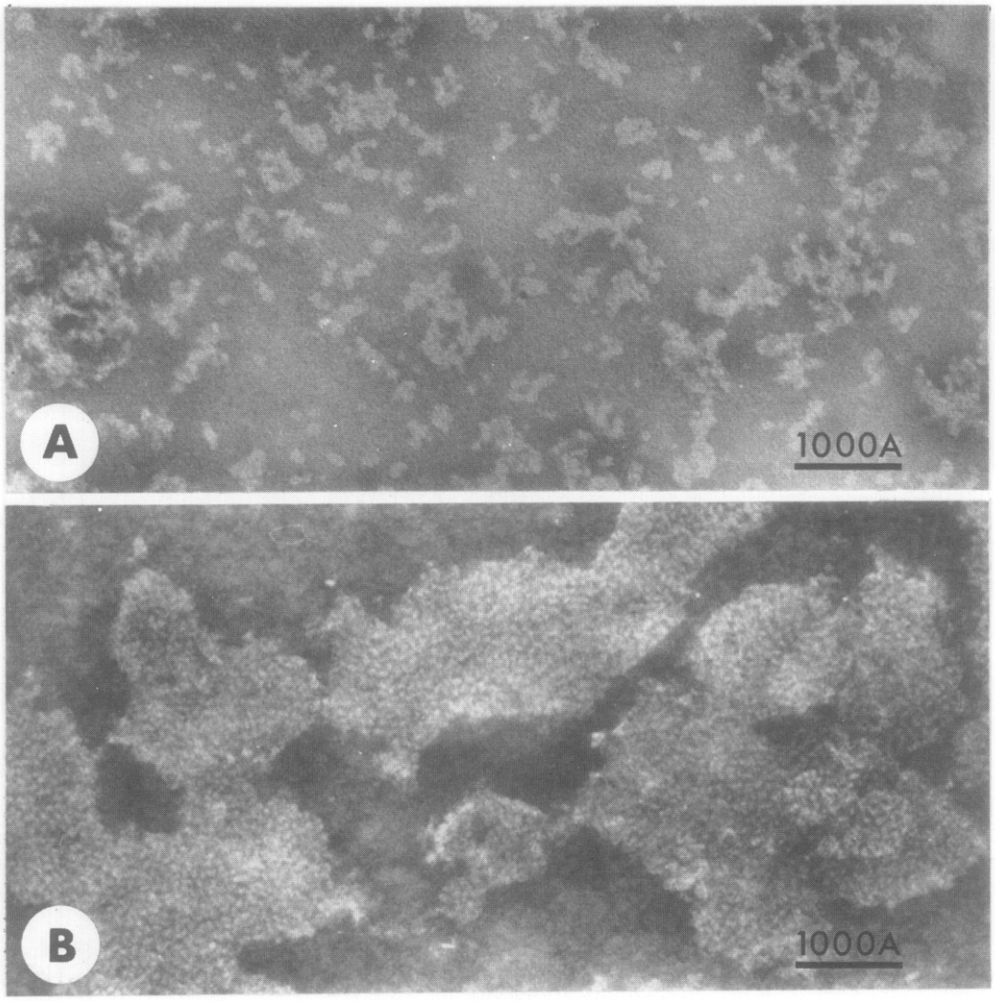

Fig. 3. Electron micrographs of the cytochrome oxidase in different media. A: Conditions were the same as in Fig. 1. B: Conditions were the same as in Fig. 2, trace VI.

uptake by cytochrome oxidase induced by ascorbate alone is inhibited by cyanide. Trace V shows the autoxidation of ascorbate. Trace VI shows the oxygen uptake by the oxidase suspended in phosphate-EDTA medium (2) with ascorbate alone.

When cholate was used in place of deoxycholate, a slightly turbid solution was obtained. The solution showed the spectral changes essentially same as Fig. 1 The rate of oxygen uptake was linear with respect to time. Therefore, the slowing down of oxygen uptake in the medium containing deoxycholate may be ascribed to the

specific inhibitory action of deoxycholate on the mitochondrial enzymes.

Both spectrophotometry and SDS-disc electrophoresis could not detect cytochrome *c* in the oxidase prepared here. So, it is considered that the oxidase preparation does not contain cytochrome *c* or, if contains, the amount of cytochrome *c* should be less than 1/20 mole per mole of heme *a*, since the methods could detect cytochrome *c* more than 1/20 of it per heme *a*. Moreover, the addition of such amount of cytochrome *c* did not enhance the oxygen uptake by the oxidase. However, the significant rise in oxygen uptake was observed upon addition of excess cytochrome *c* (see Fig. 2, trace II).

Distinct differences between previous reports and ours may be ascribed to the difference in the reaction system. We have used bile acid and KCl in order to ensure that cytochrome oxidase is solubilized. Orii *et al.* (2) measured oxygen uptake by the oxidase in phosphate-EDTA medium without any detergent. We also suspended the oxidase in phosphate-EDTA medium (similar to that of Ref. 2) and obtained a turbid solution. The addition of ascorbate to the oxidase suspended in this medium caused essentially similar spectral changes to those observed with the solubilized enzyme. However, the oxygen uptake by cytochrome oxidase in phosphate-EDTA medium (Fig. 2, trace VI) was similar to the rate of autoxidation of ascorbate. This observation is consistent to that reported by Orii *et al.* (2). The difference in turbidity of these samples led us to the examination by electron microscopy. The electron micrograph of the solubilized sample in deoxycholate, Fig. 3-A, shows no vesicular structures, but random aggregates of the oxidase as observed by McConnel *et al.* (11). On the other hand, the oxidase suspended in phosphate-EDTA medium shows vesicular structures of the oxidase (Fig. 3-B).

These results indicate that the topological characteristic of cytochrome oxidase and its reactivity with ascorbate depend on the presence of bile acid, and that ascorbate (in the absence of cytochrome *c*) induces oxygen uptake where there are no vesicular structures, but not where the vesicular structures are present.

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