# Purification of Cytochrome Oxidase by using Sepharose-bound Cytochrome *c*

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SUMMARY

Cytochrome oxidase, a typical membrane enzyme, was purified by a simple procedure using immobilized cytochrome *c*. Sepharose-bound cytochrome *c* packed in a column was reduced with ascorbate. A cytochrome oxidase sample prepared from mitochondrial inner membrane was charged onto the column. Proteins containing no heme *a* were eluted from the column in the void volume. The oxidase was then eluted with increasing concentration of deoxycholate. The purified oxidase contains 13.6 nmoles heme *a* per mg protein, *i.e.* has a minimum mass of 73,500 daltons per heme *a*.

## INTRODUCTION

Cytochrome oxidase, the terminal member of the mitochondrial electron transfer chain, is a typical membrane-bound enzyme and has been studied extensively. However, the tight association between the oxidase and the other components of mitochondrial inner membrane has made isolation and purification of the enzyme difficult. The enzyme is soluble only in the presence of detergents. When the membrane is disintegrated by detergents into four electron transfer complexes and ATPase (1), an oxidase preparation containing 8-8.5 nmoles heme *a* per mg protein is obtained. However, further purification of the oxidase has been difficult due to its aggregation and to interference from detergent micelles, resulting in a rather confusing picture of its molecular weight. Orii and Okunuki (2) carried out extensive studies of the molecular weight of the oxidase, and reported the minimum molecular weight (termed monomer weight in their paper) to be 128,300 or 93,000 on a lipid free basis. However, a variety of cytochrome oxidase preparations differ considerably in

the molecular weight based on heme *a* content; 72,000 (3), 110,000 to 117,000 (4), 100,000 (5,6), 124,000 (7), 91,000 (8), 81,500 (9), 94,000 to 106,000 (10) and 69,000 to 76,000 (11). The variation in the molecular weight seems to be caused by the different kinds of detergents employed. For example, the tetramer rather than the monomer of the enzyme was observed in Emasol solution (2). The monomeric form was observed only in a medium containing sodium dodecyl sulfate (3), which rendered the enzyme inactive. Also, the purification procedures employed suffer from a major defect in that the detergents used in the first step of purification solubilized a number of impurities that were subsequently difficult to remove. Consequently, repeated fractionations with ammonium sulfate in the presence of detergents or denaturating reagents such as sodium dodecyl sulfate had to be carried out. These operations are not only inadequate and poorly reproducible, but also time-consuming.

The rationale of the present method differs from all of these approaches. We have succeeded in purifying cytochrome oxidase by a simple and mild method using immobilized substrate, reduced cytochrome *c*. With low concentration of detergent, impurities that do not bind with immobilized cytochrome *c* are eluted first. With increasing concentration of deoxycholate, which has been reported to disperse cytochrome oxidase vesicles and to change the reactivity of the enzyme (12), the oxidase is separated from immobilized substrate. The active enzyme without impurities allows the identification of its subunit structure and also makes possible a better understanding of its reaction mechanism and its role in the mechanism of energy conservation.

## MATERIALS AND METHODS

Salt-free cytochrome *c* was supplied by Sigma (Type VI). Seventy mg of cytochrome *c* were dissolved in 5 ml of 0.1 M bicarbonate, pH 8.0, containing 0.5 M NaCl. Five g of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) were swollen and washed with 1 liter of 1 mM HCl for 15 minutes

on a glass filter, and suspended in the bicarbonate buffer. The Sepharose was mixed with cytochrome *c* solution, and the mixture was gently rotated for 2 hours at room temperature. At the end of the incubation, red-colored particles were sedimented and the supernatant was almost colorless. Unbound material was washed out with the bicarbonate buffer. Any remaining active groups were reacted with 1 M ethanolamine at pH 8.0 for 1 hour. Three washing cycles were used to remove non-covalently adsorbed cytochrome *c*, each cycle consisting of a wash at pH 4.0 with 0.1 M acetate buffer containing 1 M NaCl followed by a wash at pH 8.0 with 0.1 M borate buffer containing 1 M NaCl. The yield of coupled cytochrome *c* was 95%. Deoxycholate was recrystallized three times from 50% ethanol. Protein was estimated by the biuret method (13), phospholipid by phosphate determination (14), and heme *a* by direct spectrophotometric measurement of the α band of dithionite reduced enzyme using an excitation coefficient (Δ605-630 nm) of 16.5 $mM^{-1}$ $cm^{-1}$ (15). Activity of cytochrome oxidase was measured polarographically at 20°C by the method of Wharton and Griffiths (16).

## RESULTS AND DISCUSSION

Cytochrome *c*-Sepharose was packed in 0.9 × 25 cm column (Pharmacia K 9/30). The column top was protected by overlaying 1 ml of Sephadex G-25. The bound cytochrome *c* was reduced by passing 0.1 M ascorbate, pH 7.4, through the column, followed by washing with the bicarbonate buffer. The color of the column was changed from red to pink, characteristic of the reduced form of cytochrome *c*. This conversion was essential, since neither the oxidized form of immobilized cytochrome *c* nor Sepharose 4B alone showed significant affinity for cytochrome oxidase, resulting in the appearance of both the oxidase and impurities in the void volume. Cytochrome oxidase preparation was obtained from beef heart mitochondria by the method of Flower, Richardson and Hatefi (1). The sample contained 8.5 nmoles heme *a* per mg protein. The solution containing 10 mg protein was passed through a column (2 × 10 cm) of Sephadex

G-25 (coarse) equilibrated with the bicarbonate buffer to reduce the concentration of deoxycholate in the sample. The eluate was charged onto the cytochrome *c*-Sepharose column. The bicarbonate buffer alone and the same buffer containing 0.25% deoxycholate were passed successively through the column followed by the same buffer containing 0.75% deoxycholate. Elution speed was controlled by a peristaltic pump (LKB 10,200 Perpex). The ultraviolet absorption of the eluate was monitored by a UV spectrometer (TOYO Uvicon 540 M). A typical elution pattern is shown in Fig. 1. Protein eluted in the void volume contains less than 0.1 nmole of heme *a* per mg protein and negligible catalytic activity (Table I). With 0.75% deoxycholate, a highly puri-

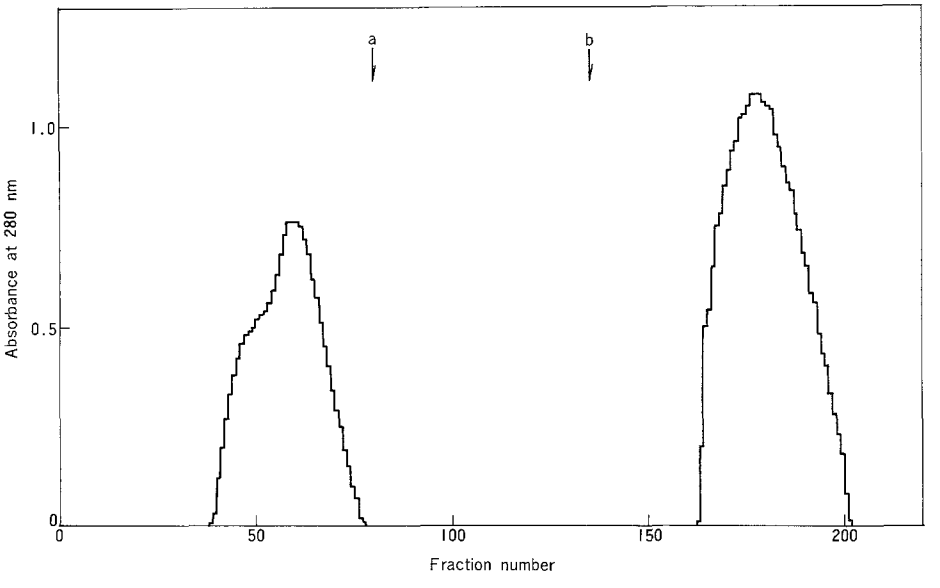


Fig. 1. Separation of cytochrome oxidase from contaminants by Sepharose-bound cytochrome *c*. Cytochrome oxidase sample prepared from beef heart mitochondria (1) was passed through a column (2 × 10 cm) of Sephadex G-25 (coarse) equilibrated with 0.1 M bicarbonate, pH 8.0, containing 0.5 M NaCl, and then charged onto the column (0.9 × 25 cm) of Sepharose-bound cytochrome *c* which was previously reduced by ascorbate and washed with 0.1 M bicarbonate, pH 8.0, containing 0.5 M NaCl. The bicarbonate buffer alone and the same buffer containing 0.25% deoxycholate (arrow a) and 0.75% deoxycholate (arrow b) were successively used as the eluting agents. Elution speed was set to be 5.4 ml/hr by using a peristaltic pump. The ultraviolet absorption of the eluate was monitored. Each fraction was 0.25 ml.

Table I

Purification of cytochrome oxidase by Sepharose-bound cytochrome *c*

| Sample | Specific activity (μmoles cytochrome *c* oxidized/min/mg protein) | Heme *a* (nmoles/mg protein) | Phospholipid (μg P/mg protein) |
|---|---|---|---|
| Starting Material | 3.6 | 8.5 | 13.5 |
| Fraction No. 46-66 | < 0.1 | < 0.1 | 14.5 |
| Fraction No. 171-183 | 5.8 | 13.6 | 13.0 |

fied cytochrome oxidase (62.3% of the original protein) was eluted as the main peak. In this enzyme sample, heme *a* was quantitatively concentrated to 13.6 nmoles per mg protein. The minimum molecular weight (73,500) calculated on the basis of the heme *a* content is close to that (72,000) reported by Criddle and Bock (3) for the molecular weight of the monomeric form of the enzyme in the presence of sodium dodecyl sulfate. Table I shows that the purified sample has higher specific activity than the starting material.

Judging from the separation capacity of the column, a part of immobilized cytochrome *c* molecules is bound with Sepharose in a suitable way and/or in an appropriate environment to interact with cytochrome oxidase, while some of the cytochrome *c* molecules appear to be bound incorrectly or denatured during the coupling process.

The present results indicate that affinity chromatography with a membrane component as ligand is a useful and simple procedure for the specific purification of the membrane-bound enzyme. The further characterization of the purified enzyme will be published elsewhere.

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