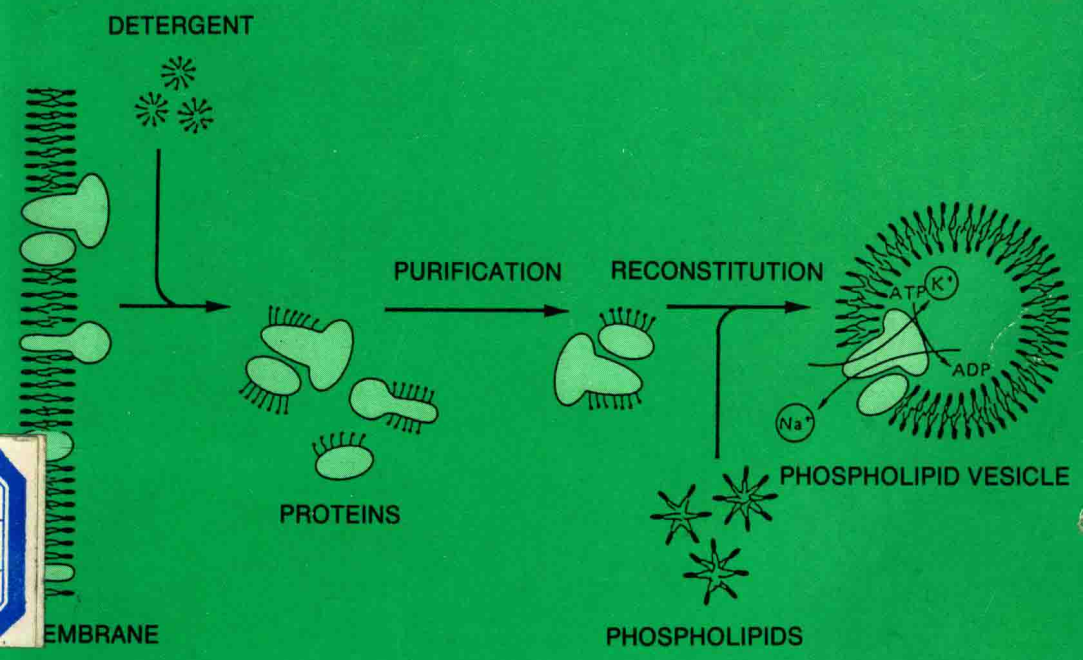
Membrane Proteins

Isolation and Characterization

Edited by
Angelo Azzi, Lanfranco Masotti, Arnaldo Vecli



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Preface

This volume is the third of a series on Membrane Proteins and, like the preceding manuals, is the result of an International Advanced Course entitled *Isolation and Characterization of Membrane Proteins: Biochemical and Biophysical Aspects* sponsored by the Federation of European Biochemical Societies (FEBS) and the Italian Research Council (CNR).

The success of the course and the continuous development in the field of membrane biology has prompted me to publish also in this case the protocols of the experiments which were carried out by the students.

The students have been able not only to perform the experiments published in this manual without help from the instructors, but also to suggest improvements, which have been incorporated in the published version.

Care has been taken in making the planning and the execution of the experiments as simple as possible, by listing in detail all the necessary pieces of equipment, test tubes, pipettes, chemicals, etc. At the same time the introduction and the "philosophy" have been limited to the essential, as also the references, only those having been listed which may help in a better understanding of the principles and of the biological background of a given experiment.

Like the previous manuals, this one can be useful in the research laboratory where new experiments can be based on carefully checked routine protocols. It can be useful also in a laboratory of clinical analysis, where scientists may be looking for new, simple methodologies suitable to be applied to the field of human clinical testing. Finally, it can be used as a textbook for a practical course at graduate level of biochemistry and of biophysics.

October 1986

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I. Membrane Proteins: Function, Purification, and Characterization

Purification of Cytochrome c Reductase and Oxidase by Affinity Chromatography

REINHARD BOLLI, CLEMENS BROGER and ANGELO AZZI

I. Introduction

Cytochrome c reductase and oxidase have traditionally been purified from a large number of species and tissues by techniques based on detergent solubilization of these membrane enzymes and subsequent fractionation by ammonium sulfate precipitation [Nelson (1978), Yu et al. (1979); for a review on the enzymes see Azzi et al. (1985) and Rich (1984)]. These procedures are useful only for large-scale preparations and they have to be adapted for the isolation of the enzymes from different sources.

In the present experiment a more recently developed method is described. It makes use of the specific interaction between cytochrome c and the two enzymes which act as electron acceptor and electron donor for cytochrome c, respectively. The use of cytochrome c as an affinity ligand for the purification of the two enzymes was proposed already in 1960 and subsequently developed further (Weiss et al. 1978). It was known that cytochrome c contains many lysine residues, through which the protein could be attached to a cyanogen bromide-activated Sepharose 4B gel. Later, however, it was found that these lysine residues are located mainly on one side of the cytochrome c molecule ("front side") and that they are important for the interaction between this protein and its electron donor and acceptor (Margoliash and Bosshard 1983). This could have been the reason why such an affinity chromatography procedure worked only in some cases.

Here, an affinity resin is used, where cytochrome c is attached to the solid support via its "back side". Cytochrome c from *Saccharomyces cerevisiae* contains a cysteine residue near its C-terminus, which is located on the rear side of the molecule. The cysteine can be linked to Activated Thiol Sepharose 4B (Glutathion attached to cyanogen bromide activated Sepharose 4B) via a disulfide bridge (Fig. 1), thus leaving the front side of the protein exposed for the interaction with its electron donor and acceptor (Azzi et al. 1982).

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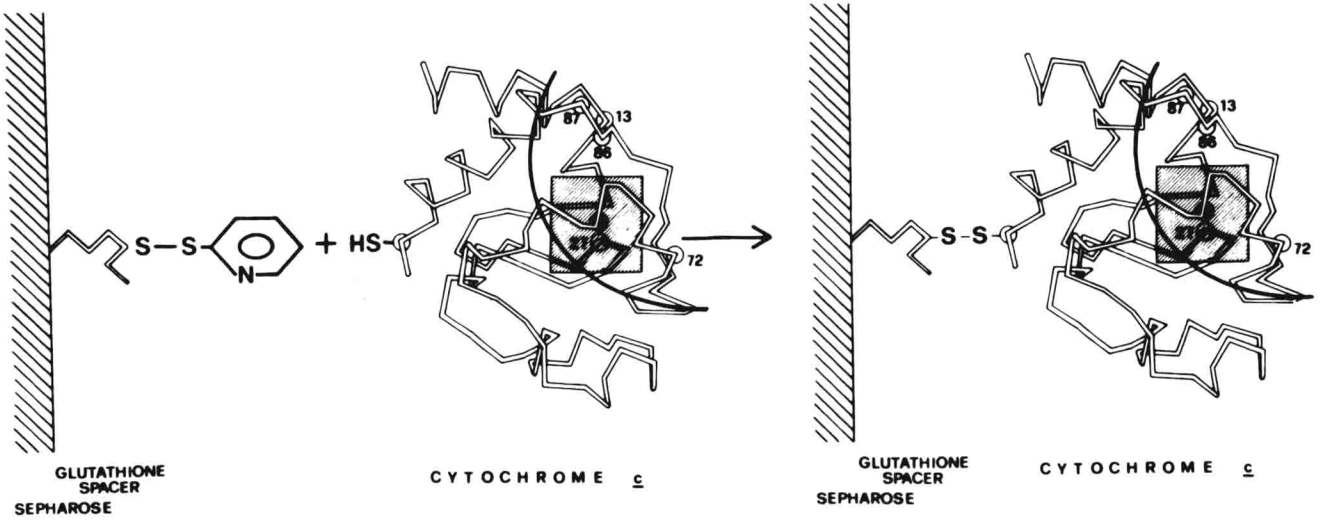

Fig. 1. Cytochrome c from *Saccharomyces cerevisiae* linked to activated thiol Sepharose 4B via a disulfide bridge

Since the surface domain on cytochrome c, which interacts with cytochrome c reductase and oxidase, is almost the same (Capaldi et al. 1982) both enzymes will bind to the affinity resin although with different affinity. Further, since the interaction is mainly electrostatic, the enzymes can be eluted from the resin by increasing the ionic strength of the buffer.

II. Equipment and Solutions

A. Equipment

- Refrigerated high speed centrifuge or ultracentrifuge recording spectrophotometer
- Fraction collector
- Pipettes
- Sintered glass filter (G3)
- Small columns for chromatography
- Peristaltic pump

B. Chemicals and Solutions

- Beef heart mitochondria
- Activated Thiol Sepharose 4B (Pharmacia or prepared in the laboratory from Sepharose 4B and glutathion)
- Sephadex G-25 (Pharmacia)
- Buffer: 40 mM Tris/HCl, 1 mM EDTA, pH 7.4
- Acetate buffer: 50 mM Na-acetate, 1.5 mM 2-mercaptoethanol, pH 4.5
- Cytochrome c (yeast) (Sigma)
- Cytochrome c (horse heart) (Sigma)
- Triton X-100, 20% (w/v) in water
- Tween 80, 20% (w/v) in water
- 1 M NaCl
- Sodium dithionite, solid
- 10 mM KCN
- Antimycin A (Sigma), lmg ml^{-1} in ethanol
- Sodium borohydride, solid
- Quinole: DBH (reduced form), 10 mM in ethanol or Q238 (Aldrich) (DBH: 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) (Q238: 2-methyl-3-undecyl-1,4-naphtoquinone)

III. Experimental Procedures

A. Preparation of the Affinity Column

2 ml of swollen Activated Thiol Sepharose 4B are washed with 100 ml buffer on a sintered glass filter and incubated over night in the same buffer with 6 mg yeast cytochrome c (3 mg ml^{-1} of swollen gel). The total volume should be about 4 ml. In order to be sure that the cysteine residue of yeast cytochrome c is not blocked, it is advised to reduce the cytochrome c prior to the coupling to the gel: about 10 mg of cytochrome c is dissolved in 0.5 ml of buffer and reduced with 0.3 M 2-mercaptoethanol. After 10 min of incubation, excess reagent is removed by passing the solution through a short Sephadex G-25 column (15 x 1 cm) and collecting only the red portion of the eluate. The next morning, the affinity gel is washed free of noncovalently bound cytochrome c with 100 ml of 1 M NaCl. Activated thiol groups of the resin which did not react with cytochrome c are de-activated by washing the gel three times with 10 ml of acetate buffer containing the SH-group reagent 2-mercaptoethanol. About 60% of the added cytochrome c is bound to the gel. The gel may be stored in 1 M NaCl at 4°C for some weeks. Before use, the gel is washed with 100 ml of buffer, filled into a small column and equilibrated with 50 ml of buffer containing 1% Triton X-100.

B. Purification of Cytochrome c Oxidase and Reductase

Mitochondria (about 40 mg of protein) are diluted with the buffer to a protein concentration of 2 mg ml^{-1} and Triton X-100 is added to a final concentration of 1%. The solution is incubated for 30 min at 4°C. After centrifugation at 100,000 *g* for 30 min or 45 min at 50,000 *g*, the pellet is discarded and 10 ml of the supernatant is loaded on the column. Subsequently the column is washed with 20 ml of buffer containing 0.1% Triton X-100 at a flow rate of ca. 25 ml h^{-1}, until no heme absorbance is detected in the eluate. Fractions of 5 ml are collected.

Now the ionic strength of the elution buffer is increased to 50 mM NaCl. Cytochrome c oxidase is eluted next and four fractions of 5 ml are collected. Upon further increasing the ionic strength to 150 mM NaCl, cytochrome c reductase is eluted from the column. Again four fractions of 5 ml are collected. Spectra of all the fractions are recorded and the cytochrome content is determined as described under "measurements".

After washing extensively with lM NaCl containing 1% Triton X-100, the column can be reused although the binding capacity will be slightly reduced.

C. Measurements

1. Spectra

Difference spectra (reduced minus oxidized) of the Triton extract and of the fractions from the column are recorded in the range of 400 to 650 nm. The reduced sample is obtained by dissolving few grains of Na-dithionite directly in the cuvette. Before recording, the sample is incubated for 2 min. The cytochrome b, c_1, and aa_3 are calculated according to the law of Beer-Lambert using the extinction coefficients indicated in Table 1.

Table 1. Extinction coefficients

Cytochrome		Wavelenth	Extinction coefficient (mM)
Oxidase	aa_3	605–630 nm	27
Reductase	b	562–575 nm	25.6
Reductase	c_1	554–540 nm	20
Cytochrome	c	550–540 nm	19
		550 nm	21

2. Cytochrome c Oxidase Activity

The substrate of cytochrome c oxidase, reduced cytochrome c (ferrocytochrome c), is obtained by adding a few grains of sodium dithionite to a concentrated solution of horse heart cytochrome c in buffer. The dithionite is removed subsequently by passing the solution through a short column (15 x 1 cm) of Sephadex G-25 equilibrated in buffer. The red cytochrome c fraction of the eluate is collected and the heme c concentration is determined spectrophotometrically.

The activity of cytochrome c oxidase is measured by adding small aliquots of the fractions (10 to 50 μl) from the affinity column to a cuvette filled with 1 ml of buffer containing 0.5% Tween 80 and 5 μM ferrocytochrome c. The absorbance decrease is measured at 550 minus 540 nm or at 550 nm only, depending on the spectrophotometer available. The molecular activity is calculated as mol of cytochrome c oxidized per mol of hema aa_3 per second (turnover number).

3. Cytochrome c Reductase Activity

Reduction of Quinone (Q238). 5–10 mg of quinone are dissolved in a small volume of a 1:1 mixture of ethanol and DMSO. Reduction is performed by adding small portions of sodium-borohydride until the solution stays brown. Not reacted reagent is destroyed by adding dropwise 1 M HCl, until the gas evolution ceases. The concentration is determined from the weight of the originally dissolved quinone and the final volume of the solution.

Activity. A cuvette is filled with 1 ml of buffer containing 0.5% Tween 80, 5 μM oxidized horse heart cytochrome c, 0.1 mM KCN and 10 μM quinole. The rate of nonenzymatic reduction of cytochrome c is recorded as an absorbance increase at 550 minus 540 nm (or 550 nm). The reaction is then started by adding aliquots of the fractions from the affinity column and after some time 2 μl of antimycin A is added. Only the antimycin-sensitive rate of cytochrome c reduction is measured. The cytochrome c reductase activity is given as mol of cytochrome c reduced per mol of heme b per second (turnover number).

IV. Results, Interpretation and Comments

Purification. The concentration of heme aa_3, b, and c_1 in the Triton extract is about 0.65, 0.55, and 0.26 μM, respectively. After loading and washing of the affinity column, 70% of cytochrome b and 55% of cytochrome oxidase is bound, which corresponds to a binding capacity of the affinity gel of 3.5–4 nmol enzyme per ml of packed resin (2 nmol of bc_1 and 1.7 nmol of heme aa_3). Recovery after chromatography for both enzymes is about 80%. The cross-contamination in the eluted fractions is normally not more than 10%. The separation, however, can be improved to zero cross-contamination by applying a linear salt gradient between 0 and 175 mM NaCl (50 ml volume total) instead of the described elution in steps.

Enzyme Activity. A molecular activity of 100 mol mol^{-1} s^{-1} for cytochrome c oxidase and 10 mol mol^{-1} s^{-1} for the purified bc_1-complex using Q238 as substrate can be expected.

The results may be best represented in a table as proposed in Table 2.

Enzyme Purity can be further analyzed by a protein determination in the fractions according o Lowry et al. (1951) and calculation of the heme to protein ratio. Pure bc_1-complex and cytochrome c oxidase isolated by traditional procedures contain about 6.5–7 nmol heme b mg^{-1} protein and 5 nmol heme aa_3 mg^{-1}, respectively. This purity can also be achieved by the method described here. Polypeptide analysis by polyacrylamide gel electro-