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AFFINITY CHROMATOGRAPHY PURIFICATION OF CYTOCHROME *c* OXIDASE AND *b*-c_1 COMPLEX FROM BEEF HEART MITOCHONDRIA

USE OF THIOL-SEPHAROSE-BOUND *SACCHAROMYCES CEREVISIAE* CYTOCHROME *c* *

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A method for simultaneous purification of cytochrome *c* reductase and cytochrome *c* oxidase using a cytochrome *c* affinity column is presented. Cytochrome *c* from *Saccharomyces cerevisiae* was linked to an activated thiol-Sepharose gel via its Cys-102 residue located far from the lysine residues on the front side of the molecule, responsible for the interaction with the reductase and oxidase. In previously reported affinity chromatography techniques these lysine residues most probably reacted with the column. Cytochrome *c* oxidase and reductase from bovine heart mitochondria bind specifically to the affinity column and can be recovered separately at different ionic strength in the elution buffer. The enzymes are highly pure and active.

Introduction

In the mitochondrial respiratory chain, cytochrome *c* is the electron carrier between cytochrome *c* reductase and cytochrome *c* oxidase. Purification of these complexes has been traditionally achieved with procedures having in common the use of detergents for solubilizing the mitochondrial membranes and salts for precipitating the proteins. All the complications implicit in the multistep isolation of delicate enzymes are to be expected in these procedures (for reviews see Refs. 1 and 2). Attempts have been made in the past to isolate cytochrome *c* oxidase and reductase using an affinity chromatography technique with horse heart cytochrome *c* bound covalently to a Sepharose gel via some of its lysine side chains [3,4]. With this technique only the reductase and the oxidase of *Neurospora crassa*, and not those of beef heart or yeast [5], can be purified. Another useful technique employed to isolate cytochrome *c* oxidase from beef heart is that of Rosen [33] based on the application of hydrophobic chromatography.

The molecular aspects of the interaction of cytochrome *c* with cytochrome *c* reductase and oxidase have been elucidated [6–9]. Both enzymes interact with the same part of the surface of cytochrome *c* during electron transfer, namely with the area containing the top of the exposed haem edge and lysine side chains 13, 27, 72, 86 and 87, the binding partner in the oxidase being subunit II [10] and in the reductase cytochrome c_1 [11].

Cytochromes *c* from different sources are known to have a highly conservative amino acid sequence [12] and a similar three-dimensional structure. The area which is important for binding to the oxidase and reductase is located in the front part of the molecule, whereas the amino and carboxyl termini are located in the rear part [13].

Since the ϵ-amino group of the Lys-13 residue

Abbreviation: SDS, sodium dodecyl sulphate.

* A preliminary report of part of this work has been published [15].

appears to be the most reactive to chemical modification [12], it is probable that, in the attempt to prepare an affinity column with immobilized cytochrome *c*, this amino group, essential for the interaction with partner enzymes, was blocked by the reaction with CNBr-activated Sepharose 4B. This could explain why we did not succeed in repeating the experiments of Ozawa et al. [3] and Weiss and Kolb [5] and why applications of this method are not found in the literature. Cytochrome *c* from *Saccharomyces cerevisiae* contains a cysteine residue at the penultimate position before the C-terminus (residue 102 according to the numbering of Dickerson and Timkovich [12]). Such a residue, not functionally important [14], has been used to attach covalently *Saccharomyces* cytochrome *c* to an activated thiol-Sepharose gel. The disulphide bond formed in the reaction left the front part of the molecule free to interact with the reductase and the oxidase (Fig. 1).

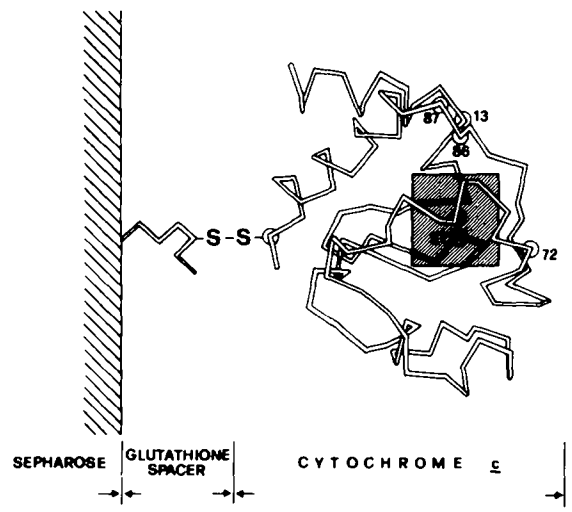

Fig. 1. Schematic drawing of cytochrome *c* bound to thiol-Sepharose 4B. The backbone is that of horse heart cytochrome *c* seen from the side [31]. A cysteine residue has been drawn at position 102 (to simulate the situation with *S. cerevisiae* cytochrome *c*) and linked through a disulphide bond with the cysteine of the Sepharose 4B-bound glutathione. The square on the plane of the page represents the haem, the closed circle the iron, the open circles are important amino acids: lysine residues (with a number) involved in the interaction with the oxidase and reductase or the cysteine residue (102) (unnumbered). At position 13 in yeast cytochrome *c*, instead of a lysine residue an arginine residue is present. The amino terminal end is near the upper part of the molecule, the carboxyl terminus is near the cysteine residue.

Materials and Methods

Materials

Triton X-100 was obtained from Fluka (Buchs, Switzerland), cytochrome *c* type VIII (*S. cerevisiae*) and type VI (horse heart) from Sigma, activated thiol-Sepharose 4B from Pharmacia. Activated thiol-Sepharose 4B (Sepharose-glutathione-2-pyridyl disulphide) is a mixed disulphide formed between 2,2'-dipyridyl disulphide and glutathione coupled to CNBr-activated Sepharose 4B. The hydrophilic glutathione residue acts as a spacer group, thereby decreasing steric interference with exchange reactions at the terminal thiol group. Activated thiol-Sepharose 4B contains approx. 1 μmol 2-pyridyl disulphide groups/ml swollen gel. Affigel 10 was obtained from BioRad. Coenzyme Q_1 was a gift from Hoffmann-La Roche (Basel, Switzerland). All other reagents used were of the highest purity commercially available.

Methods

Preparation of cytochrome c-depleted mitochondria. All manipulations were performed at 0–4°C. Mitochondria were prepared from bovine heart according to Ref. 16 and stored at −80°C. They were depleted of cytochrome *c* using the method of Jacobs and Sanadi [17]. Cytochrome *c*-depleted mitochondria when not used immediately were stored at −80°C. In this case before use they were homogenized in a glass-Teflon homogenizer. The mitochondria were suspended in 50 mM Tris-HCl, pH 7.2, containing 1% (w/v) Triton X-100 at a protein concentration of 2 mg/ml. After stirring for 1 h the solution was centrifuged for 1 h at 27 000 × *g* in a Sorvall GSA rotor. The pellet was discarded.

Preparation of yeast-cytochrome c-thiol-Sepharose-4B gel. 25 ml of swollen and washed activated thiol-Sepharose 4B were suspended in 50 ml of 50 mM Tris-HCl buffer, pH 7.2. To this suspension were added 75 mg of cytochrome *c* from *S. cerevisiae* and the suspension was incubated with stirring overnight. After this incubation the suspending buffer was almost colourless. Thiol groups of the gel which did not react with cytochrome *c* were blocked by washing the gel with 50 ml of 50 mM acetate buffer, pH

4.5, containing 1.5 mM 2-mercaptoethanol. Under these conditions, no covalently bound cytochrome *c* was removed from the gel. The gel was poured into a column (2 X 20 cm). The column was washed with 1 l of 50 mM Tris-HCl, pH 7.2, containing 1% Triton X-100, 1 M NaCl to remove non-covalently bound cytochrome *c* and 1 mM ferricyanide to oxidize fully cytochrome *c*. The column was finally equilibrated with 50 mM Tris-HCl, pH 7.2, containing 1% Triton X-100.

Affinity chromatography. In a typical experiment, 400 ml of Triton X-100-extracted mitochondria were applied to the column at a flow rate of 35 ml/h. Fractions of 15 ml were collected. Alternatively, the gel was loaded in batches and afterwards poured into the column. After loading, the column was washed with 50 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100 until no protein could be detected in the eluate. Subsequently, a linear gradient between 0 and 175 mM NaCl in the washing buffer was applied at the same flow rate (total 600 ml). Fractions of 5 ml were collected and analyzed spectrophotometrically using an Aminco DW-2a spectrophotometer.

Miscellanea. The concentrations of cytochromes aa_3, *b* and c_1 were calculated using extinction coefficients (reduced minus oxidized) of 12 (605–630 nm), 25.6 (562–577 nm) and 20.1 $mM^{-1} \cdot cm^{-1}$ (553–540 nm), respectively. The protein concentration was estimated using either the method of Wang and Smith [18] or Spector [19]. Polyacrylamide gel electrophoresis was carried out for cytochrome *c* oxidase according to the method of Swank and Munkres [20] on 10% gels or according to that of Merle and Kadenbach [21] on 16% gels and for cytochrome *c* reductase according to that of Laemmli [22] on 12% gels. The unstained gels were scanned in quartz tubes at 280 minus 300 nm and for the reductase also at 400 minus 430 nm in a special gel scanner attached to the Aminco DW-2a spectrophotometer [23]. Bovine serum albumin, carbonic anhydrase, myoglobin and cytochrome *c* were used as molecular weight standards.

Phosphorus was determined using the method of Chen et al. [24]. The enzymatic activity of cytochrome *c* reductase was measured according to the method of Wan and Folkers [25]. The activity of the cytochrome *c* oxidase was assayed spectrophotometrically at 550 minus 540 nm in a medium containing 75 mM choline chloride, 25 mM KCl, 0.5% Tween 80 and 20 μM reduced cytochrome *c*. Variable amounts (1–3 μg) of enzyme were added.

Results

Activated thiol Sepharose 4B was reacted with cytochrome *c* from *S. cerevisiae.* 0.125 μmol were bound per ml of swollen gel. The non-reacted activated thiol groups were blocked with 2-mercaptoethanol to prevent SH-group-containing mitochondrial proteins from binding covalently to the column. Mitochondria were washed with 150 mM KCl to extract endogenous cytochrome *c* until the absorbance peak at 550 nm disappeared. In a typical experiment mitochondria were solubilized at a concentration of 2 mg/ml with 1% Triton X-100. After centrifugation the protein concentration in the supernatant was 1.1 mg/ml and the concentrations of haems aa_3, *b* and c_1 were 1.5, 0.5 and 0.25 μM, respectively (Fig. 2). 400 ml of this solution were applied to the column. At the beginning of the loading no haem absorbance was found in the eluate, which had a yellowish colour and a negative absorbance in the region of 460 nm (oxidized minus reduced), indicating the presence of flavoproteins. Towards the end of the loading more and more of the haem absorbance appeared in the eluate (Fig. 3). After washing, 29% of

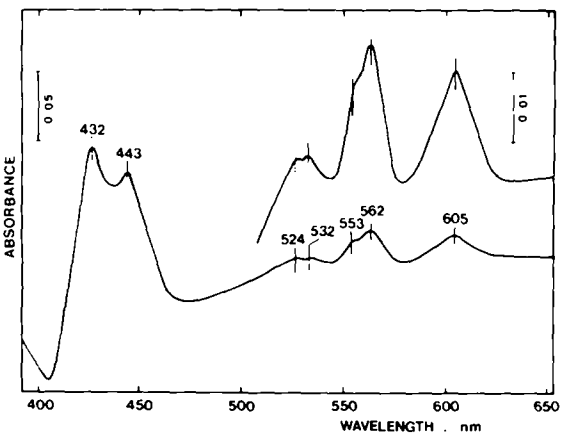

Fig. 2. Absorbance spectrum of the Triton X-100-extracted mitochondria. The spectrum is a difference spectrum (dithionite reduced minus oxidized). The sensitivity at which the upper trace was recorded was 5-times higher than for the lower.

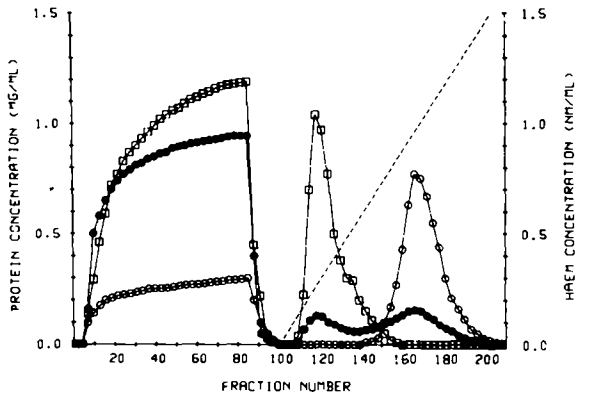

Fig. 3. Elution of cytochrome b-c_1 complex and cytochrome c oxidase from a Sepharose 4B-yeast cytochrome c column. Fractions 1–83: effluent recovered during loading of a 1% Triton X-100 extract of mitochondria in 50 mM Tris-HCl, pH 7.2. Fractions 84–100: effluent obtained after washing the column with buffer containing 0.1% Triton X-100. Fractions 101–210: elution profile of the cytochrome c oxidase and reductase using the same buffer with linearly increasing NaCl concentration. The salt concentration is indicated by the dashed line and its concentration (mM) is calculated by multiplying the numbers of the y-axis by 100. Fractions were collected as described in Materials and Methods and analysed for protein (•), cytochrome aa_3 (□) and cytochrome b (○).

the applied protein, 37% of the haem aa_3 absorbance and 53% of the haem b and c_1 absorbance were retained by the column and no more haem absorbance and protein could be detected in the eluate. Using a linear NaCl gradient between 0 and 175 mM, first cytochrome c oxidase and later cytochrome c reductase could be eluted from the column separately. Haem aa_3 absorbance appeared in fractions 109–157 simultaneously with a protein peak when the NaCl concentration was approx. 30 mM. Fractions 115–125 were pooled and concentrated using an Amicon filter PM 30. The spectrum in the visible range of the pooled oxidase is shown in Fig. 4. No haems other than haem aa_3 were detectable. The haem-to-protein ratio reached 9 nmol/mg protein. Polyacrylamide gel electrophoresis of the oxidase-containing fractions in the presence of SDS was carried out. 30–50 μg protein/sample were loaded. The profile of the polypeptide pattern as obtained at 280–300 nm is shown in Fig. 5. Seven subunits were visible, the molecular weights of which are shown in

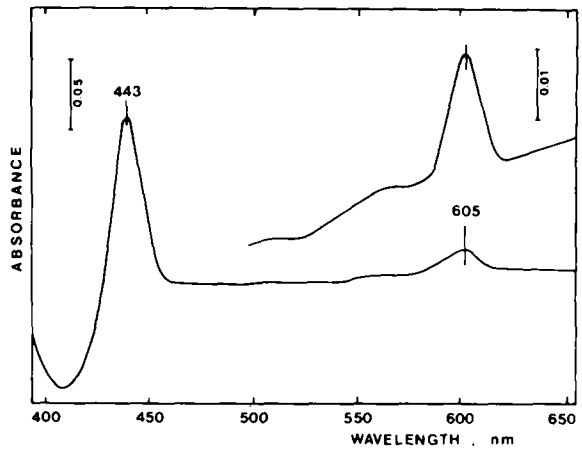

Fig. 4. Absorbance spectrum of cytochrome c oxidase purified by affinity chromatography. This spectrum (dithionite reduced minus oxidized) was obtained from the pooled fractions 115–125. The sensitivity at which the upper trace was recorded was 5-times higher than for the lower.

Table I. The gels showed no major contamination by polypeptides not belonging to the oxidase. The phospholipid content was measured to be 280 nmol/mg protein (35 mol lipid/mol oxidase assuming a molecular weight of 125 000 for the enzyme). The enzymatic activity was 100 mol cytochrome c oxidized/s per mol oxidase. The absorbance of haems b and c_1

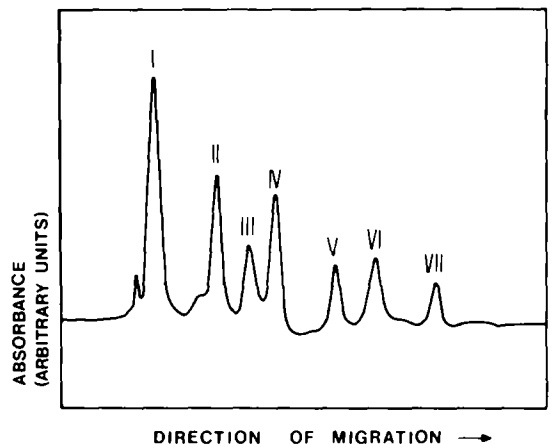

Fig. 5. Polypeptide pattern of affinity chromatography-purified cytochrome c oxidase. Densitometric trace of an SDS-polyacrylamide gel [20] of purified cytochrome c oxidase (pooled fractions 115–125) in the ultraviolet at 280 minus 300 nm. For conditions, see Materials and Methods.

TABLE I

APPARENT MOLECULAR WEIGHTS OF CYTOCHROME *c* OXIDASE AND REDUCTASE SUBUNITS PREPARED BY AFFINITY CHROMATOGRAPHY OBTAINED BY POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE PRESENCE OF SDS

Subunit	Molecular weight	
	Cytochrome *c* oxidase	Cytochrome *c* reductase
I	35 000	50 000
II	26 000	48 000
III	23 000	40 000
IV	18 000	30 000 (cytochrome c_1)
V	12 000	25 000
VI	8 500	13 500
VII	6 000	12 500
VIII		12 500

appeared in the eluate also associated with protein, in fractions 143–210. The NaCl concentration at which the elution occurred was about 95 mM. Fractions 160–180 were also pooled and concentrated. The spectrum in the visible range of the pool is shown in Fig. 6. No contamination by haems aa_3 was visible. The haem-to-protein ratios were 6.5 nmol haem *b*/mg protein and 3.3 nmol haem c_1/mg protein. The polypeptide composition of the cytochrome *b*-c_1 complex was analyzed on polyacrylamide gels in the presence of SDS using between 30 and 50 μg of protein. The polypeptide profile as detected in unstained gels at 280–300 nm showed eight polypeptides (Fig. 7). Cytochrome c_1 was identified as subunit IV by scanning the gel in the Soret region of the haem absorption spectrum. All the polypeptides detected are typical of the reductase as obtained by conventional techniques and their molecular weights are shown in Table I. Subunit V, corresponding to the iron-sulphur protein, was present in relatively small amounts. The phospholipid content was 70 nmol/mg protein (18 mol lipid/mol reductase assuming a molecular weight of 250 000 for the enzyme). The enzymatic activity reached 5 mol cytochrome *c* reduced/s per mol reductase. The enzymatic activity was inhibited completely by antimycin at a concentration of 1 μg/ml. Intermediate fractions 126–159 were a mixture of cytochrome *c* reductase and oxidase.

A cytochrome *c* column similar to that described in Refs. 3 and 4 was prepared by reacting the ε-amino groups of lysine side chains of horse heart cytochrome *c* with affigel 10. No haem aa_3- and bc_1-containing proteins remained associated with this column when a Triton X-100 extract of mitochondria was applied to it. A control was also carried out by employing a thiol-Sepharose 4B column deactivated by

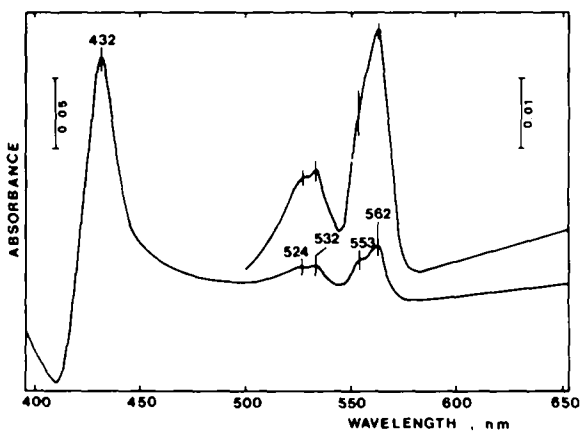

Fig. 6. Absorbance spectrum of cytochrome *c* reductase purified by affinity chromatography. This spectrum (dithionite reduced minus oxidized) was obtained from the pooled fractions 160–180. The sensitivity at which the upper trace was recorded was 5-times higher than for the lower.

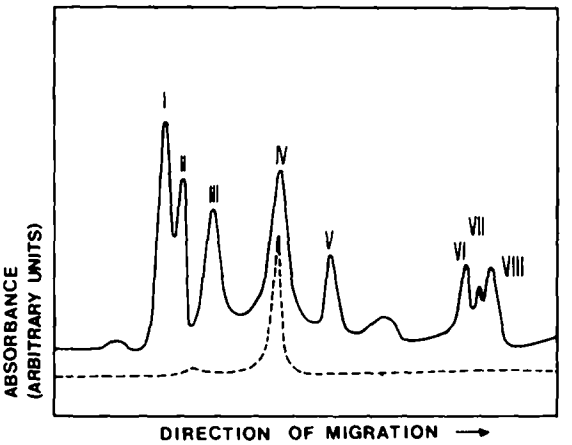

Fig. 7. Polypeptide pattern of cytochrome *b*-c_1 complex. Densitometric traces of an SDS-polyacrylamide gel of purified cytochrome *c* reductase (pooled fractions 160–180) in the ultraviolet at 280 minus 300 nm (———) and in the visible at 400 minus 430 nm (- - - - -). For conditions, see Materials and Methods.

the reaction with 50 mM cysteine. No haem-containing proteins were adsorbed by such a column [15].

In order to establish whether the gel interacted specifically with the cytochrome *c* oxidase the following test was carried out. The spectrum of a suspension of a yeast cytochrome *c*-Sepharose 4B gel reduced with ascorbate and carefully washed was taken. After addition of cytochrome *c* oxidase a second spectrum was taken, indicating full oxidation of the ferrocytochrome *c* bound to the gel. In a control experiment without oxidase no cytochrome *c* was oxidized.

Discussion

Cytochrome *c* is probably the protein of which the primary structure has been determined in the greatest number of animals, plants and bacteria. X-ray crystallographic analysis has shown that the three-dimensional structure was highly conservative during evolution. The very important function of cytochrome *c* as an electron carrier in the mitochondrial respiratory chain between cytochrome *c* reductase and cytochrome *c* oxidase has stimulated a large number of scientific studies. Chemical modification techniques have indicated that the same part of the surface of cytochrome *c* binds to the reductase and the oxidase, namely the front part of the molecule including the top of the exposed haem edge and the region of the residues Lys-13, -27, -72, -86 and -87 [6–9]. Later, our own studies showed that cytochrome *c* could be cross-linked with the reductase or oxidase by a photolabelling technique only if the photolabel was attached to the above-mentioned area on the surface of cytochrome *c*. With the label attached outside of that area no cross-linking occurred with cytochrome c_1 and with subunit II of cytochrome *c* oxidase [10, 11].

Cytochrome *c* from *S. cerevisiae* contains, in addition to the two conserved cysteines which covalently bind to the haem via thioether bonds, a third cysteine near the C-terminus. In analogy with all the known three dimensional-structures of cytochrome *c* species, it is probable that the C-terminus of *S. cerevisiae* cytochrome *c* is located in the rear side of the molecule. This is in agreement with the present findings that *S. cerevisiae* cytochrome *c* can be bound to an activated thiol-Sepharose gel through its Cys-102 residue, leaving the front side of the molecule free for interaction with cytochrome *c* reductase and oxidase. It is known that the most reactive amino groups are located in this part of the molecule and that their modification prevents the high-affinity interaction of cytochrome *c* with cytochrome *c* reductase and oxidase [32].

Previously designed affinity columns had cytochrome *c* bound to the gel through some of the ϵ-amino groups of its lysine residues, probably the most reactive ones. The reported purification properties of these columns therefore, cannot be based on the specific liganding of the substrate to its binding site, but perhaps can be interpreted by invoking ion exchange or hydrophobic interactions.

In our hands cytochrome *c* oxidase and reductase from bovine heart mitochondria solubilized in Triton X-100 in agreement with the results of Weiss and Kolb [5], but contrary to Ozawa et al. [3], could not be bound to horse heart cytochrome *c* reacted with a Sepharose column activated by CNBr. Instead, they could be easily and reproducibly bound and eluted from a column where *S. cerevisiae* cytochrome *c* was linked via its free SH group to a thiol-Sepharose gel.

Although the difference in ionic strength at which cytochrome *c* oxidase and reductase were eluted was relatively small, nevertheless the two enzymes could be separated well by using a not very steep NaCl gradient. The eluted enzymes in this case were, however, rather diluted. If the gradient was steeper the eluted enzymes were more concentrated but cross-contamination was greater. The subunit composition of cytochrome *c* oxidase isolated by the present technique showed on SDS-polyacrylamide gels [20] seven subunits, as traditionally observed. The cytochrome b-c_1 complex, except for the smaller intensity of band V, also had its characteristic polypeptide pattern. It was found that omitting EDTA from the buffers, at variance with the procedure described in Ref. 15, led to an increase in this band, which was even more pronounced with about 1 mM $MgCl_2$. This is in agreement with Weiss and Kolb [5], whose cytochrome b-c_1 complex prepared in the absence [4] but not in the presence [5] of EDTA contained the iron-sulphur protein. In addition, it seems [5] that the content of the iron-sulphur protein depends on the concentration of the cytochrome b-c_1 complex eluted from the column. The lipid content

of the oxidase is relatively high, comparable to 'lipid-rich' enzymes purified according to traditional procedures [2], and that of the cytochrome b-c_1 complex is relatively low, compared with certain procedures [1] but high compared to others [4].

The technique presented here is mild and no salt precipitation step is involved. Both enzyme complexes are purified at the same time in a procedure which can be highly automated. The column can also be used for further purification of enzymes isolated by other techniques, or for the purification of cytochrome c oxidase and reductase separately, after selective detergent extraction from the mitochondrial membrane [26]. The enzymes, prepared in a very pure form by affinity chromatography, might be the material of choice to attempt crystallization.

There exists a family of proteins which interact specifically with cytochrome c. Apart from cytochrome c oxidase and reductase, sulphite oxidase [27], cytochrome b_5 [28] and cytochrome c peroxidase [29] were also found to bind to the same part of the surface of cytochrome c as the oxidase and the reductase. It is probable that other proteins, which interact with cytochrome c, like reaction centers of photosynthetic bacteria [30], may share this property. The technique described here could therefore be more widely applicable for purification purposes.

Preliminary data from our laboratory indicate that the present technique is adequate to purify other proteins such as the oxidase and reductase from rat liver mitochondria, the oxidase from Maize mitochondria and those from *Rhodopseudomonas spheroides* and *Paracoccus denitrificans*.

Acknowledgements

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