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Identification of different quaternary structures of beef heart cytochrome-*c* oxidase by two-dimensional polyacrylamide gel electrophoresis

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A two-dimensional gel electrophoresis is decribed to identify different quarternary structures of the heart cytochrome-*c* oxidase. Bovine enzyme was purified and separated by discontinuous gradient polyacrylamide gel electrophoresis under nondenaturing conditions in the 1st dimension into several discrete complexes and thereupon shown to be heterodisperse in Triton X-100 and dodecyl maltoside. A discontinuous SDS-polyacrylamide gel electrophoresis in the 2nd dimension was used to determine the subunit composition of the isolated complexes. One of these represents the intact enzyme with 12 different polypeptides while the others have an incomplete subunit composition.

Cytochrome-*c* oxidase; Nondenaturing gradient polyacrylamide gel electrophoresis; Nonionic detergent; 2D-PAGE

1. INTRODUCTION

The complexity as well as the hydrophobicity of mitochondrial cytochrome-*c* oxidase (EC 1.9.3.1) have hindered efforts to elucidate a conclusive model for its quaternary structure. One prominent source of controversy is the available information concerning the number and operational role of the enzyme subunits. Since it is generally agreed that cytochrome-*c* oxidase from mammalian sources contains at least 12 different subunits of known amino acid sequences [1,2] (or even more [3,4]), the discussion has turned to the question which of these polypeptides are required for the catalytic activity of the enzyme [5,6] and which have just structural or regulatory functions [7–9].

Another substantial problem in the quaternary structure investigation is the polydispersity of bovine heart oxidase [10,11] which leads to contradictory results on the molecular mass and aggregation state of the active complex. The 'intermolecular polydispersity' of the enzyme as a consequence of different aggregation states that depend on experimental conditions, is well described. High-molecular-mass aggregates can be obtained [12,13], but the mammalian enzyme is mainly dimeric at physiological pH in the presence of nonionic detergents [11,12,14–17]. Dissociation into monomers occurs at increasing pH and high detergent concentrations [14], particularly coincident with protein chemical disintegration [13] causing 'intramolecular polydispersity'. Interconversion between the monomeric and dimeric form of the bovine enzyme in dodecyl maltoside has been demonstrated [18,19]. Moreover, it has been reported that complexes of different subunit composition exist in lauryldimethylamine *N*-oxide

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Abbreviations: native DGPAGE, discontinuous gradient polyacrylamide gel electrophoresis under nondenaturing conditions; SDS-DPAGE, discontinuous SDS-polyacrylamide gel electrophoresis

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and removal of subunit III tends to monomerize the enzyme [20]. Monomers containing subunit III were detected in dodecyl maltoside by analytical ultracentrifugation in mixtures of H_2O and D_2O [21].

In this article we demonstrate by a two-dimensional polyacrylamide gel electrophoresis that one of the main reasons for the polydispersity of cytochrome-*c* oxidase either in Triton X-100 or in dodecyl-β-D-maltoside is the particular protein-chemical disintegration of the enzyme. Conventional nongradient and continuous gradient separation systems that have been developed for membrane proteins [22,23] are disadvantageous in separating cytochrome oxidase complexes. Two-dimensional techniques that use nongradient cylindrical gels in the 1st dimension have been applied to cytochrome oxidase [10,13,24] but yielded insufficient resolution. We have adapted and combined two discontinuous gel electrophoretic techniques: a detergent-supplemented gradient separation system (native DGPAGE) in the 1st dimension with a nongradient SDS-PAGE system in the 2nd dimension. By this method several distinct cytochrome oxidase complexes can be identified and their subunit composition characterized. Besides the intact enzyme, stable quaternary structures exist in which several subunits, number III and up to five of the smaller polypeptides, are lacking.

2. MATERIALS AND METHODS

Cytochrome-*c* oxidase was isolated from bovine heart mitochondria essentially as in [25], with omission of the last dialysis step. The enzyme was dissolved in standard buffer 63 mM Tris-phosphate, pH 7.3, containing between 0.1% (w/v) and 0.5% (w/v) nonionic detergent Triton X-100 or dodecyl-β-D-maltoside. For removal of remaining cholate, cytochrome oxidase was dialyzed 24 h into the same buffer. These preparations were stored in small aliquots at −80°C and used for native DGPAGE after addition of glycerol to a final concentration of 10% (v/v). Preparations containing Triton X-100 were further used as raw material either for alkaline treatment according to [11] or for anion-exchange chromatography. Cytochrome oxidase was bound to a

Table 1

Stock solutions and mixtures for native DGPAGE

Stock solution (% by wt/v)	Percentage of gel soln (by vol.)		
	3.75% sepn gel soln	30.0% sepn gel soln	Stacking gel soln
Sepn gel buffer, 0.88 M Tris-HCl (pH 7.2)	12.5	12.5	–
Stck gel buffer, 0.50 M Tris-H_3PO_4 (pH 7.3)	–	–	12.5
38.96% acrylamide and 1.04% bisacrylamide	9.4	75.0	–
09.25% acrylamide and 0.75% bisacrylamide	–	–	31.3
Detergent soln, 1% per 0.1% in the gel	10.0	10.0	10.0
Water, double-distilled	67.5	2.3	33.4
1 mM Riboflavin	–	–	12.5
10% ammonium persulfate	0.60	0.20	0.25
N,N,N',N'-Tetramethylethylenediamine	0.029	0.029	0.058

Solutions were not degassed. The mixture of appropriate volumes of separation gel solution was poured into the bottom of the separation cell performing a linear gradient, topped by a solution of 1:8 diluted separation gel buffer, followed by polymerization within 45 min at room temperature. After removal of the topping solution the separation gel surface was rinsed with stacking gel solution. Mixed redox- and photopolymerization of the stacking gel occurred within 30 min using a DESAGA light box. Electrode buffer was 8.25 mM Tris, 38.3 mM glycine, pH 8.5; cathode buffer was supplemented with detergent

DEAE-Sepharose column (Pharmacia SR 10/50) equilibrated with a standard buffer containing 0.1% (w/v) Triton X-100 and eluted with a 50–350 mM NaCl gradient. Alkaline-treated material was neutralized by dialysis into the same buffer prior to the application onto the column. The green peak fractions were used for native DGPAGE after removal of salt by dialysis into equilibration buffer and further addition of glycerol.

All electrophoretic separations were performed by using a Desaphor VA thermoconstant vertical slab gel apparatus. Running gel dimensions for native DGPAGE were 110 mm × 230 mm × 1.5 mm. The buffer system used was similar to system 1/4 described in [26] with modifications for proteins of low electrophoretic mobility: stacking gel pH was 7.3, and the pH during separation was 8.6. Stock solutions, mixtures and preparation of the native DGPAGE gel are given in table 1. Electrophoresis was run at 4°C for 30 min at 50 V (constant) and for 45–50 h at 300 V (constant). Gels were stained overnight with 1% (w/v) amido black 10 B in 29% (v/v) methanol and 7% (v/v) acetic acid and destained in 45% (v/v) methanol and 9% (v/v) acetic acid.

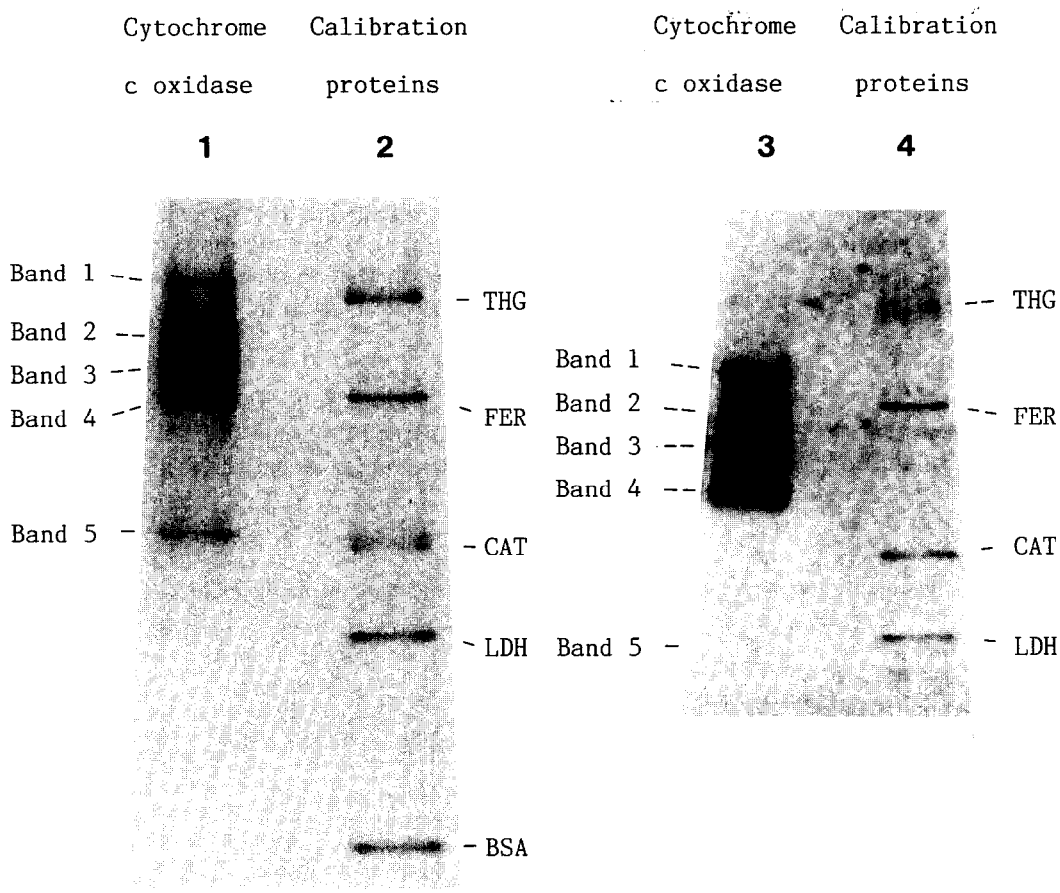

Fig.1. Discontinuous electrophoresis of the bovine heart cytochrome-*c* oxidase on a gradient polyacrylamide gel under nondenaturing conditions (native DGPAGE). Separation was performed in the presence of nonionic detergent as described in section 2. (A) Lanes 1 and 3, 30 μg isolated cytochrome oxidase, separated in the presence of 0.1% (w/v) Triton X-100 and 0.1% (w/v) dodecyl-β-D-maltoside, respectively; lanes 2 and 4, 6 μg mixture of calibration proteins, containing thyroglobulin, ferritin, catalase, lactate dehydrogenase and in lane 2 only bovine serum albumin (molecular masses: 669000, 440000, 232000, 140000 and 67000 g × mol^{-1}). A linear calibration curve could be established using these proteins within the native DGPAGE under the same conditions as described for cytochrome oxidase.

SDS-DPAGE was carried out as described in [27]. Running gel dimensions were 200 mm × 230 mm × 1.5 mm. Unstained single bands of cytochrome oxidase (4 mm in width) were punched out of the native DGPAGE gel. The enzyme was dissociated by incubation of the gel slices as well as the reference oxidase solutions in sample buffer according to [27] at 37°C for 1 h. It contained 4% (w/v) SDS and 1% (v/v) mercaptoethanol. The excised gel slices were inserted into the wells in the stacking gel following polymerization. Running conditions were at 25°C for 30 min at 50 V (constant) and for 18–28 h at 160–250 V (constant). The product of voltage and time did not exceed 4500 V × h. Gels were fixed in 12% (w/v) trichloroacetic acid for 1 h followed by 3 washes in 7% (v/v) acetic acid each for 10 min. Staining and destaining were performed as described for native DGPAGE gels. Additional silver staining was performed essentially as described in [28] after the gel was soaked 3 times in 50% (v/v) ethanol for 24 h or even longer to remove interfering substances (for further details see [29]).

3. RESULTS

3.1. *First dimension*

The native DGPAGE of beef heart cytochrome-*c* oxidase reveals several heme A-containing discrete bands in Triton X-100 as well as in dodecyl maltoside. These would not be expected if the enzyme were monodispersed. As shown in fig.1, the conventionally isolated bovine cytochrome oxidase preparation is heterodispersed showing typically four bands of relatively high apparent M_r values ranging from 410000 to 670000 g × mol^{-1} in Triton X-100 and 265000 to 530000 g × mol^{-1} in dodecyl maltoside, respectively. They stain heavily for protein. In addition a minor staining band with a lower apparent M_r value (230000 g × mol^{-1} in Triton X-100 and 130000 g × mol^{-1} in dodecyl maltoside) is detected. The relative protein content of cytochrome oxidase bands on DGPAGE gels is listed in table 2, which demonstrates three points. First, the relative protein content of cytochrome oxidase bands depends on the kind and concentration of the detergent used in the DGPAGE system. Secondly, band 5 which represents a fragment of the enzyme (see below) can be removed by ion-exchange chromatography. Finally, alkaline treatment of the enzyme causes a shift of protein distribution from higher to lower apparent M_r values.

3.2. *Second dimension*

Polypeptide analysis of the excised DGPAGE bands on a denaturing gel demonstrates the

Table 2

Relative protein contents of cytochrome-*c* oxidase bands on native DGPAGE gels

Cytochrome oxidase preparation:	Standard				Ion-exchange chromatogr. (IEC)	Alkaline treatment and IEC
Detergent:	Dodecyl maltoside		Triton X-100		Triton X-100	Triton X-100
Detergent concn:	0.5%	0.1%	0.3%	0.1%	0.1%	0.1%
Band 1	22%	41%	24%	7%	9%	2%
Band 2	25%	10%	28%	30%	28%	13%
Band 3	47%	31%	34%	41%	42%	58%
Band 4	5%	17%	12%	18%	21%	27%
Band 5	1%	1%	2%	4%	–	–

Standard preparation, ion-exchange chromatography and alkaline treatment of cytochrome oxidase were performed as described. The relative protein content was determined after staining of the native DGPAGE gels on a LKB Ultro-Scan 2202 laser densitometer setting total protein content at 100%. All determinations were done at least three times. Relative standard deviations of protein content did not exceed 18% even though different enzyme charges were used

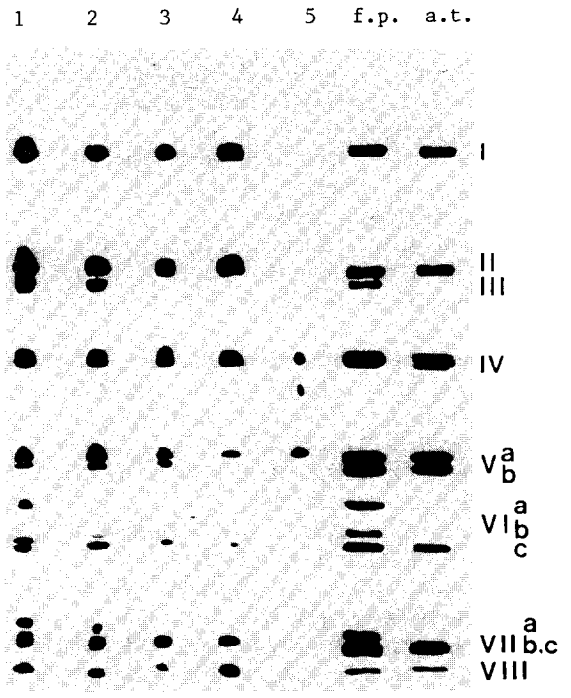

Fig.2. Two-dimensional polyacrylamide gel electrophoresis of the bovine heart cytochrome-*c* oxidase; silver stained. First dimension, 30 μg cytochrome oxidase were separated by native DGPAGE in the presence of 0.1% (w/v) Triton X-100 (cf. fig.1, lane 1). Second dimension, excised bands 1–5 of the native DGPAGE gel were denatured and polypeptides were resolved on a discontinuous polyacrylamide gel containing SDS as described in section 2; for comparison the polypeptide patterns of freshly prepared (f.p.) and alkaline-treated (a.t.) cytochrome oxidase are shown in the reference lanes, either containing 5 μg of protein; nomenclature of the subunits according to [27].

presence of incomplete enzyme complexes. As the 2-D pattern shows (fig.2) bands 1 and 2 represent the intact enzyme containing 12 different polypeptides and a complex with 10–11 polypeptides (subunit VIa totally and subunit VIb particularly lacking, nomenclature according to [27]). The next two complexes, bands 3 and 4, are identical with the main species of alkaline-treated enzyme containing 8 polypeptides (subunits III, VIa, VIb and VIIa lacking) and 6 polypeptides (subunits Vb and VIc further lacking). The fifth band on the DGPAGE represents a fragment of the enzyme containing only subunits IV and Va and probably a crosslinked product. A synopsis of the polypeptide compositions of native DGPAGE bands is given in table 3.

4. DISCUSSION

The solubilization of a highly organized membrane protein may be involved with some undesirable effects based on protein-detergent interactions. The predominant effects causing polydispersity of the solubilized membrane protein are either aggregation of the protein-detergent complexes or partial denaturation of the protein. Native DGPAGE is the method of choice to distinguish between these two kinds of polydispersity assuming that the observed enzyme species exist prior to electrophoresis. The M_r values of cytochrome oxidase bands cannot be determined exactly due to the unknown value of protein bound detergent within the gel matrix, but we can,

Table 3

Polypeptide composition of cytochrome-*c* oxidase complexes separated by native DGPAGE

Native DGPAGE gel bands	Presence of subunits (nomenclature according to [27])											
	I	II	III	IV	Va	Vb	VIa	VIb	VIc	VIIa	VII^b_c	VIII
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	0	(+)	+	+	+	+
3	+	+	0	+	+	+	0	0	+	0	+	+
4	+	+	0	+	+	0	0	0	(+)	0	+	+
5	0	0	0	+	+	0	0	0	0	0	0	0

Symbols used: +, subunit present; (+), subunit present in smaller amounts than in 1:1 stoichiometry; 0, subunit not present in the native DGPAGE band. Subunits VIIb and VIIc could not reproducibly be separated from each other and therefore are considered together in this table

however, conclude that bands 1–4 represent intramolecular polydispersity. Even though some smaller subunits are not in a 1:1 stoichiometry within the bands (cf. table 3), monomer-dimer interconversion of intact or fragmented oxidase as a source of intermolecular polydispersity in this case can also be excluded. Polydispersity and subunit heterogeneity in the form of reduced amounts of subunit III [10,13,24] and some smaller polypeptides [10,13] could be demonstrated by native nongradient rod gel electrophoresis in the presence of Triton X-100 and subsequent SDS electrophoresis. However in contrast to our improved method, a separation into distinct complexes was not obtained earlier.

The polypeptide compositions of bands 1–4 are similar to those of complexes separated by anion-exchange FPLC [20]. The FPLC-fractions are deficient in subunits III, VIa, VIb, possibly VIIa and (in contrast to native DGPAGE) in subunit II [20]. Furthermore the polypeptide compositions of bands 3 and 4, which mainly appear after the alkaline treatment when the polydispersity is also reduced [11], is in accordance with the SDS-PAGE subunit pattern obtained in [7] using an alkali-treated sample kindly supplied by Professor Wikström. The polypeptides most sensitive to disintegration seem to be subunit III [7,10,13,20,24,30] and some smaller peptides [13,20], some of which are supposed to be tissue-specific components of the enzyme (e.g. VIa and VIIa [4,7]).

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