AFFINITY CHROMATOGRAPHY PURIFICATION OF CYTOCHROME *c* OXIDASE

Use of a yeast cytochrome *c*–thiol-sepharose 4B column

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1. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is a membrane-bound enzyme which catalyses the reduction of molecular oxygen to water. The reducing equivalents for this reaction are provided by the natural substrate of the enzyme, ferrocytochrome *c*.

Cytochromes *c* have largely conservative structures, suggesting that they are descendants of a common ancestor and have analogous functions [1]. They form reversible associations with their reductases and oxidases, that are mediated by complementary charge interactions [2,3]. Separation of cytochrome *c* oxidase by affinity chromatography on a cytochrome *c* column has been attempted [4] although the technique proved to be effective only with the *Neurospora* enzyme [5].

The inability of a Sepharose 4B–cytochrome *c* column to easily and reproducibly separate cytochrome *c* oxidase may be due to the fact that the lysine residues, through which cytochrome *c* crosslinks to CNBr-activated Sepharose 4B, are most probably the residues which are necessary for the cytochrome *c* oxidase and reductase binding [2,3,6].

Here we describe a technique which allows cytochrome *c* oxidase purification in one step, starting from a mitochondrial Triton X-100 extract. It is based on the use of *Saccharomyces cerevisiae* cytochrome *c* which, through its cysteine residue located close to the N-terminus [1] can be covalently linked to a thiol-activated Sepharose column, thus leaving the important lysine residues free for binding cytochrome *c* oxidase and reductase.

2. Materials and methods

Beef-heart mitochondria were prepared according to a standard procedure [7]. For the spectral analysis an Aminco-DW 2a spectrophotometer was used. Polyacrylamide gel electrophoresis was carried out according to [8]. The unstained gels were scanned in a special scanning attachment using a 280–300 nm wavelength pair [9]. Cytochrome *c* oxidase activity was measured spectrophotometrically in the following medium: 75 mM choline chloride; 25 mM KCl; 0.5% Tween-80; 2.8 μM reduced cytochrome *c*. Variable amounts of cytochrome *c* oxidase (0.3 – 1.5 μM heme) were added and the reaction followed at 550–540 nm. Protein was determined by the method in [10]. Activated thiol-Sepharose 4B was obtained from Pharmacia, cytochrome *c* type VIII from Sigma and Triton X-100 from Fluka. The other substances were of the purest grade commercially available.

3. Results and discussion

Activated thiol-Sepharose 4B was swollen (4 g in 30 ml distilled water) for 30 min and washed on a sintered glass filter (G3) with 1.2 l distilled water to yield 12 ml hydrated gel. This was subsequently equilibrated with 200 ml medium containing 0.050 M Tris–HCl and 1 mM EDTA (pH 7.4) then divided into two 6 ml aliquots. One of these was packed into a column (10 cm × 1 cm) and through this was passed a solution of *S. cerevisiae* cytochrome *c* (25 mg in 0.5 ml above medium). The two ends of the column were connected through a peristaltic pump and the solution of cytochrome *c* was circulated for 8 h at 4°C. After washing with 50 ml buffer, a further 25 mg cytochrome *c* were passed through the column and the reaction and washing were repeated. More than 90% of the added cytochrome *c* was bound to the resin. The other 6 ml swol-

len gel was treated identically, except that instead of cytochrome *c* it was reacted with 100 ml 0.025 M cysteine to deactivate the resin. This column was used to establish that the separation observed with the cytochrome *c* column indeed occurred through affinity chromatography and was not simply based on interaction with the resin itself.

Beef-heart mitochondria were depleted of cytochrome *c* by the procedure in [11]. The amount of cytochrome *c* remaining associated with the resulting inner membrane preparation was less than that which could be detected spectrophotometrically.

The mitochondria, depleted of cytochrome *c* were diluted to 2 mg protein/ml in the buffer described and Triton X-100 was added to 1% final conc. After stirring for 1 h at 0–4°C, the mitochondrial suspension was centrifuged at 28 000 X *g* for 40 min in a Sorvall RC-5B centrifuge.

The protein remaining in the supernatant was 75% of the total, and gave an absorbance spectrum (dithionite reduced minus oxidized) shown in fig.1. The maxima are characteristic of the cytochromes of the mitochondrial respiratory chain. After equilibration of the cytochrome *c* column (and the inactivated column) overnight with the buffer described containing 1% Triton X-100 (pH 7.4) at a pump flow rate of 12 ml/h, 30 ml mitochondrial Triton X-100 extract was loaded. The elution was started using the equilibration buffer and 3 ml fractions were collected.

In fig.2, the elution profiles of protein, cytochromes aa_3 and bc_1 are shown. The column adsorbs only ~50% of the solubilized oxidase. The remainder is eluted with the void volume. The capacity of the column was 0.03 nmol oxidase/nmol cytochrome *c*.

On increasing the ionic strength by addition of 50 mM NaCl to the elution buffer, cytochrome *c* oxidase alone was eluted. Cytochrome bc_1 complex was eluted, beginning with fraction 33, after [NaCl] had been increased to 100 mM. The fractions containing separated cytochrome *c* oxidase have the spectrum shown in fig.3. No evident contamination of other absorbing species is present. The activity in the original Triton

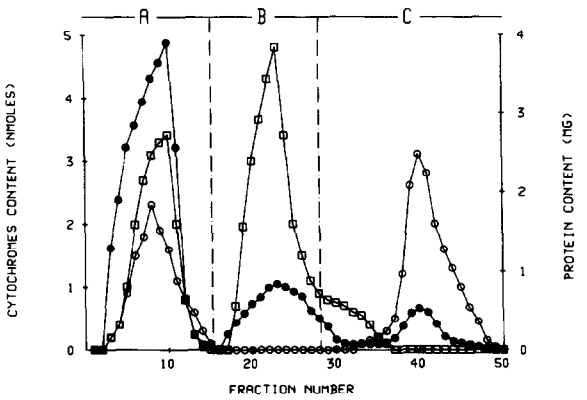

Fig.2. Elution of mitochondrial cytochromes and protein from a Sepharose 4B–cytochrome *c* column. Media used for elution were 50 mM Tris, 1 mM EDTA, 0.1% Triton X-100, (pH 7.4) containing either: (A) no further additions; (B) 50 mM NaCl; or (C) 100 mM NaCl. Fractions were collected as described in the text and assayed for protein content (•) and spectrophotometrically for content of cytochromes aa_3 (□) and bc_1 (o).

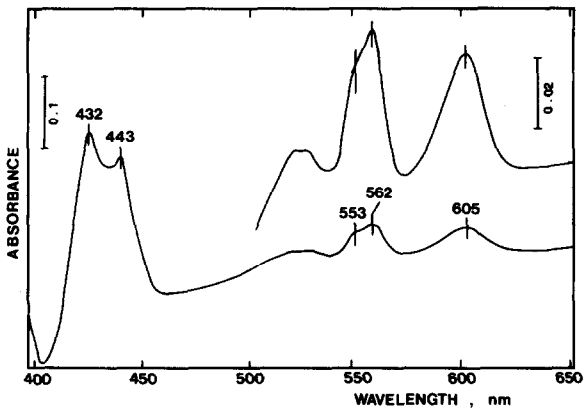

Fig.1. Absorbance spectrum (dithionite reduced minus oxidised) of a 1% Triton-solubilized extract from cytochrome *c*-depleted mitochondrial inner membranes.

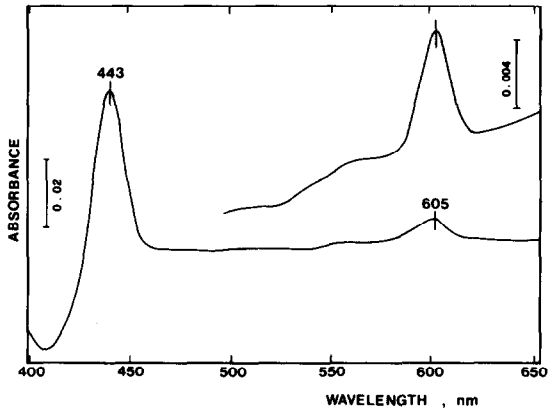

Fig.3. Absorbance spectrum (dithionite reduced minus oxidised) of cytochrome *c* oxidase separated from a 1% Triton X-100-solubilized mitochondrial extract by affinity chromatography (fraction 23 of fig.2).

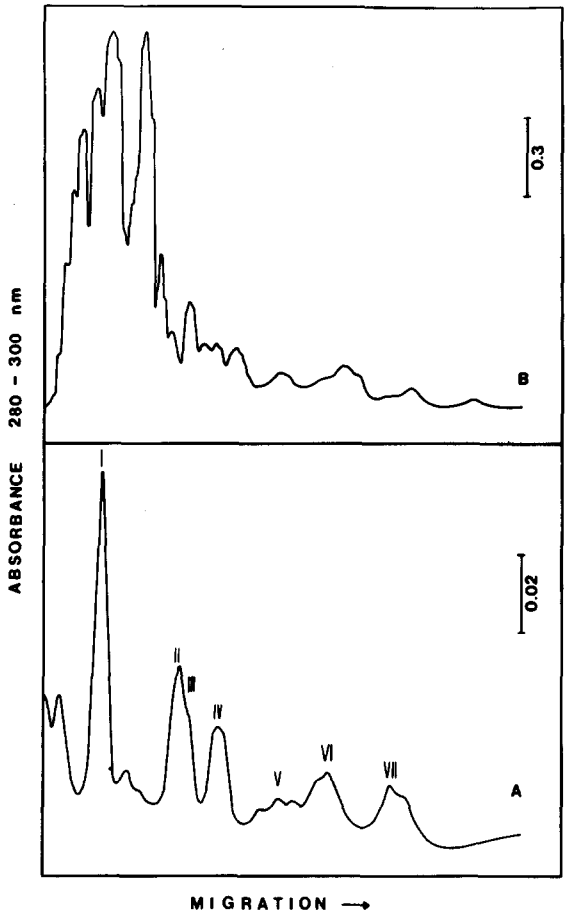

Fig.4. Densitometric traces of SDS–polyacrylamide gels following electrophoresis of: (A) a 1% Triton X-100 solubilized mitochondrial extract; and (B) cytochrome *c* oxidase separated from this extract by affinity chromatography.

X-100 extract was 61 mol cytochrome *c* oxidised. s^{-1}. mol cytochrome *c* oxidase^{-1}. After chromatography it became 97.1. The heme to protein ratio reached the value of 9.5 nmol/mg protein.

The purity of the enzyme was analyzed by SDS – polyacrylamide gel electrophoresis. A typical profile is shown in fig.4, which shows 7 major bands and some minor lower molecular weight contaminants, normally present in most preparations of this enzyme. The yield of pure cytochrome *c* oxidase relative to the amount of enzyme which was retained by the column was 50%. That relative to the total added Triton X-100 extract was 25%. The column has a long lifetime and does not release cytochrome *c* unless disulfide reducing agents are employed. It is, however, easy to regenerate.

4. Conclusion

A simple, one-step preparation of cytochrome *c* oxidase by affinity chromatography is described which offers a new and flexible tool for the studies of this important enzyme. The quasi-identity of the cytochrome *c*-binding surface for cytochrome *c* reductases and peroxidase, the ubiquitous nature of soluble cytochrome *c*, present in respiratory and photosynthetic electron-transfer chains, make this technique an excellent candidate for the isolation of a number of enzymes from very different sources, having in common only their reactivity to cytochrome *c*.

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