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Subunit Structures of Purified Beef Mitochondrial Cytochrome bc_1 Complex from Liver and Heart

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The existence of tissue-specific isozymes of cytochrome *c* oxidase has been widely documented. We have now studied if there are differences between subunits of mitochondrial bc_1 complexes isolated from liver and heart. For this purpose, we have developed a method for the purification of an active ubiquinol–cytochrome *c* oxidoreductase from adult bovine liver that includes solubilization of submitochondrial particles with deoxycholate, ammonium acetate fractionation, resolubilization with dodecyl maltoside, and ion exchange chromatography. The electrophoretic pattern of the liver preparation showed the presence of 11 subunits, with apparent molecular weights identical to the ones reported for the heart complex. Western blot analysis and isoelectric focusing followed by two-dimensional gels of bc_1 complexes from liver and heart were compared, and no qualitative differences were observed. In addition, the high-molecular-weight subunits of the purified complexes from both tissues, subunits I, II, V, and VI, were isolated by PAGE in the presence of Coomasie Blue and subjected to limited proteolysis and to chemical digestion with cyanogen bromide and BNPS-skatol, and the peptide patterns were compared. Finally, two of the small-molecular-weight subunits from the liver complex were isolated (subunits VII and X), partially analyzed by amino terminal sequencing, and found to be identical with the reported sequence of their heart counterparts. The data suggest that, in contrast to the case of cytochrome *c* oxidase, bc_1 complexes from liver and heart do not exhibit tissue-specific differences.

KEY WORDS: Liver and heart bc_1 complexes; ubiquinol–cytochrome *c* oxidoreductase; tissue specificity; isoelectric focusing; mitochondrial respiratory complexes; subunit structure of bc_1 complexes; BNPS-skatol cleavage of subunits.

INTRODUCTION

Ubiquinol–ferricytochrome *c* oxidoreductase (EC 1.10.2.2), or bc_1 complex, is an oligomeric protein formed by 11 different polypeptides (Schägger *et al.*, 1986). Three of these polypeptides are associated with prosthetic redox groups (Rieske, 1986): subunit III (cytochrome *b*); subunit IV (cytochrome c_1) and subunit V (a Rieske-type iron-sulfur protein). Topological models for the arrangement of the 11 subunits of the bc_1 complex in relation to the mitochondrial inner membrane have been proposed (González-Halphen *et al.*, 1988, Hemrika and Berden, 1990). Homologous oligomeric proteins to the mitochondrial bc_1 complex are widely distributed in nature, and are usually referred to as bc_1 or *bc* complexes in fungi and bacteria, and as b_6f complexes in chloroplasts and cyanobacteria (Hauska *et al.*, 1983; Cramer *et al.*, 1987; Hauska *et al.*, 1988; Trumpower, 1990). In bacteria, these electron transfer complexes have a simpler polypeptide composition, and are constituted by only three or four subunits, three of which carry the same prosthetic redox groups (Trumpower, 1990; Andrews *et al.*, 1990).

With the appearance of membrane compartmentalization in eukaryotes during evolution, the

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respiratory cytochrome complexes seem to have increased in complexity: four to five subunits are found in the chloroplast b_6f complex, 10 subunits in the mitochondrial bc_1 complexes of yeast and *Neurospora crassa*, and 11 subunits in that of higher mammals (Kadenbach, 1983; Capaldi *et al.*, 1988). The function of the subunits which do not have redox groups has not been definitively established, and different suggestions for their structural and functional role have been put forward, i.e., interaction with substrates (Kim and King, 1983; Japa and Beattie, 1989; Usui *et al.*, 1990), regulatory functions (Schoppink *et al.*, 1988, 1989), and participation in the biogenesis and assembly of the complex (Crivellone *et al.*, 1988; Schmitt and Trumpower, 1990; Fu and Beattie, 1991). For the case of cytochrome *c* oxidase the tissue-specific origin of some of the low-molecular-weight subunits has been widely documented (Kuhn-Nentwig and Kadenbach, 1985; Stroh and Kadenbach, 1986). This differential expression of cytochrome *c* oxidase isozymes in vertebrates is both tissue- and species-specific (Kadenbach *et al.*, 1990). Unambiguous evidence for the existence of tissue-specific subunits in cytochrome *c* oxidase has been obtained by amino acid sequence analysis of three polypeptides from bovine heart and liver enzymes (Yanamura *et al.*, 1988). In the case of the bc_1 complexes, only species-specific subunits have been tentatively identified from comparison of SDS-PAGE[3] electrophoretic patterns (Engel *et al.*, 1983; Degli Esposti *et al.*, 1986; Berry *et al.*, 1991). In addition, a comparison between calf liver and beef heart bc_1 complexes was found to be indistinguishable in their subunit composition (Engel *et al.*, 1983). However, the polypeptide composition of the complexes was not analyzed with a technique that allowed optimal electrophoretic resolution of components.

We thought it was of interest to ascertain if there are tissue-specific differences between the noncatalytic subunits of the bc_1 complex isolated from different tissues of the same organism (*Bos primigenius taurus*). Here we report the isolation and characterization of the bc_1 complex from adult bovine liver, as well as a comparison of its polypeptide electrophoretic and isoelectric focusing patterns with that of the heart enzyme. The peptide patterns of subunits I, II, V, and VI of the liver and heart complexes were also compared. The results obtained indicate that these subunits are identical as judged by their electrophoretic migration, reaction with antibodies, isoelectric focusing properties, peptide patterns, and amino terminal sequence analysis, strongly suggesting that tissue-specific subunits are not present in the mitochondrial bc_1 complex.

[3] Abbreviations: BNPS-skatol, 2-(2′-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DEAE-, diethylaminoethyl-; IEF, isoelectric focusing; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloracetic acid; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

EXPERIMENTAL PROCEDURE

Purification of Beef Heart bc_1 Complex

Beef heart mitochondrial bc_1 complex was obtained as described by Rieske (1967), or alternatively by the method of Ljungdahl *et al.* (1989) and kept at −70°C until used.

Activity and Reconstitution Measurements

Cytochrome *b*, cytochrome c_1, cytochrome *c*, and ubiquinone concentrations were determined spectrophotometrically as described before (González-Halphen *et al.*, 1991). Ubiquinol–cytochrome *c* oxidoreductase activity was measured as described by Kubota *et al.* (1992) using an SLM-Aminco DW-2C spectrophotometer in the dual wavelength mode (550–540 nm). Horse heart cytochrome *c* reduction was recorded after the addition of 0.015 mM DBH. The quinone was reduced in ethanolic solution with sodium dithionite and sodium borohydride as reported by Trumpower and Edwards (1979) and subsequently extracted with cyclohexane (Rieske, 1967); after evaporation of the solvent under a stream of nitrogen, the reduced quinone was dissolved in dimethyl sulfoxide in the presence of 1 mM HCl and kept at −70°C until used.

Purification of Beef Liver bc_1 Complex

All steps were carried out at 4°C and in the presence of protease inhibitors: 1 mM PMSF and 50 μg/ml of TLCK. Mitochondria and submitochondrial particles were prepared from fresh adult bovine liver following the method described by Yanamura *et al.* (1988). Submitochondrial particles were resuspended in 0.05 M Tris-HCl (pH 8.0), 0.67 M sucrose, and 1 mM EDTA to a protein concentration of 23 mg/ml and solubilized with potassium deoxycholate as

described by Hatefi and Rieske (1967). Thereafter, the same procedure was followed for the preparation of the red pellet referred to as S-1; the step that involved dilution of the red supernatant with 0.25 volumes of cold water was omitted. The preparation of crude bc_1 complex was done according to Rieske (1967), except that the S-1 pellet was resolubilized with twice the amount of potassium deoxycholate (1 mg per mg of protein at a concentration of 10 mg of protein/ml). Further purification of the complex was achieved by ion-exchange chromatography on a DEAE-Biogel A column, as described by Ljungdahl *et al.* (1989); in our protocol, the crude bc_1 complex was taken to a protein concentration of 10 mg/ml with 50 mM Tris-HCl (pH 8.0)/1 mM $MgSO_4$ and incubated with gentle stirring in the presence of 0.2 mg of dodecyl maltoside per mg of protein in an ice bath for 1 hour. The mixture was centrifuged at 30,000 rpm for 10 minutes in a Beckman 50 Ti rotor. The supernatant was dialyzed for 2 hours against 50 mM Tris-HCl (pH 8.0) and 1 mM $MgSO_4$, and loaded into a DEAE-Biogel A column equilibrated with the same buffer that in addition contained 0.2 mg/ml of dodecyl maltoside. The column was washed with one column-volume of the same buffer followed by a linear gradient that ranged from 0 to 150 mM NaCl, where complex II and IV were excluded. Subsequently, pure bc_1 complex was eluted with a linear gradient from 150–400 mM NaCl, concentrated by ultrafiltration on an Amicon YM100 filter, dialyzed for 1 hour against 100 volumes of 20 mM Tris-HCl (pH 8.0), and stored at −70°C until used.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Schägger *et al.* (1986), using 1.2 mm thick slab gels (16% acrylamide). Gels were fixed and stained as described in the same work.

Electrophoretic Isolation of Subunits. Cyanogen Bromide and BNPS-Skatol Cleavage

Subunits of the bc_1 complex were purified by polyacrylamide gel electrophoresis, transferred to a ProBlott membrane (Applied Biosystems), eluted from the membrane, and subjected to chemical cleavage with BNPS-skatol or cyanogen bromide. For this purpose, a Coomassie Blue–SDS-PAGE was run according to Schägger *et al.* (1988), except that 2 μl/ml of 100 mM thioglycolic acid (Sigma) was included in the upper buffer and gels were prerun for 30 min at 50 V constant voltage. Fifty micrograms of protein were loaded into each well. The directly visualized polypeptides were excised from the gel and transferred to a ProBlott membrane for 4 hours at 250 mA and 4°C, in the presence of 25 mM Tris-HCl (pH 8.3)/192 mM glycine as described by Szewczyk and Summers (1988). Proteins were eluted by gentle orbital rotation in the presence of 25 mM Tris-HCl (pH 8.5) containing 1% Triton X-100 and 2% SDS, as described by the same authors. After elution, the proteins were treated with 0.6 g/ml of Biobeads SM-2 (20–50 mesh, BioRad) under gentle shaking for 24 hours to remove the excess of Triton X-100, and subsequently lyophilized. The resulting powder was resuspended in 70% formic acid containing 10 mg/ml of cyanogen bromide (Scott *et al.*, 1988), and the mixture was incubated in the dark for 24 hours at room temperature in an Eppendorf mixer. Alternatively, the lyophilized powder was transferred to a 1.5 ml Eppendorf tube to which 100 μl of 1 mg/ml of BNPS-skatol in 75% acetic acid was added (Crimmins *et al.*, 1990). The tube was incubated for 1 hour at 47°C, and for a further 12 hours at room temperature in the dark. After chemical cleavage by either of the two methods, the samples were dried in a Speed Vac (Savant Instruments) and resuspended in a small volume of 0.2% trifluoroacetic acid (HPLC grade, Pierce), neutralized with saturated Tris and run in SDS-PAGE.

Amino Terminal Sequence Analysis of Subunits

When polypeptides were isolated for amino terminal sequence analysis, gels were run as described by Schägger *et al.* (1986) and transferred into a ProBlott membrane at 400 mA for 2.5 hours (4°C) in the presence of 10 mM CAPS (pH 11.0) and 10% methanol following the procedure for Matsudaira (1987). The membranes were stained, destained, and air dried as described by the same author. Amino-terminal sequence analysis was carried out on a Model 476A Microsequencer with on-line PTH analysis (Applied Biosystems, Foster City, California). The data were analyzed using a Model 610A system.

Isoelectric Focusing

Isoelectric focusing was modified from Cabral and Schatz (1979), and carried out in continuous slab gels (20 × 15 cm, 1.2 mm thick). The gel solution was made by mixing urea (23.8 g), 10 ml of 30% acrylamide/0.98% bisacrylamide, 9.8 ml of 10% Nonidet P-40, 7 ml of distilled water, and the following mixture of ampholines (Sigma Chemical Co.):

pH 3–10 (400 μl); pH 2.5–5 (200 μl); pH 7–9 (200 μl) and pH 9–11 (200 μl). The mixture was degassed and polymerized in the presence of 120 μl of TEMED and 120 μl of fresh 10% ammonium persulfate. The samples were solubilized as follows: 100 μg of protein were taken to 45 μl with distilled water, and the following components were added: 60 mg of solid urea, 30 μl of 20% Triton X-100, 2.5 μl of 1M Tris-HCl (pH 7.0), 1 μl of 0.2 M EDTA, 1.2 μl of 2-mercaptoethanol, and 5 μl of glycerol per every 50 μl of the final mixture.

The upper reservoir contained previously degassed 0.2% H_2SO_4, while the lower one had degassed 0.4% ethylenediamine. As the alkaline buffer exchanged less CO_2 with the ambient when located in the lower chamber, the electrodes were attached in an inverted fashion (negative in the lower reservoir and positive in the upper one).

Before adding the sample, 25 μl of a mixture containing 1% ampholines (pH 9–11) and 5% glycerol was added to each lane, and gels were prerun at 250 V (constant voltage) for 1 hour. The solubilized samples were applied (100 μg of protein in each lane), and the gels run at constant voltage successively at 300 V for 30 min, 350 V for 30 min, and 400 V for 15 hours. Before staining, the gels were fixed for 30 min in 50% methanol/10% acetic acid followed by incubation in 10% TCA/33% ethanol for 1 hour (at this stage, a white precipitate appeared). The gels were then washed with 5% TCA/33% ethanol for 30 min followed by a second wash in 30% methanol/10% acetic acid for 30 min and stained for 2 h in 30% methanol/10% acetic acid containing 0.2% of Coomassie Brilliant Blue and destained overnight in 30% methanol/10% acetic acid. For two-dimension analysis, the IEF gel-lanes were incubated in 0.025 M Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2.5% SDS, and 10% glycerol for 2 hours. Electrophoresis was carried out as described by González-Halphen *et al.* (1988) with the system described by Schägger *et al.* (1986).

Antibodies and Western Blots

Antibodies against the beef heart bc_1 complex subunits were raised and partially purified by ammonium sulfate precipitation as described before (González-Halphen *et al.*, 1991). Immunoblotting was carried out as described by Towbin *et al.* (1979) with the modifications described by González-Halphen *et al.* (1988).

Other Methods

Protein concentrations were determined according to Lowry *et al.* (1951) with the modifications of Markwell *et al.* (1978).

RESULTS

In order to explore the possible existence of tissue-specific subunits in the mitochondrial bc_1 complex, a method for the isolation of this oligomeric protein from adult beef liver mitochondria was developed. The method includes deoxycholate solubilization of submitochondrial particles, ammonium acetate fractionation, resolubilization with dodecyl

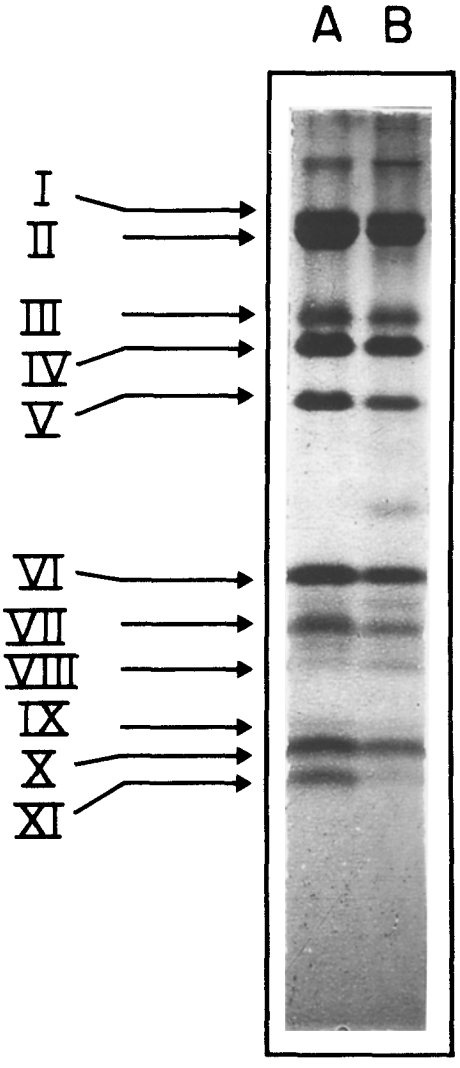

Fig. 1. Comparative SDS-polyacrylamide gel electrophoresis of purified mitochondrial bc_1 complex from heart (A) and liver (B) (30 μg of protein per lane). The subunit nomenclature used in the text is indicated on the left.

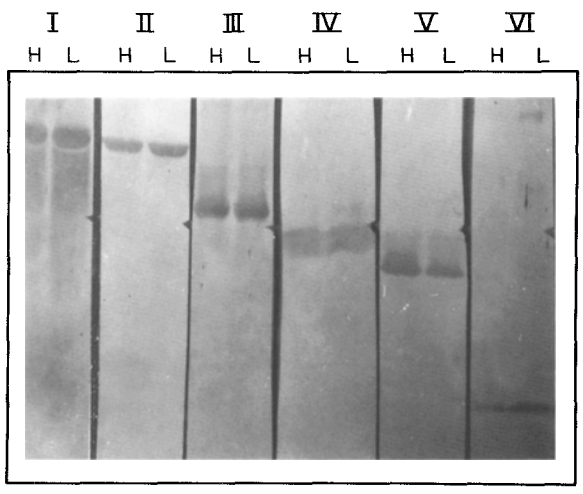

Fig. 2. Western blot of heart (H) and liver (L) bc_1 complexes (30 μg of protein per lane) decorated with antibodies raised against the purified subunits I, II, III, IV, V, and VI from beef heart bc_1 complex.

maltoside, and ion exchange chromatography. Liver submitochondrial particles were prepared as described by Yanamura *et al.* (1988). This preparation was solubilized and fractionated according to Rieske (1967), resolubilized with the nonionic detergent dodecyl maltoside, and further purified according to Ljungdahl *et al.* (1989). In a typical preparation, 1 kg of liver yielded between 4.8 to 6.2 mg of pure bc_1 complex, representing a 20-fold purification from submitochondrial particles (based on heme c_1 enrichment). The complex exhibited a b/c_1 stoichiometry between 1.3 and 1.7, lower than in the heart preparation, where values between 1.8 and 2.0 were obtained. The liver bc_1 complex had ubiquinol–cytochrome *c* activity fully sensitive to antimycin. A turnover number (V_{max}) of 67.5 s^{-1} and a K_m for ferrycytochrome *c* of 1.1 μM were estimated when measured under the conditions described by Kubota *et al.* (1992). The turnover number was lower than the one obtained for the heart complex in the same experimental conditions (364 s^{-1}), while the K_m had similar values (3.2 μM in the case of the heart bc_1 complex).

With respect to the number of subunits and electrophoretic migration, the purified bc_1 complexes from heart and liver exhibited a similar polypeptide pattern (Fig. 1). However, differences in the stoichiometry of some of the polypeptides, (subunits VII, VIII, and XI) were observed. Also, an additional band with an apparent molecular weight of 19 kDa was sometimes observed in the liver preparation. The latter was

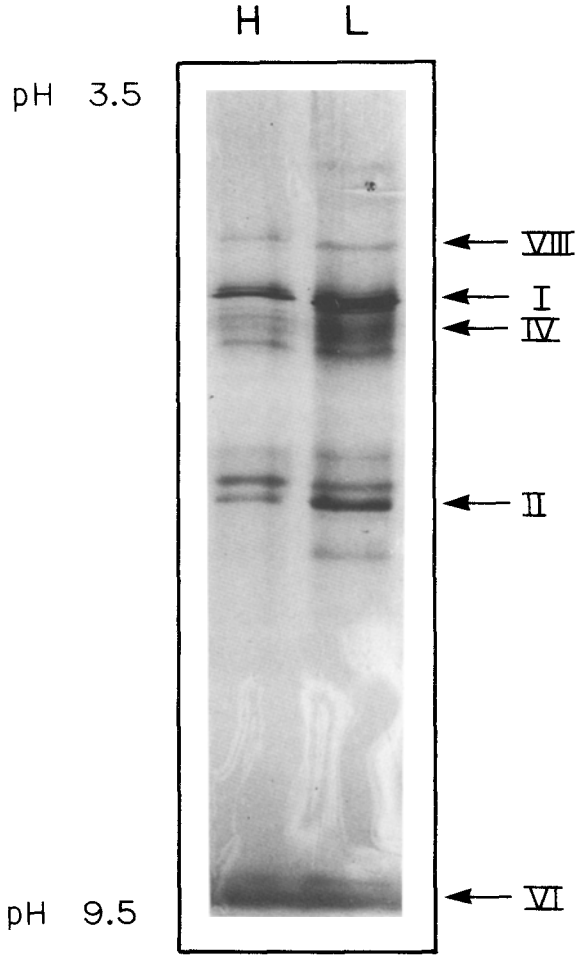

Fig. 3. Isoelectric focusing gels comparing bc_1 complex from heart (H) and liver (L) bc_1 complex (100 μg of protein per lane). The pH gradient obtained was linear, ranging from 3.5 to 9.5. The subunits identified by two-dimension analysis are indicated on the right.

considered a contaminant, as its stoichiometry varied in several preparations and some lacked this polypeptide. In addition, it is noted that this polypeptide did not exhibit cross-reaction with any of the antibodies directed against the first six high-molecular-weight subunits, indicating that it was not a proteolytic degradation product of one of these subunits (see below).

Samples run in SDS-PAGE, transferred to nitrocellulose, and decorated with antibodies raised against the first six high-molecular-weight subunits of the heart complex, showed cross reactivity with the corresponding six subunits of the liver complex (Fig. 2). The same antigenic epitopes are probably shared by the subunits of both complexes since the intensities of

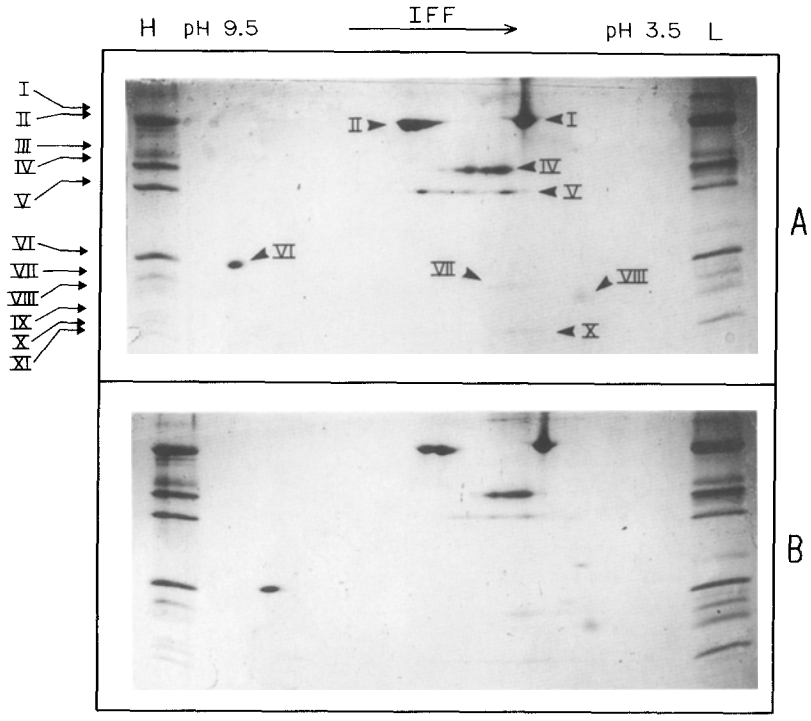

Fig. 4. Two-dimensional gels of bc_1 complex using isoelectric focusing (IEF) in the first dimension (100 μg of protein) and a Schägger *et al.* (1986) gel system (16% acrylamide) in the vertical dimension. (A) Beef heart bc_1 complex; (B) liver bc_1 complex. Controls (bc_1 complex that chromatographed only in the vertical dimension) were added in the lateral lanes of the second-dimension SDS-PAGE (30 μg of protein): liver (L) and heart (H).

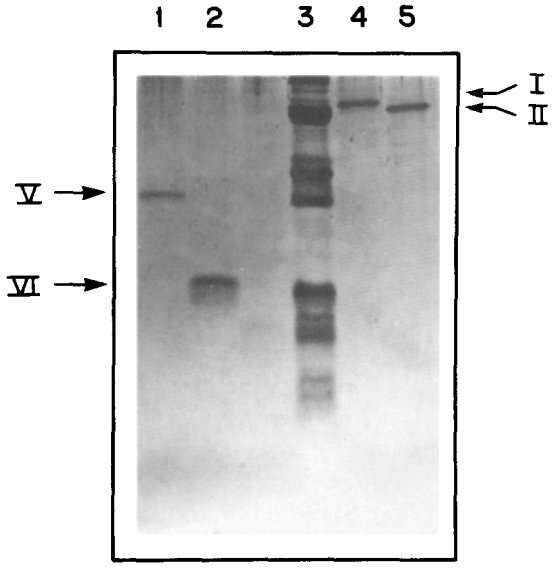

Fig. 5. SDS-PAGE (16% of acrylamide) from electrophoretically purified high molecular weight subunits from heart and liver bc_1 complex. Lane 1, subunit V (heart); lane 2, subunit VI (heart); lane 3, liver bc_1 complex; lane 4, subunit I (liver); lane 5, subunit II (liver).

immunostaining were the same when equal amounts of protein were loaded in the gel and transferred to nitrocellulose.

To further explore possible differences between subunits, isoelectric focusing of both preparations was carried out. Resolution of most of the subunits was attained by a modification of the procedure described by Cabral and Schatz (1979) (Fig. 3). With this technique the large separation between the two core proteins from both biological sources is noteworthy (an experimental isoelectric point of 4.2–4.6 was obtained for core I and of 5.9–6.6 for core II). On the other hand, several hydrophobic subunits were not resolved (subunits III, IX, and XI), probably because they aggregated and did not migrate as conspicuous bands in the IEF. An additional band, corresponding to the 19 kDa component (see above), was found in the IEF patterns of some preparations of the liver bc_1 complex. The position of the subunits in IEF was identified by two-dimensional gel electrophoresis as shown in Fig. 4. No difference in the migration of subunits from liver and heart as judged by isoelectric focusing was found, not even

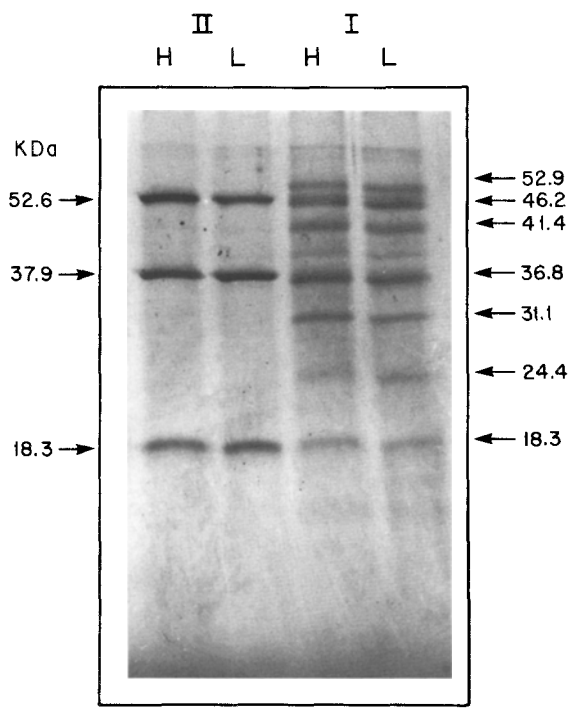

Fig. 6. Digestion of electrophoretically purified core subunits I and II from heart (H) and liver (L) treated with 1 mg/ml BNPS-skatol in 75% acetic acid. The apparent molecular weights of the peptides are indicated.

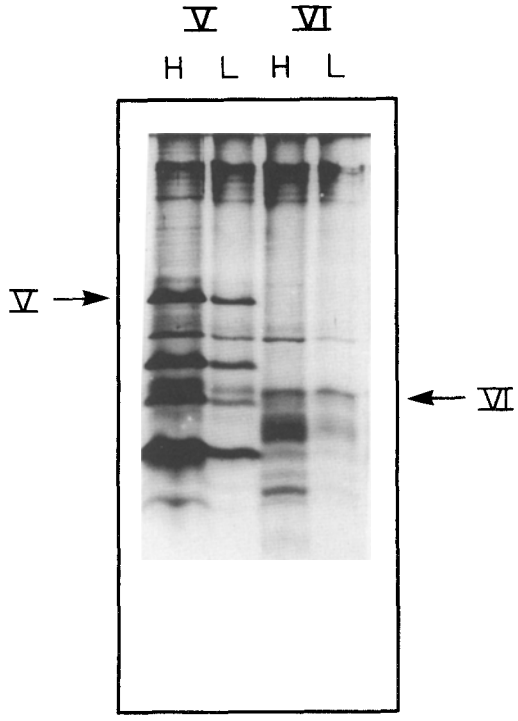

Fig. 7. Digestion of electrophoretically purified subunits V and VI from beef heart (H) and liver (L) treated with 1 mg/ml BNPS-skatol in 75% acetic acid. The position of the intact subunits is indicated.

when a mixture of 50% (w/w) of both preparations was run (results not shown).

In order to compare the polypeptide pattern of different subunits after chemical cleavage, an electrophoretic procedure for the isolation of subunits was developed. The subunits were separated by SDS-PAGE and recovered after transfer to a ProBlott membrane by elution in the presence of SDS and Triton X-100; Fig. 5 shows the polypeptide pattern of purified subunits I, II, V, and VI obtained by this method. Through this methodology sufficient material was obtained for chemical cleavage of the polypeptides and their subsequent analysis.

Peptide mapping of subunits I and II isolated from liver and heart was carried out after their digestion with BNPS-skatol (Fig. 6). For core II, two main products with apparent molecular weights of 37.9 and 18.3 kDa were obtained; more likely these polypeptides correspond to the predicted fragments in the sequence obtained by Gencic *et al.* (1991), i.e., a large fragment of 302 residues comprising Glu 136 to Leu 437 (31,691 Da) and a smaller one of 135 amino acids comprising Ser 1 to Trp 135 (14,675 Da). According to the amino acid sequence of core I reported by Gencic *et al.* (1991), one main cleavage product of 222 residues (24,543 Da) comprising Ile 41 to Trp 262, and six more polypeptides with molecular weights below 6 kDa, are predicted. Multiple bands were observed after digestion of this subunit, probably due to incomplete cleavage in the six different tryptophans, giving rise to different concatemers. In addition, the electrophoretically isolated core I protein contained residual core II polypeptide, since its cleavage products also appeared in the electrophoretic pattern. Hence, the peptide pattern generated by BNPS-skatol of core protein I was the same for the subunit isolated from both liver and heart. The peptide patterns generated by the digestion with cyanogen bromide and with trypsin of core proteins I and II were also the same in the subunits from liver or from heart (data not shown).

The products generated by BNPS-skatol cleavage of subunits V and VI were analyzed on SDS-PAGE (Fig. 7). Again, the subunits isolated from the two tissues gave the same polypeptide pattern. To establish possible differences in the small-molecular-weight subunits, their amino terminal sequence was determined after their isolation in polyacrylamide gels

Table 1. Amino-Terminal Sequence Analysis of Subunits VII and X of Beef Liver Mitochondrial bc_1 Complex

Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sub VII	X	Arg	Gln	Phe	Gly	His	Leu	Thr	Arg	Val	Arg	His	Val	Ile	Thr	Tyr	Ser	Leu	X	Pro
Sub X	X	Ala	Pro	Thr	Leu	Thr	Ala	Arg	Leu	Tyr	Ser	Leu	Leu	Phe	Arg	Arg	Thr	—	—	—

and transfer to a ProBlott membrane. The sequence obtained (Table I) of unambiguously identified residues was identical to the one reported for the 9.5 kDa protein (subunit VII) of beef heart bc_1 complex (Borchart *et al.*, 1986) and to the smallest protein of the cytochrome c_1 subcomplex (subunit X) from beef heart mitochondria (Schägger *et al.*, 1983).

DISCUSSION

The question concerning a possible tissue specificity of the subunits of mitochondrial bc_1 complex has been addressed. Of special interest are the so-called supernumerary subunits, the polypeptides from the complex that do not carry redox prosthetic groups and that are not present in the bc_1 complexes from prokaryotes, e.g., *Paracoccus denitrificans* (Trumpower, 1991). For this purpose, a reproducible procedure for the isolation of adult liver mitochondrial bc_1 complex was developed in the present work. The enzyme showed a high degree of purity, as revealed by its heme content, polyacrylamide gel electrophoresis, spectral analysis, and antimycin-sensitive ubiquinol–cytochrome *c* reductase activity.

The electrophoretic pattern of the bc_1 complex purified from bovine liver showed the presence of 11 different polypeptides. The subunits exhibited the same electrophoretic mobility as reported for the bovine heart enzyme (Schägger *et al.*, 1986). Western blot analysis revealed that antibodies raised against the heart bc_1 subunits I, II, III, IV, V, and VI cross-reacted with the corresponding liver bc_1 subunits. Nevertheless, it is noted that differences in the staining intensity by Coomassie Brilliant Blue were found in subunits VII and VIII when comparing the electrophoretic patterns of liver and heart bc_1 complexes; this could reflect the different stoichiometric content of these two polypeptides. This differences in stoichiometry may be explained by a partial dissociation of the complex by the detergent dodecyl maltoside, and it is in agreement with the lower b/c_1 stoichiometry consistently observed in the liver preparations. Liver subunit XI was also found in a variable stoichiometry from preparation to preparation, as judged by laser scanning densitometry (results not shown). In some cases, liver subunit XI was present in a larger amount than in the heart complex. It is known that this subunit dissociates easily from the rest of the complex, and may be partially lost during the isolation procedure (Schägger *et al.*, 1985; Yue *et al.*, 1991; Schägger and Von Jagow, 1991).

The modifications introduced in the present work to the isoelectric focusing technique described by Cabral and Schatz (1979) produced a high resolution of the subunits that constitute the bc_1 complex. The modifications included a combination of ampholines that lead to a linear pH gradient from 3.5 to 9.5, inversion of electrodes, and degassing of the gel, anode, and cathode solutions to avoid the cathode drift. Interestingly, those subunits that according to their hydropathy analysis lacked putative transmembrane stretches (data not shown) migrated as discrete bands during the isoelectric focusing electrophoresis (subunits I, II, VI, VIII, and X), while those with one or more putative transmembrane stretches were not resolved or tended to smear along the gel (subunits III, IV, V, VII, IX, XI). Isoelectric focusing patterns for both complexes were very similar. Subunit VI migrated toward the anode, while subunit VIII migrated toward the cathode. In addition, a very distinct separation was obtained for core proteins I and II, which usually tend to co-migrate when isolated by various chromatographic techniques. The isoelectric focusing method described in the present work may prove useful for the analysis of other oligomeric membrane proteins.

In bc_1 complexes from beef, chicken, turkey, duck, and tuna, differences in the electrophoretic mobility of the small subunits have been observed. The structure of subunits I, II, and VI in the aforementioned species appeared to be conserved as judged by their electrophoretic migration, whereas subunit VII showed structural variations between species (Degli Esposti *et al.*, 1986). The present work is in accordance with this observation, since no differences were observed when comparing the polypeptide patterns of the liver and heart subunits after chemical digestion. We also explored a possible tissue-specific difference of subunit VII, a polypeptide that

has been proposed to be the site of interaction with ubiquinol (Usui *et al.*, 1990). Amino terminus sequence analysis of this polypeptide showed that the first 17 residues are identical to those obtained by Borchart *et al.* (1986) for the heart polypeptide. The same result was obtained for the first 20 amino acids of the small subunit X from the liver bc_1 complex, the sequence corresponding exactly to that of the heart complex described by Schägger *et al.* (1983). This small subunit, as well as subunit VIII, is thought to be associated with cytochrome c_1, favoring its interaction with the soluble cytochrome *c* (Kim and King, 1983; Schägger *et al.*, 1986; González-Halphen *et al.*, 1988).

The core proteins are indispensable constituents of an active mitochondrial bc_1 complex (Gellerfors and Nelson, 1977; Bell *et al.*, 1979; D'Souza and Wilson, 1982; Sidhu *et al.*, 1983; Mendel-Hartvig and Nelson, 1983; Ho and Rieske, 1985; González-Halphen *et al.*, 1988; Berry *et al.*, 1991), and although they seem not to contribute directly to the redox and proton-motive activity of the complex (Cocco *et al.*, 1991), they participate in the import of presequences into the mitochondrial matrix (Schulte *et al.*, 1989), processing of the leader sequences (Braun *et al.*, 1992), assembly of the mature complex (Crivellone *et al.*, 1988; Schmitt and Trumpower, 1991), and in stabilizing the quaternary structure and function of the active bc_1 complex (Karlsson *et al.*, 1983; Weiss *et al.*, 1987; Gatti and Tzagoloff, 1990). From a comparison of the peptide patterns generated by cleavage of the purified core proteins from the heart and liver enzymes with a variety of chemical agents (BNPS-skatol, cyanogen bromide, and limited proteolysis with trypsin), we found that the core subunits were identical in liver and heart. These results, based on the chemical and enzymatic cleavage of the polypeptides, are in accordance with the results of Gencic *et al.* (1991) who showed that a cDNA fragment containing the full coding region of the core I protein hybridized against the poly(A)-rich RNA isolated from different bovine tissues, i.e., liver, heart, kidney, spleen, and skeletal muscle.

Definite evidence for the existence of tissue-specific subunits in mitochondrial bc_1 complex will come from the complete sequencing of the subunits from different tissues. Nevertheless, there is an interesting possibility that only cytochrome *c* oxidase, the final component of the electron-transfer chain in mitochondrial respiration, may be the sole component in which function (or assembly) may be expressed differently in different tissues (Dowhan *et al.*, 1985; Saccone *et al.*, 1991). In fact, as discussed by Capaldi (1988), there is a relationship between the tissue specificity of the respiratory complexes and the clinical manifestations of mitochondrial myopathies. Some of these diseases are related specifically with defects in the bc_1 complex (Kennaway, 1988). It is interesting to note that although cases of reversible mitochondrial myopathies related to cytochrome *c* oxidase deficiencies have been clinically identified (DiMauro *et al.*, 1988), to our knowledge no case of reversible myopathy involving the bc_1 complex has been described. This fact may be related to the absence of a tissue-specific regulation in the bc_1 complex.

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