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PURIFICATION OF BEEF-HEART CYTOCHROME *c* OXIDASE BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON OCTYL-SEPHAROSE CL-4B

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Summary

1. Hydrophobic interaction chromatography on Octyl-Sepharose CL-4B is used as a new and simple method for the preparation of large amounts of beef-heart cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1).

2. The method involves only one cycle of $(NH_4)_2SO_4$ fractionation before the material is applied to the column. After washing with 10% cholate and 1.5% Tween 80, elution of the enzyme is accomplished with 1% Triton X-100.

3. The enzyme so prepared contains about 10 nmol heme *a*/mg protein and about 0.2% phospholipid.

4. Characterization of the enzyme has been made with optical and EPR spectroscopy and polyacrylamide gel electrophoresis. The preparation appears by these criteria to be at least as good as other purified enzyme preparations.

5. The turnover rate at infinite cytochrome *c* concentration in 0.1 M sodium phosphate buffer and 0.5% Tween 80 at pH 6.1 is 80 s^{-1} per functional unit of the enzyme. A more than three-fold activation could be obtained by the addition of phosphatidylcholine at neutral pH.

Introduction

Many methods for the purification of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), all based on similar principles, have been described [1]. This paper presents an essentially different procedure, hydrophobic interaction chromatography [2,3], using selective binding of membrane proteins to Octyl-Sepharose CL-4B in the presence of ionic or non-ionic detergents.

Materials and Methods

All preparative procedures were carried out at 0—6° C. Chemicals used were of analytical reagent grade. Fresh beef hearts were obtained from Scan-Väst, Varberg, Sweden. Phenyl- and Octyl-Sepharose CL-4B came from Pharmacia Fine Chemicals, Uppsala, Sweden.

Tween 80 and Triton X-100 came from Atlas Chemical Co. and Sigma, respectively. Metal traces in Tris base (Sigma) solutions were removed by extraction with dithizone in $CHCl_3$. Cholic acid (Sigma) was recrystallized from nearly saturated solutions in hot ethanol after treatment with active charcoal (0.05 g/g bile acid) followed by filtration through Celite [4]. For the rapid estimation of the protein content during the preparation, a method described by Gornall et al. [5] was used. Determination of the protein content in the purified enzyme was made according to Lowry et al. [6] with bovine serum albumin as standard. Phospholipid content was determined by phosphorus determination [7].

Polyacrylamide gel electrophoresis was run in the presence of sodium dodecyl sulphate and 8 M urea by the method of Downer et al. [8]. Densitometric traces of the gels were made at 550 nm on a Gilford 240 spectrophotometer equipped with a Gilford linear transport attachment. Heme *a* was estimated from the difference in absorbance at 605 nm between the reduced and oxidized form of the enzyme, using an extinction coefficient for heme of 12 $mM^{-1} \cdot cm^{-1}$.

Preparative ultracentrifugation steps were carried out in a MSE Superspeed 65 centrifuge.

Optical spectra were recorded in a Beckman Acta MIV spectrophotometer. EPR spectra were obtained in a Varian E-9 spectrometer at 12 K or a Varian E-3 spectrometer at 77 K.

Results and Discussion

Purification procedure

Keilin-Hartree particles were prepared from fresh bovine hearts [9] and homogenized in 50 mM Tris-sulphate/0.66 M sucrose/1 mM histidine (pH 8.0). The pH of the homogenate was adjusted to pH 8.0 with 3 M ammonia and the protein concentration adjusted to 30 mg/ml homogenate with Tris/sucrose/histidine. 20% cholate was then added to a final concentration of 3% followed by the addition of 80 g/l KCl and stirred for 10 min. The enzyme was precipitated at 50% saturation of $(NH_4)_2SO_4$ after prior removal of the proteins precipitating at 30% saturation. The precipitate was homogenised in Potter-Elvehjem homogenisers with 2 vols. of 50 mM Tris-sulphate/2% cholate (pH 8.0), allowed to stand for 1.5 h and centrifuged at 40 000 × *g* for 40 min. The supernatant was applied to an Octyl-Sepharose CL-4B column (flow rate 4 ml/min), previously equilibrated with Tris · cholate buffer/15% $(NH_4)_2SO_4$/1 mM EDTA. Cytochrome oxidase binds strongly under these conditions. The column was eluted with 10% cholate/50 mM Tris-sulphate (pH 8.0)/1 mM EDTA until the absorbance at 550 nm, caused by unbound cytochromes, approached zero. To remove cholate (a known inhibitor of the enzyme), the column was eluted with

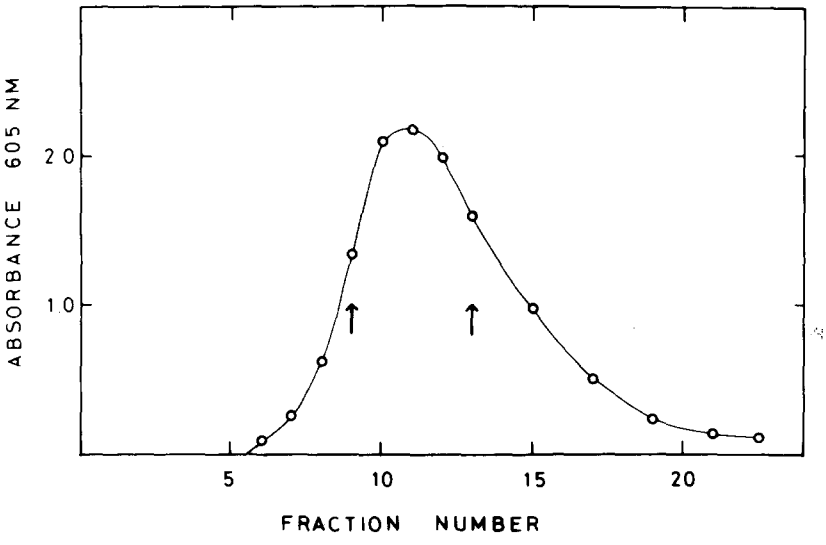

Fig. 1. Elution profile of cytochrome oxidase after desorption from Octyl-Sepharose CL-4B (4 × 26 cm) with 1% Triton X-100/50 mM Tris-sulphate (pH 8.0). The fractions within the arrows were pooled together for further characterization. The amount of enzyme applied was 2.3 g with 12 ml fractions collected.

1.5% Tween 80/50 mM Tris-sulphate. Small amounts of cytochrome oxidase were also eluted in this step. After 1.5—2 h the solution was exchanged for 1% Triton X-100 in the same medium, and the flow rate decreased to 1 ml/min.

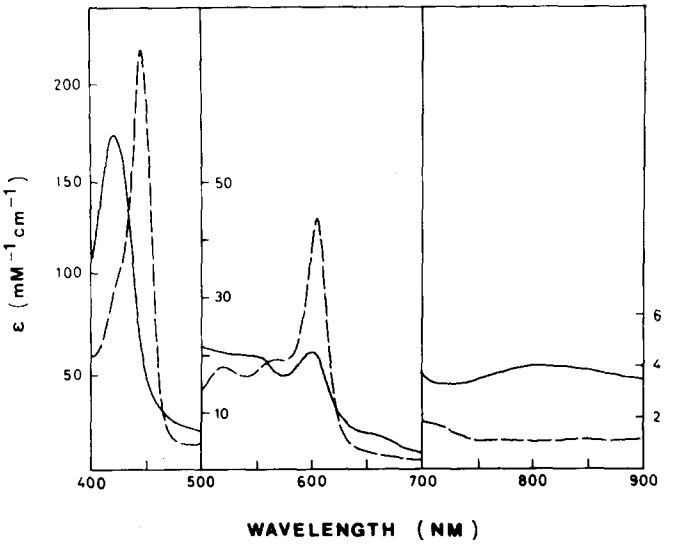

Fig. 2. Optical absorbance spectra of oxidized (———) and reduced (- - - - -) cytochrome oxidase after elution from Octyl-Sepharose CL-4B with 1% Triton X-100/50 mM Tris-sulphate (pH 8.0). The spectra were recorded on the pooled fractions from Fig. 1. The enzyme concentration was 11.5 μM in the Soret and visible region and 25.4 μM in the near infrared region. Dilution of the enzyme was made with 50 mM Tris-sulphate buffer (pH 8.0)/0.5% Tween 80.

Cytochrome oxidase was released from the column and eluted as a concentrated peak (Fig. 2). A spectral investigation of the last portion of the peak reveals a small cytochrome contamination in the α-region.

Characterization of the protein

The peak fractions were pooled and Fig. 3 shows the optical spectrum between 400 and 900 nm of the oxidized and reduced forms of the enzyme. The preparation was stable at low detergent concentrations. A sample of the enzyme was first applied to a Sephadex G-25 column equilibrated with 50 mM Tris-sulphate (pH 8.0) to decrease the detergent concentration and could then stand two weeks dialysis at 6°C against the same buffer with frequent changes without precipitation.

Fig. 4 shows the EPR spectrum at 12 K of the oxidized and reduced forms of a 120 μM enzyme solution. The oxidized spectrum (Fig. 4A) has the appearance typical of a purified preparation, with only very minute amounts of high-spin heme iron. The low- and high-field peaks of the low-spin heme are shown at a higher gain in Figs. 5A and B. The reduced spectrum (Fig. 4B) shows a contamination of Fe-S centers from the NADH dehydrogenase complex [10], but the preparation seems to compare favourably with conventionally prepared enzyme in this respect. Fig. 5C, which is recorded under conditions where the signal of extraneous copper is not saturated (77 K, 5 mW), shows that this type of copper can only be present in negligible amounts, as there is no indication of the low-field peak at 0.2675 T.

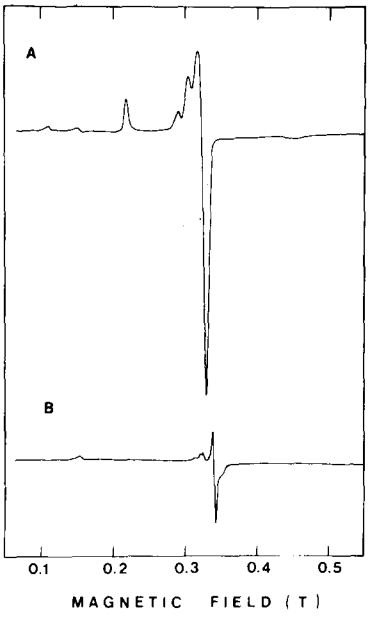

Fig. 3. EPR spectra of oxidized (A) and dithionite-reduced (B) cytochrome oxidase obtained from Octyl-Sepharose CL-4B after elution with 1% Triton X-100/50 mM Tris-sulphate (pH 8.0) and subsequent concentration to 120 μM. Microwave frequency 9.13 GHz; microwave power, 2 mW; modulation amplitude, 2 mT; gain $8 \cdot 10^2$ and temperature, 12 K.

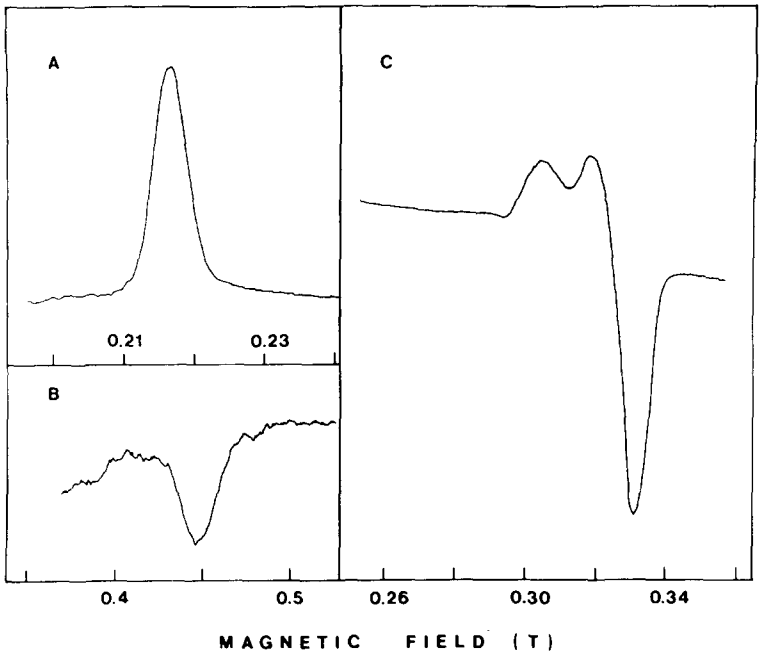

Fig. 4. EPR spectra obtained in the (A) $g3$, (B) $g1.5$ and (C) $g2$ regions of oxidized cytochrome oxidase. The spectra were recorded on the same sample as shown in Fig. 4. In A and B the instrument settings were the same as in Fig. 4 except that the gain = $6.3 \cdot 10^3$ in A and $2.5 \cdot 10^4$ in B. In C the microwave power was 5 mW and the temperature 77 K.

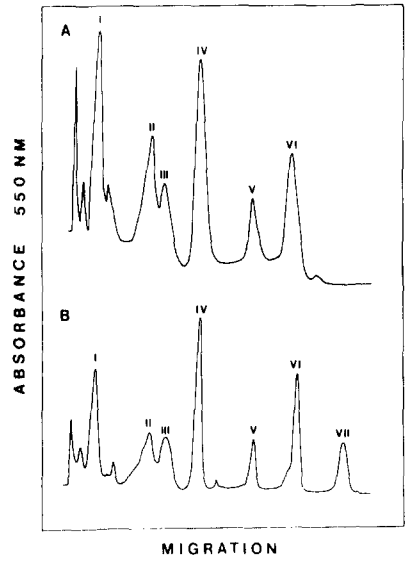

Fig. 5. Densitometric traces at 550 nm of sodium dodecyl sulphate urea polyacrylamide gels of cytochrome oxidase after staining with Coomassie Brilliant Blue. A shows the enzyme isolated according to this paper and B purified according to van Buuren [4]. Subunits are labeled I—VII.

Polyacrylamide gel electrophoresis performed under dissociating conditions indicated that the smallest subunit, with a molecular weight of about 4500, is missing as compared to a cytochrome oxidase sample prepared according to the method of van Buuren [4].

The phospholipid content of the enzyme, based on determination of organic phosphorous [7] and with the use of an average molecular weight of 700 for the phospholipids, is only about 0.2% (w/w). This low content is probably a consequence of the cholate-washing step. The activity of the enzyme, determined as the molecular activity at infinite cytochrome *c* concentration, MA_{0max}, in 0.1 M sodium phosphate buffer (pH 6.1)/0.5% Tween 80 was 80 s^{-1} per functional unit consisting of two heme components and two copper atoms. The value is in the same range as that usually obtained for preparations with low lipid contents. The enzyme could, however, be activated more than three-fold by the addition of phosphatidylcholine at neutral pH. This result implies, that if the smallest subunit actually is missing, it does not seem to affect the catalytic properties of the enzyme.

The amount of bound octyl groups on the Sepharose material is about 40 μmol/ml gel, but the binding capacity for proteins is considerably lower. Calculations made for three different preparations show that approx. 100 nmol of cytochrome oxidase are bound per ml gel. Thus, with 30% saturation of the bed material, only 150 ml gel is needed for preparation of 1 g of protein.

The present procedure, starting with Keilin-Hartree particles, is completed in less than 30 h. The purification procedure has also been tested batch-wise with slow stirring of the gel and the protein solution for 3 h, but the binding efficiency was much lower, so that a longer time is needed for binding of the enzyme as compared to the column procedure. After use, the bed material could be easily regenerated by washing the column with Tris buffer containing 2% cholate.

The results presented here show that different non-ionic detergents such as Tween 80 and Triton X-100 behave quite differently in their interactions with Octyl-Sepharose and the enzyme. Thus, though both detergents are non-ionic, Triton X-100 is far more active as a desorbing agent. The mode of action is, however, not yet known. Lowering of the polarity by addition of ethylene glycol to the Tween 80 medium did not result in an appreciable desorbing effect until as much as 30% ethylene glycol was added, and the effect was still inferior to that caused by Triton X-100.

Purification by cytochrome oxidase by the described procedure has also been tested on Phenyl-Sepharose CL-4B, but the separation of cytochromes *b* and c_1 from the oxidase was not so good.

Acknowledgements

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