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Purification of all thirteen polypeptides of bovine heart cytochrome *c* oxidase from one aliquot of enzyme

Characterization of bovine fetal heart cytochrome *c* oxidase

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A protocol has been worked out for separating all thirteen different polypeptides in the beef heart cytochrome *c* oxidase complex from a single aliquot of enzyme. This involves an initial separation of polypeptides by gel filtration on a Biogel P-60 column in SDS, a step which purifies subunits C_{IV} and C_{VIII} and gives mixtures of $C_V + C_{VI}$, ASA, AED and STA, as well as C_{VII}, C_{IX} and IHQ. These mixtures are then resolved by reverse-phase high-performance liquid chromatography. The separation procedures have been applied to fetal heart cytochrome *c* oxidase of gestation between 100 and 200 days. No differences were found in the N-terminal sequences of any of the cytoplasmically made subunits or in the entire sequence of C_{IX} between late fetal and adult forms of the enzyme.

Cytochrome-*c* oxidase; Purification; N-terminal sequence; Subunit sequence; Polypeptide separation

1. INTRODUCTION

The emerging picture of mammalian cytochrome *c* oxidase is of a large protein complex subject to physiological regulation, allowed for by developmentally different, and tissue-specific, isoenzyme forms [1,2].

The simplest cytochrome *c* oxidases in structural terms are those from prokaryotes, such as *Paracoccus denitrificans*, which are composed of two hemes, two copper atoms and three different subunits [3–5]. Cytochrome *c* oxidases from eukaryotes contain polypeptides highly homologous to the three subunits of the prokaryote enzyme. These are coded for on mitochondrial DNA; they presumably contain the prosthetic groups and are the catalytic core [1,2]. In addition, cytochrome *c* oxidase in eukaryotes contains a number of cytoplasmically made polypeptides, ranging from as few as four in lower eukaryotes such as *Dictyostelium* [6], to six in yeast or *Neurospora* [7], and as many as ten in mammals [8,9]. It is these cytoplasmically made polypeptides which show tissue-specific and possibly, developmentally different isoenzyme forms, as demonstrated by differences in size and N-terminal sequences [11] and different immunoreactivities [12] of some subunits. Studies of mitochondrial myopathies are also consistent with isoenzyme forms of cytochrome *c* oxidase. Thus, patients have been described with cytochrome *c* oxidase deficiencies localized to specific tissues [13–15]. Also, benign forms of mitochondrial myopathy have been reported in which patients have little or no cytochrome *c* oxidase activity in muscle at birth, but this activity appears spontaneously after several months [15,16].

It has not been established how many of the

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cytoplasmically made polypeptides of mammalian cytochrome *c* oxidase are tissue-specific, and how many have developmentally different forms. We have begun such an analysis. This requires methods for separating the individual subunits for peptide analysis and sequencing. Here we describe a protocol that allows purification of all 13 polypeptide components of beef cytochrome *c* oxidase from a small aliquot of protein. The approach was worked out with adult heart enzyme and then applied to fetal heart cytochrome *c* oxidase. According to Kuhn-Newtwig and Kadenbach [12], the adult and fetal forms of at least five of the ten subunits of the heart enzyme are very different, based on their immunoreactivity to subunit specific polyclonal antibodies.

2. MATERIALS AND METHODS

Beef heart cytochrome oxidase was prepared according to Capaldi and Hayashi [17]. The fetal hearts used were from fetuses of 100–200 days gestation.

2.1. *Separation of polypeptides by gel filtration on Biogel P-60*

Beef heart cytochrome oxidase, dissolved in 0.1 M potassium phosphate buffer (pH 7.4) containing 2% cholate, was precipitated by addition of saturated ammonium sulfate solution (final 33% saturation). The precipitated enzyme was collected by centrifugation (6000 × *g*, 10 min), dissolved and dissociated in 0.4 ml of 20% SDS containing 2% (v/v) β-mercaptoethanol. The dissociated enzyme was allowed to stand for 30 min at room temperature, loaded on the top of a Biogel P-60 (<400) column (1.5 × 116 cm), previously equilibrated with 2% SDS, and eluted at 1 ml/h. The eluent was monitored at 280 nm.

2.2. *Purification of subunits using reversed-phase high-performance liquid chromatography (HPLC)*

HPLC was performed on a Beckman gradient liquid chromatograph using a Brownlee Labs column (type RP-300, Aquapore Octyl, 300 Å pore size, 7 μm spherical, 100 × 4.6 mm) and guard column (type RP-8, Aquapore Octyl, 7 μm spherical, 15 × 3.2 mm) at a flow rate of 0.7 ml/min.

All water used for HPLC analyses was purified by passage through a NANO pure II (Sybron/Barnstead, Boston, MA). The solvents used for chromatography were: ('A') 0.05% (v/v) triethylamine and 0.05% (v/v) trifluoroacetic acid in acetonitrile; ('B') 0.05% (v/v) triethylamine and 0.05% (v/v) trifluoroacetic acid in 5% (v/v) acetonitrile. An aliquot of eluent from Biogel P-60 column was diluted with 2 vols of 0.1% (v/v) trifluoroacetic acid immediately before injection.

2.3. *Determination of N-terminal sequence of purified subunits*

The N-terminal sequence of purified subunits was determined using an Applied Biosystems protein sequencer (model 470A) and PTH analyzer (model 120A).

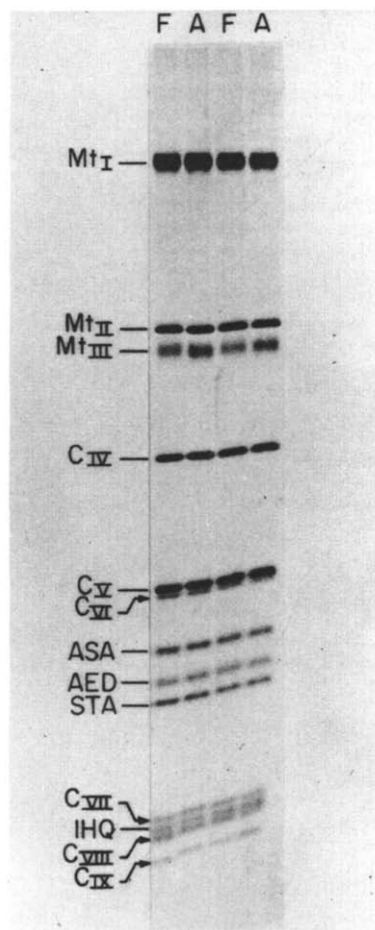

Fig.1. SDS-polyacrylamide gel electrophoresis of fetal (F) and adult (A) beef heart cytochrome *c* oxidase. 10 μg protein was loaded on each lane of the gel.

2.4. *Other methods*

SDS-polyacrylamide gel electrophoresis was performed according to Kadenbach et al. [18] except that 19.2% acrylamide and 0.5% *N,N'*-methylenebisacrylamide were used.

2.5. *Chemicals*

HPLC-grade acetonitrile and trifluoroacetic acid were purchased from Burdick and Jackson (Muskegon, MI) and Pierce (Rockford, IL), respectively. Triethylamine was obtained from Fluka. SDS (specially pure) was a product of BDH (Poole, England).

3. RESULTS

Beef heart cytochrome *c* oxidase can be resolved into 13 different polypeptides using the highly resolving gel system of Kadenbach and Merle [8], as shown in fig.1 for both adult and fetal forms of the enzyme. Component polypeptides are labeled according to our recently adopted nomenclature, in which the mitochondrially coded subunits are numbered Mt_{I-III} in order of their migration on the gel, C_{IV}–C_{IX} represent polypeptides found in both lower and higher eukaryotes, while polypeptides labeled by their N-terminal sequences are components unique to cytochrome *c* oxidase from higher eukaryotes.

Fig.2A shows the separation of the subunits of beef heart cytochrome *c* oxidase on Biogel P-60, following a procedure developed by Steffens and Buse [19] for isolation of the largest subunits of the enzyme. Eight clear peaks were resolved, the first to elute containing a small amount of aggregated protein, followed by Mt_I, Mt_{II}, Mt_{III}, C_{IV}, C_{V+VI}, a mixture of ASA, AED and STA and finally C_{VII}–C_{IX} and IHQ. The last peak to elute was broad and polyacrylamide electrophoresis gels of fractions across this peak show a partial resolution of polypeptides with IHQ eluting first, followed by C_{VII} and C_{IX} together and then C_{VIII} (fig.2B) eluting last. The elution profiles obtained with adult and fetal heart cytochrome *c* oxidase were

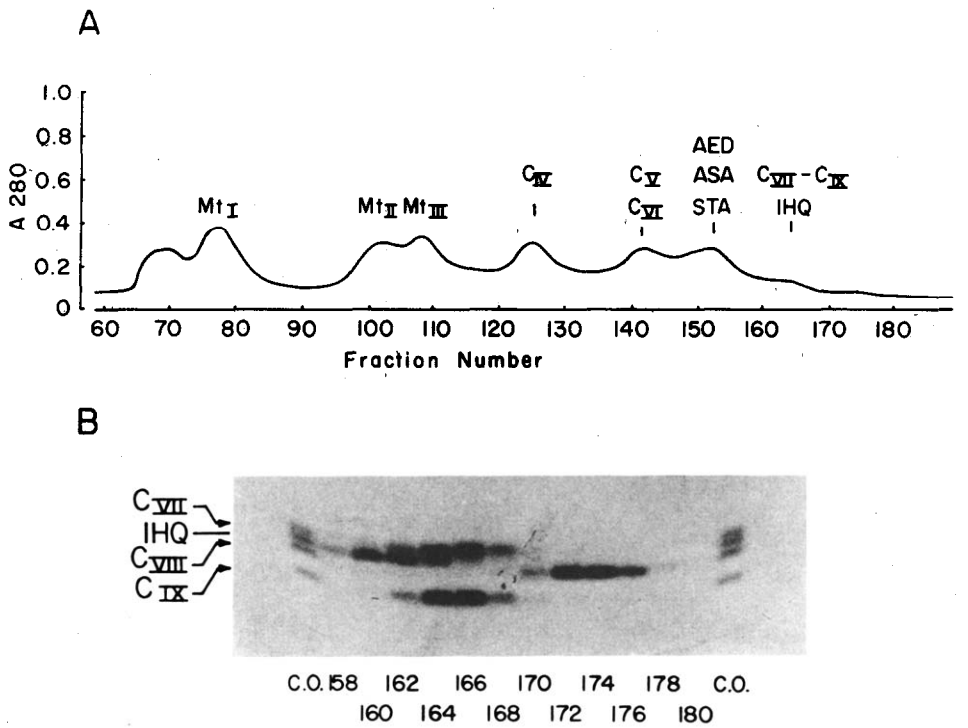

Fig.2. Separation of the polypeptide components of fetal beef heart cytochrome *c* oxidase on Biogel P-60 (<400) in 2% SDS. 24 mg of protein was applied on the column. (A) Elution profile. (B) SDS-polyacrylamide gel electrophoresis of fractions from the last broad peak to elute from the column.

identical and fig.2 shows a typical result for the fetal enzyme. Polypeptides C_{IV} and C_{VIII} were the only cytoplasmically made subunits pure enough for sequencing straight from the gel filtration column. The remaining subunits were purified by HPLC. Separations of C_V and C_{VI}, of ASA, AED and STA, and of C_{VII}, IHQ and C_{IX} are shown in figs 3 and 4, with identification of all of the purified polypeptides confirmed by SDS-polyacrylamide gel electrophoresis. The elution of the polypeptides of the adult and fetal heart enzyme was identical in all cases and the figures show the results for the fetal form of heart cytochrome *c* oxidase.

The HPLC fractionation step provided subunits in essentially pure form and each of the component polypeptides of the fetal enzyme was subjected to N-terminal sequencing. In the case of subunit C_{IX} the entire sequence was obtained. These data are presented in table 1. In all cases, the sequence of the fetal form of the subunit was identical to that reported for the adult form.

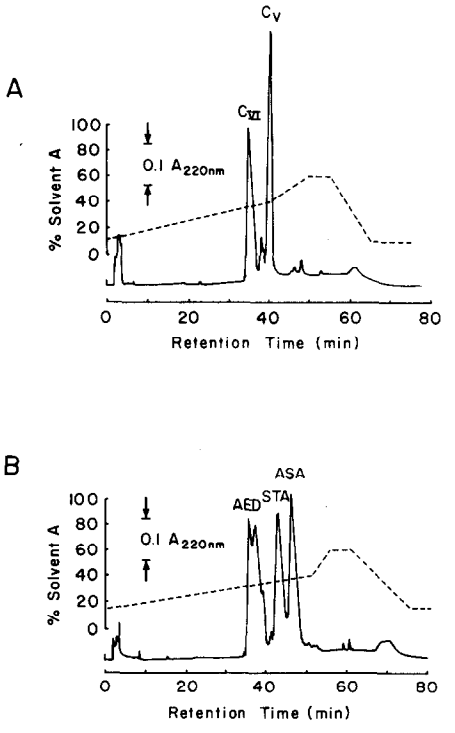

Fig.3. Separations of subunits C_V and C_{VI} (A) and of subunits ASA, AED and STA (B) by HPLC. Dashed lines show the pattern of gradients used for protein elution. Solvent A: 0.05% (v/v) triethylamine, 0.05% trifluoroacetic acid in acetonitrile; solvent B: 0.05% triethylamine, 0.05% trifluoroacetic acid in 5% (v/v) acetonitrile.

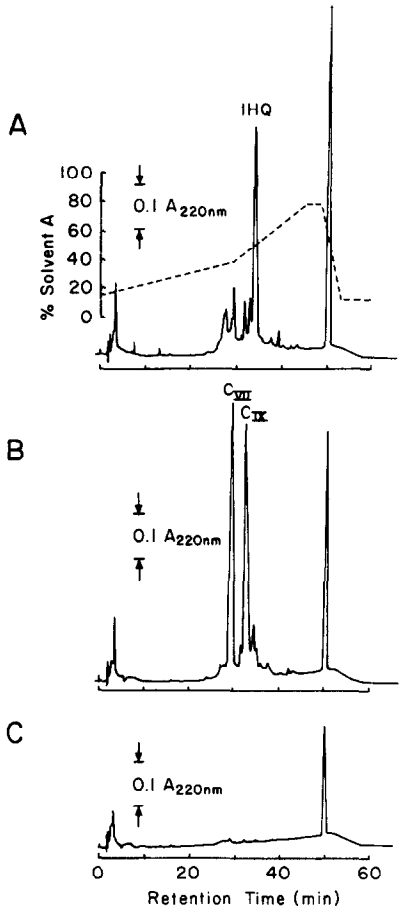

Fig.4. Separations of subunit IHQ and of subunits C_{VII} and C_{IX} on HPLC. The dashed line shows the pattern of gradient employed in all cases. Solvent A: 0.05% (v/v) triethylamine, 0.05% trifluoroacetic acid in acetonitrile; solvent B: 0.05% triethylamine, 0.05% trifluoroacetic acid in 5% (v/v) acetonitrile. (A) Biogel P-60 fraction 160; (B) P-60 fraction 166; (C) P-60 fraction 180 (non-protein fraction) (see fig.2B).

4. DISCUSSION

The question of how many subunits of mammalian cytochrome *c* oxidases are involved in tissue and developmental specificity remains to be worked out completely. Kadenbach et al. [11] have

Table 1

N-terminal sequence of subunits of fetal beef heart oxidase

C_{IV}	AHGSVVKSEDYALPSYVDRRDYPLPDVAHVKNLSASQ - - - - -
C_{V}	SHGSHETDEEFDARWVT - - - - -
C_{VI}	ASGGGVPTDEEQATGLEREV - - - - -
ASA	ASAAKGDHGGTGARTWRFLTFGL - - - - -
AED	Blocked
STA	STALAKPQMRGLLARRLRFHIVG - - - - -
C_{VIII}	FENRV - - - - -
IHQ	IHQKRAPDFHD - - - - -
C_{VIII}	SHYEE - - - - -
C_{IX}	ITAKPAKTPTSPKEQAIGLSVTFLSFLLPAGWVLYHLDNYKKSSAA

concluded that all of the cytoplasmically made subunits have tissue-specific forms based on their comparisons of enzyme from heart, brain, diaphragm and liver. Kuhn-Nentwig and Kadenbach [12] also found major differences in five of the cytoplasmically made subunits of both adult and fetal heart and adult and fetal liver in rats. According to the data of Kadenbach and colleagues [11,12], the number of nuclear genes coding for cytochrome *c* oxidase subunits would be close to 100.

The approach we have adopted involves a separation of subunits first by size which gives C_{IV} and C_{VIII} pure and $C_V + C_{VI}$, ASA + AED + STA, and C_{VII}, C_{IX} and IHQ as mixtures. The polypeptides in these mixtures are then resolved by reverse-phase HPLC. Our protocol allows isolation of all 13 polypeptide components from a single aliquot of enzyme, something not achieved before. It also provides an approach to purifying polypeptide IHQ for the first time. The approach was worked out using adult bovine heart cytochrome *c* oxidase and was then applied to fetal bovine heart enzyme. The protocol has also been used successfully to separate polypeptides of bovine adult liver cytochrome *c* oxidase (Yanamura, W., unpublished). The present data are for bovine fetal heart cytochrome *c* oxidase using enzyme purified from hearts of fetuses ranging from 100 to 200 days of gestation. The N-terminal sequence of each of the cytoplasmically made subunits was determined. No differences were observed between the sequences of the fetal and adult forms of any of the subunits. Particular attention was paid to polypeptide C_{IX}. The immunological studies of Kuhn-Nentwig and Kadenbach [12] showed C_{IX} to be the most different of the subunits when adult and fetal forms of rat heart or liver cytochrome *c* oxidase were compared. For this reason we sequenced the entire polypeptide without finding any amino acid substitutions.

Our results do not rule out that there are fetal forms of some of the polypeptides of heart cytochrome *c* oxidase, but do indicate that the switch from fetal to adult form must occur before around 100 days of gestation, at least for subunit C_{IX}. There can be no late switch in C_{IX} as implied for subunit C_{IV} in human muscle tissue by the studies of Miranda et al. [20] with patients having benign hereditary cytochrome *c* oxidase deficiency.

In summary, a protocol has now been developed for separating all of the polypeptides of cytochrome *c* oxidase from a single aliquot of enzyme. This allows a systematic examination of tissue and developmentally different forms of the bovine enzyme, for which sequences of all the subunits of adult heart are available for comparison.

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REFERENCES

[1] Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135–148.
[2] Kadenbach, B., Stroh, A., Ungibauer, M., Kuhn-Nentwig, L., Buge, U. and Jarausch, J. (1985) Methods Enzymol. 126, 32–45.
[3] Poole, R.K. (1983) Biochim. Biophys. Acta 726, 205–243.

[4] Saraste, M., Raitio, M., Jalli, T. and Peramaa (1986) FEBS Lett. 206, 154–156.
[5] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[6] Bisson, R. and Schiavo, G. (1986) J. Biol. Chem. 261, 4373–4376.
[7] Power, S.D., Lochrie, M.A., Patterson, T.E. and Poyton, R.O. (1984) J. Biol. Chem. 259, 6564–6570.
[8] Kadenbach, B. and Merle, P. (1981) FEBS Lett. 135, 1–11.
[9] Capaldi, R.A., Gonzales-Halphen, D. and Takamiya, S. (1986) FEBS Lett. 207, 11–17.
[10] Montecucco, C., Schiavo, G. and Bisson, R. (1986) Biochem. J. 234, 241–243.
[11] Kadenbach, B. (1983) Angew. Chem. Int. Ed. Engl. 22, 275–282.
[12] Kuhn-Nentwig, L. and Kadenbach, B. (1985) Eur. J. Biochem. 149, 147–158.
[13] Darley-Usmar, V.M., Kennaway, N.G., Buist, N.R.M. and Capaldi, R.A. (1983) Proc. Natl. Acad. Sci. USA 80, 5103–5106.
[14] Bresolin, N., Zeviani, M., Bonilla, E., Miller, R.H., Leech, R.W., Shanske, S., Nakagawa, M. and DiMauro, S. (1985) Neurology 35, 802–812.
[15] DiMauro, S., Bonilla, E., Zeviani, M., Nakagawa, M. and DeVivo, D.C. (1985) Ann. Neurol. 17, 521–538.
[16] DiMauro, S., Nicholson, J.F., Hayes, A.P., Eastwood, A.B., Papadimitriou, A., Koenigsberger, R. and DeVivo, D.C. (1983) Ann. Neurol. 14, 226–234.
[17] Capaldi, R.A. and Hayashi, H. (1972) FEBS Lett. 26, 261–263.
[18] Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1982) Anal. Biochem. 29, 517–521.
[19] Steffens, G.J. and Buse, G. (1976) Hoppe Seyler's Z. Physiol. Chem. 357, 1125–1137.
[20] Miranda, A.F., Nakagawa, M., Bonilla, E. and DiMauro, S. (1985) J. Cell Biol. 101, 449a.