A new procedure for the purification of monodisperse highly active cytochrome *c* oxidase from bovine heart

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A simple and rapid method for the isolation of a large quantity of cytochrome *c* oxidase from bovine heart mitochondria was developed, based on selective solubilization of mitochondrial protein with first Triton and then lauryl maltoside. Gel filtration shows that the lauryl maltoside-solubilized oxidase preparation is in a hydrodynamically homogeneous state with a Stokes radius of 7.5 ± 0.2 nm. It contains $8.0 \mu\text{mol}$ of haem (with an a/a_3 ratio of 1)/g of protein. The catalytic constant (maximum turnover number) with respect to cytochrome *c* approaches 600 S^{-1} . After further purification of the solubilized enzyme on a sucrose-gradient centrifugation, the purified enzyme has a haem content of $10.3 \mu\text{mol/g}$ of protein and eight major polypeptide bands shown on SDS/polyacrylamide-gel electrophoresis.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal oxidase of the mitochondrial respiratory chain, has been the focus of numerous studies because of its central role in mitochondrial energy transduction. There are four redox centres per functional unit of cytochrome oxidase: two iron atoms (cytochromes *a* and *a*₃) and two copper atoms (*Cu*_a and *Cu*_{a3}) (Wikström *et al.*, 1983). It has been reported that cytochrome oxidase also contains zinc and magnesium (Einarsdottir & Caughey, 1985). Cytochrome oxidase catalyses the electron transfer from cytochrome *c* to *O*₂ (for a review see Naqui *et al.*, 1986). This electron-transfer process produces a proton gradient across the membrane, which in turn drives the production of ATP.

In our laboratory we are interested in the structure-function relationship in cytochrome oxidase and other related enzyme systems by using the time-resolved X-ray-absorption spectroscopy coupled with rapid mixing and flow-flash techniques. The X-ray-absorption technology at the present time requires large amounts and high concentrations of enzymes. For this reason current available purification methods of mammalian cytochrome oxidase are not satisfactory for our purposes. The conventional published methods are based on either cycles of detergent solubilization and $(\text{NH}_4)_2\text{SO}_4$ fractionation (e.g. Yonetani, 1961; Kuboyama *et al.*, 1972; Yu *et al.*, 1975) or affinity chromatography (e.g. Weiss & Kolb, 1979; Azzi *et al.*, 1982; Thompson & Ferguson-Miller, 1983). The first group of methods is extremely time-consuming (at least 3 days for a single preparation), and usually cytochrome oxidase prepared by these methods is heterogeneous or polydisperse (Kuboyama *et al.*, 1972) and shows low activity (Vanneste *et al.*, 1974). The latter methods are useful only for preparation of small amounts of cytochrome oxidase.

In the present paper we describe a very simple and rapid method for preparing homogeneously disperse cytochrome oxidase from bovine heart. The method is based on selective solubilization of mitochondrial protein, first by Triton (Sun *et al.*, 1968; Frey *et al.*, 1978) and subsequently in lauryl maltoside. The enzyme prepared by this method is in a physically stable state, is highly active and satisfies biochemical and biophysical criteria of purity.

MATERIALS AND METHODS

Materials

Cytochrome *c* from horse heart (type III), Triton and molecular-mass markers for gel filtration (thyroglobin, catalase, immunoglobulin G, conalbumin, haemoglobin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c*) was purchased from Sigma Chemical Co., lauryl maltoside was from Calbiochem and *NNN'*-tetramethyl-*p*-phenylenediamine was from Kodak. Sephacryl S-300 was from Pharmacia. SDS/polyacrylamide-gel-electrophoresis materials were obtained from Bio-Rad Laboratories. All of the other chemicals were of the best commercially available quality.

Enzyme preparation

Mitochondria from bovine heart were prepared by using the procedure of Smith (1967), except that a solution containing 8% (w/v) sucrose, 50 mM-Tris/HCl buffer, pH 8.0, 1 mM-EDTA and 0.2 mM-phenylmethanesulphonyl fluoride was used. All steps were performed at 4 °C.

The membranous oxidase was prepared essentially as described by Sun *et al.* (1968) and Frey *et al.* (1978) with the following modification. Mitochondria (approx. 9.5 g of protein) prepared from 1 kg of bovine heart were suspended in the above solution and adjusted to a protein concentration of 25 mg/ml, Triton X-114

Abbreviation used: r.R., resonance Raman.

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Table 1. Enrichment and yield of cytochrome oxidase during sequential solubilization with Triton X-114, Triton X-100 and lauryl maltoside with haem *a* as a marker

	Protein (mg)	Haem <i>a</i> (nmol)	Haem <i>a</i> /protein ($\mu\text{mol/g}$)	Yield (%)	Enrichment (fold)
Mitochondria	9500	8469	0.89	100	1.0
Cytochrome-protein-enriched membrane	3584	6093	1.7	72	1.9
Oxidase 'membrane'	2498	5376	2.2	63	2.5
Lauryl maltoside-solubilized oxidase	627	4953	8.0	58	9.0

(0.6 mg/mg of protein) and KCl (0.2 M) were added with constant stirring. After incubation with stirring for 20 min, the suspension was centrifuged at 100 000 *g* for 45 min. The mixture separated into a clear orange-reddish supernatant, a soft red-brown cytochrome-rich middle layer and a tightly packed yellow-beige pellet. The supernatant and the hard pellet were discarded, the soft middle cytochrome-enriched red-brownish fraction was suspended in 5% (w/v) sucrose in 10 mM-sodium phosphate buffer, pH 7.0, and adjusted to 30 mg of protein/ml, Triton X-100 (0.7 mg/mg of protein) and KCl (1 M) were added, and the suspension was stirred slowly for 20 min before centrifugation at 12 000 *g* for 40 min. The red-brown supernatant containing cytochrome *bc*₁ and some cytochrome oxidase was discarded. The greenish pellets were adjusted to 22 mg of protein/ml with 25 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-EDTA, and 1.0–1.2 mg of lauryl maltoside/mg of protein was added. After being stirred for 20 min, it was centrifuged at 12 000 *g* for 40 min. The off-white pellets were discarded, and the clear dark-greenish-brown supernatant (oxidase concentration 25–30 μM) was concentrated by ultrafiltration in an Amicon Diaflow (filter XM 300) to 4–5 ml. The lauryl maltoside concentration was decreased by adding 5 vol. of 25 mM-Tris/HCl buffer, pH 7.0, to 1 vol. of concentrated enzyme preparation and then continuing the ultrafiltration until the original volume of the concentrated preparation was reached. The excess lauryl maltoside passed through this filter. The concentrated enzyme preparation was stored at -78°C .

Gel filtration

The Stokes radius was determined by a conventional method (Nazaki *et al.*, 1976) on Sephacryl S-300 (1.0 cm $\times$ 54 cm). The column was equilibrated with 50 mM-Tris/HCl buffer, pH 7.0, containing 0.025% lauryl maltoside. The column was calibrated with thyroglobin (660 kDa, 8.2 nm), catalase (240 kDa, 5.2 nm), immunoglobulin G (160 kDa, 5.1 nm), haemoglobin (64.5 kDa, 3.2 nm), ovalbumin (45 kDa, 3.0 nm), myoglobin (17.8 kDa, 1.9 nm) and cytochrome *c* (12.4 kDa, 1.7 nm). The void volume (V_0) was determined with Blue Dextran (2000 kDa) and the total volume (V_t) with *N*-(2,4-dinitrophenyl)alanine.

Spectroscopy

The optical absorption spectra were recorded on a Johnson Foundation dual-wavelength spectrophotometer. The following absorption coefficients were used: (a) for determining haem *a* content,

$\Delta\epsilon_{605-630} = 12 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the reduced-minus-oxidized spectra (Van Gelder, 1966); (b) for oxidase concentration determination, $\Delta\epsilon_{605-630} = 20.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Yonetani, 1960) for the reduced-minus-oxidized spectra; (c) for determining the amount of a_3^{2+} -CO, $\Delta\epsilon_{590-606} = 9.9 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the reduced-CO-minus-reduced spectra (Vanneste, 1966).

E.p.r. spectra were recorded on a Varian E109 spectrophotometer equipped with an Air Products liquid-helium cryostat.

R.R. spectra were obtained with an He/Cd laser with an excitation wavelength at 441.6 nm. The sample was in a spinning n.m.r. cell.

Atomic absorption was performed in a Varian 575 model AA spectrophotometer.

Kinetic studies

Reduced cytochrome *c* was prepared by reducing the cytochrome *c* solution with excess dithionite and then passing through a Sephadex G-25-50 column. The concentration of cytochrome *c* was determined from the optical spectra by using $\Delta\epsilon_{550-575} = 27 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

The rate of O₂ uptake was measured polarographically by using an oxygen electrode.

Cyanide-binding kinetic studies and data analysis were performed as described by Naqui *et al.* (1984).

Phospholipid content

Phospholipid was determined by the method of Rousor & Fleischer (1967) and Chen *et al.* (1956) on the basis of phosphate content, assuming an average molecular mass of 750 kDa for the phospholipid molecule.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was based on the system described by Kadenbach *et al.* (1983) with a 14% polyacrylamide gel slab (16 cm $\times$ 14 cm $\times$ 0.15 cm). Gel were stained with Coomassie Blue.

Protein concentration

Protein was measured by the biuret method (Gornall *et al.*, 1949), except in the case of the purified enzyme, when the method of Lowry *et al.* (1951) was used in order to avoid interference for lauryl maltoside. Bovine serum albumin was used as the standard for both methods.

RESULTS AND DISCUSSION

Preparation of cytochrome oxidase

The cytochrome-protein-enriched membrane and oxidase 'membrane' were obtained from freshly prepared

Fig. 1. Gel filtration of lauryl maltoside-solubilized bovine heart cytochrome *c* oxidase (0.4 ml, 100 μ M) on a Sephacryl S-300 column (1.0 cm $\times$ 55 cm)

The column was equilibrated in 50 mM-Tris buffer, pH 7.0, containing 0.025% lauryl maltoside. The inset shows the elution profile of the cytochrome *c* oxidase at a flow rate of 3.5 ml/h: $\bullet$ — $\bullet$, $A_{605} - A_{630}$ for dithionite-reduced minus oxidized cytochrome oxidase; $\circ$ — $\circ$, A_{280} ; $\triangle$ — $\triangle$, protein determined by the Lowry method. The column was calibrated with the following standards: Blue Dextran (2000 kDa), thyroglobin (660 kDa, 8.2 nm), catalase (240 kDa, 5.2 nm), immunoglobulin G (160 kDa, 5.1 nm), conalbumin (77 kDa), haemoglobin (64.5 kDa, 3.2 nm), ovalbumin (45 kDa, 3.0 nm), myoglobin (17.8 kDa, 1.9 nm), cytochrome *c* (12.4 kDa, 1.7 nm) and *N*-(2,4-dinitrophenyl)alanine.

mitochondria by sequential extraction with Triton. About 99% of cytochromes *bc*₁ and *c* were removed, and 63% of the oxidase remained in the membrane. In the presence of 2.2% lauryl maltoside and low ionic strength (25 mM-Tris buffer, pH 8.0, containing 5 mM-EDTA), cytochrome oxidase was released from the oxidase 'membrane' (22 mg of protein/ml) as a protein-detergent complex with a concentration of about 25–30 μ M. Under this condition more than 92% of the 'membrane'-bound oxidase was solubilized whereas most contaminant proteins remained in the insoluble material. The concentration of lauryl maltoside in the preparations was decreased to 0.5% by dilution and subsequent concentration of the enzyme by ultrafiltration in a Diaflow.

Table 1 presents the purification of mitochondrial cytochrome *c* oxidase extracted with non-ionic detergent Triton X-114, Triton X-100 and lauryl maltoside. About 55–60% of the mitochondrial haem *a* was recovered by lauryl maltoside with a 9.0-fold enrichment. The lauryl maltoside-solubilized concentrated enzyme (0.5 mM) with a haem *a* content of 8.0 μ mol/g of protein is stable for several months without aggregation, loss of cytochrome content or enzyme activity in 0.5% lauryl

maltoside and 25 mM-Tris buffer, pH 7.0, at -78°C . After further purification of the concentrated preparation by sucrose-gradient centrifugation, the haem *a* content reached 10.3 μ mol/g of protein (see below).

Subunit composition and gel filtration

Once the membrane-bound cytochrome oxidase was solubilized by lauryl maltoside, the enzyme-detergent complex became stable and could be handled as an ordinary water-soluble protein in micellar concentration of detergent (the critical micelle concentration of lauryl maltoside is about 0.19 mM; De Grip & Bovee-Geurts, 1979) without difficulty (Helenius & Simon, 1975). Gel filtration of isolated bovine heart cytochrome oxidase on Sephacryl S-300 (1.0 cm $\times$ 54 cm) resulted in the elution of a single symmetric peak absorbing at 605 nm (Fig. 1). The enzyme complex has a Stokes radius of 7.5 ± 0.2 nm indicating a molecular mass of about 540 kDa, which includes bound detergent in order that the result may be directly compared with those of others in this field (Suarez *et al.*, 1984). The absorbance at 280 nm revealed another peak eluted after the enzyme with a Stokes radius of about 4.5 ± 0.2 nm and molecular mass of about 100 kDa. Both

Fig. 2. SDS/polyacrylamide-gel electrophoresis of isolated cytochrome *c* oxidase from bovine heart

(a) Lauryl maltoside-solubilized enzyme was on the right gel. The standard proteins were on the left gel: myosin (200 kDa), β -galactosidase (116.3 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). (b) The enzyme was gel-filtered on Sephacryl S-300 (1.0 cm $\times$ 4 cm). Electrophoresis was carried out by the procedure of Kadenbach *et al.* (1983) with a 14% polyacrylamide gel slab (16 cm $\times$ 14 cm $\times$ 0.15 cm).

Table 2. Content of cytochrome oxidase components solubilized by lauryl maltoside

Component	Content (μ mol/g of protein)	Molecular ratio (mol/mol of enzyme)
Haem <i>a</i>	8.6	1.0
Iron	9.3	1.05
Copper	8.6	0.95
Zinc	3.9	0.45
Phospholipids	120–160	28–34

Lowry protein assay and SDS/polyacrylamide-gel electrophoresis indicated that the second peak is not a protein; it was subsequently identified as a lauryl maltoside/Triton X-100 mixed micelle by independent chromatography of detergent alone.

The SDS/polyacrylamide-gel electrophoresis showed that the lauryl maltoside-solubilized enzyme preparation contains at least ten different polypeptide subunits (Fig.

2a) with apparent molecular masses of 56, 36, 33, 29, 24, 18, 14, 12, 11 and 9 kDa. Compared with the polypeptide pattern published by other laboratories (Capaldi *et al.*, 1983; Kuhn-Nentwig & Kadenbach, 1984), two extra polypeptides with molecular masses 36 and 33 kDa are present. The 36 kDa and 33 kDa polypeptides vary in concentration after further purification steps and are removed completely by sucrose-gradient centrifugation (see below and Fig. 6), indicating that they are contaminants responsible for the lower than expected haem/protein ratios observed with this preparation. The sum of the molecular masses of polypeptide subunits is around 200 kDa, and therefore the molecular mass of the enzyme-detergent complex (540 kDa) determined by gel filtration suggests that it is possibly a dimer.

It has been reported (Suarez *et al.*, 1984) that cytochrome oxidases from both bovine heart and rat liver are monomers in lauryl maltoside. However, repeated experiments in our hands under identical conditions showed that the molecular mass of the enzyme-detergent complex is about 540 kDa, which we interpret as a dimer. (We are unable to reconcile this discrepancy at this moment.) It may be noted that Azzi and co-workers (Nalecz *et al.*, 1983) have shown that the biphasicity in the Eadie-Hofstee type plot (see below) is due to the reaction of cytochrome *c* with dimers of cytochrome oxidase, whereas in the presence of monomers the kinetic plot is monophasic. If this is the case, the biphasicity observed in our preparation (Fig. 4b) could be indicative of the presence of dimers of cytochrome oxidase in our preparation.

Chemical composition and spectral analysis

The contents of haem *a*, iron, copper, zinc and phospholipids are listed in Table 2. The Fe/Cu ratio is 1:1, which is consistent with the haem *a* concentration. The enzyme preparations contain about 30 mol of phospholipids/mol of cytochrome *aa*₃.

The reduced-minus-oxidized difference spectrum of the enzyme ($A_{400}-A_{900}$) showed that there is no cytochrome *c* reductase contaminant in the preparation (Fig. 3). Upon reaction with CO, the spectrum of the reduced form exhibited the characteristic shoulder at 590 nm and peak at 430 nm. The amount of CO-reactive haem *a*₃ was calculated from the reduced-CO-minus-reduced difference spectrum of the oxidase. The total haem *a*/haem *a*₃ ratio determined by this method gave the value 2:1.

Cytochrome *c* oxidation kinetics

The ferrocycytochrome *c* oxidation kinetic studies performed with our preparation exhibited the two well-known phenomena (Fig. 4). (1) The progress curve of cytochrome *c* oxidation as measured spectrophotometrically at 550 nm can be linearized in a semi-logarithmic plot (Smith & Conrad, 1956), suggesting that the product of the reaction (ferricytochrome *c*) binds with the enzyme with the same affinity as the substrate, i.e. $K_s = K_p$ (Minnaert, 1961). (2) The non-saturating initial velocity with increasing cytochrome *c* concentration shows clear biphasicity in an Eadie-Hofstee-type plot (Ferguson-Miller *et al.*, 1976; Sinjorgo *et al.*, 1986). It can be easily seen that our preparation is highly active, the maximum turnover number with respect to cytochrome *c* (k_{cat}^c) approaching 600 electrons/s per mol of enzyme. In accordance with published results (Ferguson-Miller

Fig. 3. Optical spectra of cytochrome oxidase in Soret, α and near-i.r. region: dithionite-reduced-minus-resting and reduced-CO-minus-reduced $13 \mu\text{M}$ -cytochrome oxidase in 10 mM -sodium phosphate buffer, pH 7.0, containing 0.05% lauryl maltoside

The temperature was 21°C ; a 10 mm optical pathlength was used. —, Reduced-minus-oxidized spectrum; ·····, reduced-CO-minus-reduced spectrum; ----, baseline (reduced-minus-reduced).

Fig. 4. Kinetics of ferrocytochrome *c* oxidation by cytochrome oxidase

(a) Progress curve of cytochrome *c* oxidation by cytochrome oxidase, plotted in a semi-logarithmic plot after normalization. The end point was determined by adding excess $\text{K}_3\text{Fe}(\text{CN})_6$. The cytochrome *c* oxidation was monitored at $550\text{--}575 \text{ nm}$. The concentrations of cytochrome *c* and cytochrome oxidase were $4.5 \mu\text{M}$ and 0.75 nM respectively. All other conditions were as indicated in Fig. 3 caption. (b) Initial rate of cytochrome *c* oxidation as a function of initial cytochrome *c* concentration shown in a Eadie-Hofstee type plot in 10 mM - (●) and 200 mM - (×) sodium phosphate buffer, pH 7.0, containing 0.05% lauryl maltoside. Cytochrome *c* concentration varied from $0.1 \mu\text{M}$ to $80.0 \mu\text{M}$, and cytochrome oxidase concentration was 0.75 nM . All other conditions were as indicated in Fig. 3 caption.

et al., 1976; Sinjorgo *et al.*, 1986), we observed a monophasic interaction of cytochrome *c* with enzyme at high ionic strength (Fig. 4).

We also studied the O_2 uptake in the presence of cytochrome *c*, ascorbate and tetramethyl-*p*-phenylenediamine. The catalytic constant with respect to oxygen ($k_{\text{cat}}^{\text{O}_2}$) had a value of 150 O_2 molecules/s per mol of enzyme. This multiplied by 4 (4 electrons are required to reduce O_2 to H_2O) corresponds to the above value derived from the rate of cytochrome *c* oxidation.

Cyanide-binding kinetics

The results of the cyanide-binding kinetic studies are presented in Fig. 5. The results are in accordance with the earlier binding studies (Van Buuren *et al.*, 1972; Naqui *et al.*, 1984) that the cyanide binding to resting oxidase is multiphasic whereas binding to pulsed oxidase is monophasic with a second-order rate constant of $2 \text{ M}^{-1} \cdot \text{s}^{-1}$.

E.p.r. and r.R.

The e.p.r. and r.R. spectra (not shown) are similar to those published in the literature. The e.p.r. signal at $g = 4.3$ is almost absent with our preparation, suggesting that no or very little non-haem iron is present in our preparation. The copper-region signal is also very 'clean', indicating the absence of adventitious or denatured copper in the preparation.

The r.R. spectrum of our preparation has all the characteristic peaks of cytochrome oxidase. The increasing background usually due to fluorescence from flavoprotein is almost absent from the spectrum.

Fig. 5. Time course of cyanide binding to cytochrome oxidase plotted in a semi-logarithmic plot

The data were normalized. Cytochrome oxidase and cyanide concentrations were $3\ \mu\text{M}$ and $1.33\ \text{mM}$ respectively. KCN solution was prepared in $1\ \text{mM-KOH}$. Experiments were carried out in a stoppered cell. Pulsed oxidase was prepared as described by Kumar *et al.* (1984). The reference wavelength was $522\ \text{nm}$. All other conditions are as in Fig. 3 legend. For further details see Naqui *et al.* (1984). ○, Resting cytochrome oxidase; ●, pulsed cytochrome oxidase.

General discussion

We have further purified the lauryl maltoside-solubilized enzyme preparation by ultracentrifugation on a 20–45% (w/v) sucrose density gradient in $50\ \text{mM-Tris/HCl}$ buffer, pH 7.0, containing $0.1\ \text{M-NaCl}$ for

removal of the excess detergent and for separation from contaminant proteins. The non-protein-bound detergent remained at the top of the gradient, whereas the multiprotein complex was recovered as a peak at about 30% sucrose (Fig. 6). The purified enzyme has a haem *a* content of $10.3\ \mu\text{mol/g}$ of protein. SDS/polyacrylamide-gel electrophoresis shows that two extra polypeptides with molecular masses of 36 and $33\ \text{kDa}$ are completely removed, and the enzyme was resolved into eight major bands. The fastest-migrating band, however, probably contains a mixture of small subunits; our gel system is not capable of resolving polypeptide chains of molecular mass lower than about $8\ \text{kDa}$ (14% polyacrylamide). Therefore the sum of the molecular masses of polypeptide subunits is around $200\ \text{kDa}$, which agrees with the haem/protein ratio of the purified enzyme about $10.3\ \mu\text{mol/g}$ of protein.

The method for cytochrome *c* oxidase preparation from bovine heart described in this paper is simple and rapid. We consider that the purification time can easily be reduced from the traditional $(\text{NH}_4)_2\text{SO}_4$ fractionation method. This, we believe, is the biggest advantage of this method. The method is simple both conceptually and practically, but the simplicity and quickness of the method do not sacrifice the quality of the final preparation. The preparation is in a highly active monodisperse stage, and all other properties studied so far do not show any deviation from the norm.

In conclusion, we consider that this procedure will help researches studying cytochrome *c* oxidase to cut down the time effort in preparation of enzyme by at least 2–3-fold.

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REFERENCES

- Azzi, A., Bill, K. & Broge, C. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2447–2450
- Capaldi, R. A., Malatesta, F. & Darley-USmar, V. M. (1983) Biochim. Biophys. Acta **726**, 135–148

Fig. 6. Sucrose-gradient centrifugation of the cytochrome *c* oxidase-detergent complex

(a) The lauryl maltoside-solubilized concentrated enzyme was layered on 20–45% (w/v) sucrose in $50\ \text{mM-Tris/HCl}$ buffer, pH 7.0, containing $0.1\ \text{M-NaCl}$ and sedimented towards the left at $195000\ g$ for 22 h at $10\ ^\circ\text{C}$ in a Beckman SW41 rotor (○—○, A_{605} ; ●—●, protein). (b) SDS/polyacrylamide-gel electrophoresis of the purified cytochrome oxidase of fraction 11.

- Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1766
- De Grip, W. J. & Bovee-Geurts, P. H. M. (1979) *Chem. Phys. Lipids* **23**, 321–335
- Einarsdottir, O. & Caughey, W. S. (1985) *Biochem. Biophys. Res. Commun.* **29**, 840–847
- Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 1104–1115
- Frey, T. G., Chan, S. H. P. & Schatz, G. (1978) *J. Biol. Chem.* **253**, 4389–4395
- Gornall, A. G., Bardawill, C. I. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Helenius, A. & Simon, K. (1975) *Biochim. Biophys. Acta* **415**, 29–79
- Kadenbach, B., Jarausch, J., Hartmann, R. & Merle, P. (1983) *Anal. Biochem.* **129**, 517–521
- Kuboyama, M., Yong, F. C. & King, T. E. (1972) *J. Biol. Chem.* **247**, 6375–6383
- Kuhn-Nentwig, L. & Kadenbach, B. (1984) *FEBS Lett.* **172**, 189–192
- Kumar, C., Naqui, A. & Chance, B. (1984) *J. Biol. Chem.* **259**, 2073–2076
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Minnaert, K. (1961) *Biochim. Biophys. Acta* **50**, 23–34
- Nalecz, K. A., Bolli, R. & Azzi, A. (1983) *Biochem. Biophys. Res. Commun.* **114**, 822–828
- Naqui, A., Kumar, C., Ching, Y. C., Powers, L. & Chance, B. (1984) *Biochemistry* **23**, 6222–6227
- Naqui, A., Chance, B. & Cadenas, E. (1986) *Annu. Rev. Biochem.* **55**, 137–166
- Nazaki, Y., Schechter, N. M., Reynolds, J. A. & Tanford, C. (1976) *Biochemistry* **15**, 3884–3889
- Rousor, G. & Fleischer, S. (1967) *Methods Enzymol.* **10**, 385–405
- Sinjorgo, K. M. C., Steinebach, O. M., Dekker, H. L. & Muijsers, A. O. (1986) *Biochim. Biophys. Acta* **850**, 108–115
- Smith, A. L. (1967) *Methods Enzymol.* **10**, 81–86
- Smith, L. & Conrad, H. (1956) *Arch. Biochem. Biophys.* **63**, 403–413
- Suarez, M. D., Revzin, A., NarLock, R., Kempner, F. S., Thompson, D. A. & Ferguson-Miller, S. (1984) *J. Biol. Chem.* **259**, 13791–13799
- Sun, F. F., Prezbindowski, K. S., Crane, F. L. & Jacob, E. E. (1968) *Biochim. Biophys. Acta* **153**, 864–818
- Thompson, D. A. & Ferguson-Miller, S. (1983) *Biochemistry* **22**, 3178–3187
- Van Buuren, K. J. H., Nicholls, P. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **256**, 258–276
- Van Gelder, B. F. (1966) *Biochim. Biophys. Acta* **118**, 36–46
- Vanneste, W. H. (1966) *Biochemistry* **5**, 838–848
- Vanneste, W. H., Ysebaert-Vanneste, M. & Mason, H. S. (1974) *J. Biol. Chem.* **249**, 7390–7401
- Weiss, H. & Kolb, H. J. (1979) *Eur. J. Biochem.* **99**, 139–149
- Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York
- Yonetani, T. (1960) *J. Biol. Chem.* **235**, 3138–3243
- Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680–1688
- Yu, C. A., Yu, L. & King, T. E. (1975) *J. Biol. Chem.* **250**, 1383–1392

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