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# Tissue- and species-specific expression of cytochrome *c* oxidase isozymes in vertebrates

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**Cytochrome *c* oxidase was isolated from brown fat tissue of the rat and compared with the isozymes from rat liver and heart, which differ at least in subunits VIa and VIII. ELISA titrations of COX from the three tissues with monospecific antisera to all 13 subunits of the rat liver enzyme showed differences between the three enzymes. The N-terminal amino-acid sequence analysis of subunits VIa and VIII from SDS-PAGE gel bands of the three enzymes indicates the occurrence of three different isozymes in the rat. N-terminal amino-acid sequence analysis of subunits VIa and VIII from cytochrome *c* oxidase of bovine and human heart demonstrates also species-specific differences in the expression of the 'liver-type' and 'heart-type' of subunits VIa and VIII.**

## Introduction

Mitochondrial respiration is known to be controlled by the concentrations of oxygen and ferrocytochrome *c*, the substrates of cytochrome *c* oxidase, and by the proton chemical gradient across the inner mitochondrial membrane [1–4]. An additional regulation of cytochrome *c* oxidase activity by allosteric effectors (e.g., nucleotides, ions, substrates, etc. was suggested [5] and recently supported by the influence of nucleotides [6–11] phosphate [9,12] and fatty acids [13,14] on the kinetics of cytochrome *c* oxidase. Allosteric effectors are assumed to bind to one of the ten subunits encoded on nuclear DNA modifying the catalytic activity, which is associated with the three subunits encoded on mitochondrial DNA [15]. A tissue-specific regulation of cytochrome *c* oxidase activity was found, based on different kinetics of the liver and heart enzyme [13,16], which differ in subunits VIa, VIIa and VIII [15,17–19]. Recently, the two genes for liver and heart subunit VIa have been cloned and the deduced amino-acid sequences show only 50% homology [20].

In the present publication, cytochrome *c* oxidase was isolated from rat brown fat tissue, and the N-terminal amino-acid sequences of subunits VIa and VIII were compared with those of the isolated enzyme from rat liver and from rat, bovine and human heart. The data indicate the occurrence of different isozymes in rat liver, heart and brown fat tissue. In addition the expression of the 'liver-type' and 'heart-type' genes for subunits VIa and VIII differs in rat, bovine and human heart.

## Methods

### *Isolation of cytochrome c oxidase*

Rats were cold-acclimated by keeping them for 6 weeks at 4°C. The interscapular, dorsal, cervical and axilar brown fat tissue was used to prepare mitochondria [21]. Cytochrome *c* oxidase was prepared from human heart [22], bovine heart, and of rat liver, heart and brown fat mitochondria as described previously [23]. SDS-polyacrylamide gel electrophoresis was performed with Tricine buffer without urea as described by Schägger and von Jagow [24].

Abbreviations: COX, cytochrome *c* oxidase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Tricine; *N*-2-hydroxy-1,1-bis(hydroxymethyl)ethyl glycine; ELISA, enzyme-linked immunosorbent assay.

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*Enzyme-linked immunoassay*

Monospecific antisera to all 13 subunits of rat liver cytochrome *c* oxidase were produced as described in Ref. 25. The ELISA was performed in microtiter plates (12 × 8 holes) with the isolated enzymes dissolved in 8 M urea, 50 mM sodium borate (pH 9.5) (2 μg cytochrome *c* oxidase/ml). To each hole 100 μl enzyme were added and incubated at 0 °C overnight. After incubation for 1 h at room temperature with 200 μl PBS (150 mM NaCl/20 mM $NaP_i$ (pH 7.4)) containing 1% BSA and twice for 10 min with 200 μl PBS, 0.5% Triton X-100, 100 μl of the specific antisera, diluted in PBS as indicated in the figure, were added and incubated for 2 h at room temperature. After washing three times for 10 min with 200 μl PBS, 0.5% Triton X-100, 100 μl of the second antibody (peroxidase-conjugated antibody to rabbit immunoglobulins from swine, Dakopatts) diluted 1 : 1000 with PBS, BSA was added and incubated for 1 h at room temperature, followed by 4 × 10 min washings with PBS/0.5% Triton X-100. The color was developed for 30 min by incubation with 100 μl of a solution containing 0.11 mg/ml 2,2'-azino-di(3-ethylbenzthiazoline)sulfonic acid in 0.1 M sodium acetate, 0.05 M sodium phosphate (pH 4.2), 2.5 mM $H_2O_2$, and the absorbance was immediately read automatically in a minireader (Titertek Multiscan Plus).

*N-terminal amino-acid sequencing of gel bands*

The subunits of isolated cytochrome *c* oxidase from rat liver, heart and brown fat and from bovine and human heart were separated by SDS-PAGE according to Schägger et al. [24]. N-terminal sequences were determined according to Eckerskorn et al. [26] as follows: the separated subunits were electroblotted in a semi-dry apparatus onto siliconized glass-fiber sheets (Glassybond, Biometra). Electrotransfer was performed in 50 mM boric acid, 10% methanol (pH 9.0) with constant current (1 $mA/cm^2$ for 5 h). The transferred proteins were stained with 0.1% (w/v) Coomassie blue R 250/30% (v/v) methanol/10% (v/v) acetic acid for 2 min. The blot was destained in water/methanol/acetic acid (60 : 30 : 10, v/v), washed with bidistilled water and dried. The deteted protein bands were excised and placed directly in a gas phase sequencer (470 A, Applied Biosystems).

## Results and Discussion

The SDS-gel electrophoretic patterns of isolated cytochrome *c* oxidase from rat heart, brown fat tissue and liver are compared in Fig. 1. By the applied gel electrophoretic system [24] the heart and liver subunits VIII show a different apparent molecular weight. The brown fat cytochrome *c* oxidase subunit VIII runs at the level of the heart subunit. A weakly stained band is also seen above it at the level of the liver subunit VIII, which

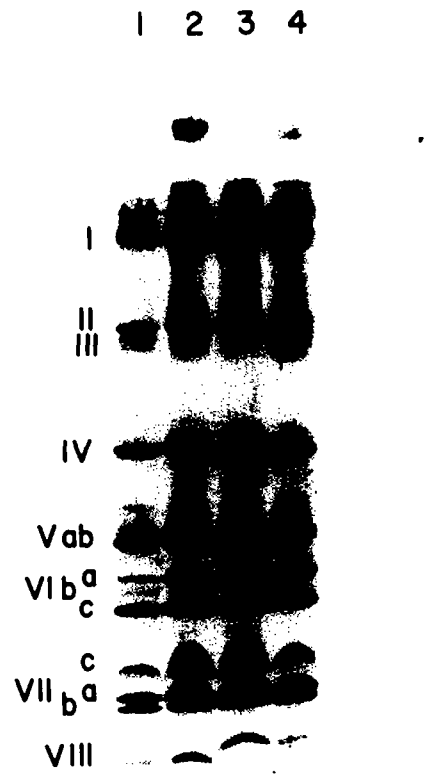


Fig. 1. Comparison of the SDS-gel electrophoretic subunit pattern of isolated cytochrome *c* oxidase from rat heart (lane 1), brown fat tissue (lane 2) and liver (lanes 3, 4). The amount of enzyme protein applied to each lane was 20 μg (lane 1), or 40 μg (lanes 2–4). Roman numbers indicate subunits of cytochrome *c* oxidase.

indicates the presence of a small amount of the liver isozyme, possibly expressed in contaminating non-induced adipose tissue. In order to identify possible immunological differences between corresponding subunits of cytochrome *c* oxidase from heart, brown fat and liver, ELISA titrations of the three isolated enzymes with monospecific antisera to all 13 subunits of the liver enzyme [25] were performed (Fig. 2). With most antisera similar titration curves are found with the three enzyme preparations. Different curves are obtained with the antisera to subunits IV, VIa, VIc, VIIa and VIII. Some antisera, prepared against the liver cytochrome *c* oxidase subunits, gave a stronger reaction with the brown fat than with the liver enzyme (subunits IV, VIa, VIc, VIIa). The stronger reaction with subunits of the brown fat enzyme is also found with an antiserum to the mitochondrial-encoded subunit II, which is assumed to be identical in all isozymes [25]. This could be due to an influence of the variable nuclear-encoded subunits in the ELISA.

To characterize further the variable subunits of cytochrome *c* oxidase in different tissues, the N-terminal amino-acid sequences of polypeptides were determined with an automatic gas-phase sequencer after blotting onto glass-fiber sheets [26]. The initial yields of the

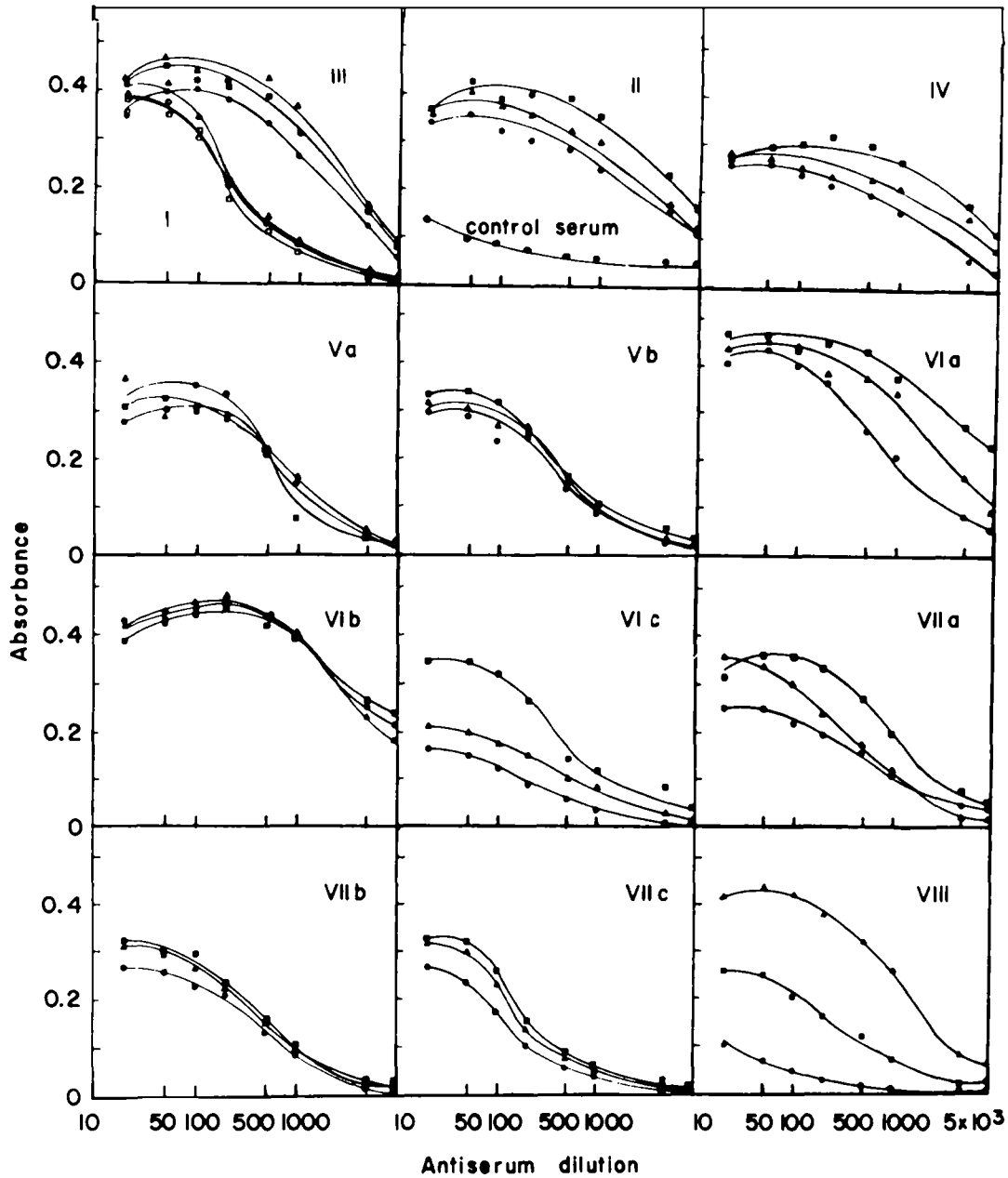


Fig. 2. Comparison of cytochrome *c* oxidase from rat brown fat (■), liver (▲) and heart (●) by ELISA. The three isolated enzymes were titrated with monospecific antisera to rat liver cytochrome *c* oxidase subunits [25], as indicated in the figure. ○, Rat heart cytochrome *c* oxidase titrated with non-immune serum. For better clarity, open symbols were also used for titration with antiserum to subunit I.

sequence degradations are given in the legends to Figs. 3 and 4; the repetitive yields for all degradations were between 92 and 95%. In Fig. 3 are presented the N-terminal amino-acid sequences of subunits VIa of isolated cytochrome *c* oxidase from rat liver, heart and brown fat tissue and from bovine heart, together with the deduced amino-acid sequences of cDNAs, isolated from rat liver and rat heart cDNA libraries, respectively [20]. Whereas in rat liver and brown fat tissue the 'liver-type' subunit VIa is found, in the bovine heart only the 'heart-type' of subunit VIa was detected. No liver-type subunit VIa [18,19] was found in bovine heart subunits VIb and VIc as well.

Surprisingly in cytochrome *c* oxidase isolated from rat heart, both sequences, the liver-type and the heart-type were found in equal amounts in the position of subunit VIa. In the position of subunit VIb, containing an acetylated N-terminus, only the sequence of heart-type subunit VIa was found. In another experiment of equal material in the position of subunit VIa, only the heart-type and in position of subunit VIc only the liver-type was found. It should be pointed out that

COX subunit VIa

| | | 10 | 20 | 30 |
|---|---|---|---|---|
| Rat liver, cDNA | | SSGAHGEEG- | -SARIWKALT | YFVALPGVGV... |
| Rat liver* | | SSGAHG... | | |
| Rat brown fat* | | SSGAHGEEG- | -SARIWKALT | YFVA... |
| Rat heart*, | 35 % | SSGAHGEEG- | -S... | |
| | 65 % | ASASKGDHGG... | | |
| Rat heart. cDNA | | ASASKGDHGG | AGANTWRLLT | FVLALPSVAL... |
| Bovine heart* | | ASAAKGDHGG... | | |

Fig. 3. Comparison of the N-terminal amino acid sequences of cytochrome *c* oxidase subunits VIa from rat liver, brown fat tissue and heart and from bovine heart, as determined from gel bands after blotting onto glass-fiber sheets (*) with the deduced amino-acid sequences of cDNAs, isolated from rat liver and rat heart cDNA libraries [20]. The initial yields obtained with the gas-phase amino-acid sequencer were: rat liver, 250 pmol; rat brown fat, 70 pmol; rat heart, first blot: 50 pmol (liver-type) + 50 pmol (heart-type) in position of subunit VIa and 50 pmol (heart-type) in position of subunit VIb, second blot: 180 pmol (heart-type) in position of subunit VIa and 100 pmol (liver-type) in position of subunit VIc; bovine heart, 120 pmol (heart-type).

in the gel system of Schägger et al. [24] the position of subunits VIa, b and c as well as of subunits VIIa, b and c are partly exchanged, depending on the amount of mercaptoethanol in the sample buffer. Thus the exact identification of gel bands is difficult and requires N-terminal amino-acid sequencing. We assume that the blotting efficiency is high and equal for all subunits. This is supported by the facts that (i) the stained glass-fiber resembles quite well the pattern observed in the gel without blotting, (ii) the gel was empty after blotting and (iii) in our experience only a very small portion (few percent) of proteins in this molecular weight range is lost by blotting through the membrane [26]. From the quantity of phenylthiohydantoin amino acids obtained in each cycle of the Edman degradation it can be estimated that the amount of heart-type VIa is about double the amount of liver-type subunit VIa in cytochrome *c* oxidase of rat heart. The detection of liver-type and heart-type of subunit VIa in rat heart by amino-acid sequencing corroborates the previous observation of similar amounts of liver-type and heart-type mRNA for subunit VIa in rat heart, as concluded from Northern blots [20].

The N-terminal amino-acid sequences of subunits VIII of isolated cytochrome *c* oxidase from rat liver, heart and brown fat tissue and from human heart are presented in Fig. 4 together with the amino-acid sequences determined from the isolated bovine heart [27] and bovine liver [19] subunit VIII, and the deduced amino-acid sequences of cDNAs, isolated from rat liver [28] and human liver [29] cDNA libraries, respectively. In the human heart enzyme the liver-type subunit VIII was exclusively found by sequencing the gel band corresponding to subunit VIII. This result corroborates the results of van Kuilenberg et al. [30], who determined the complete amino-acid sequence of isolated subunit VIII of human heart. In that publication the possibility could not be excluded that both a liver-type and a heart-type subunit VIII were expressed in human heart, because during isolation by HPLC the liver-type subunit VIII could have been enriched. Our results almost exclude this possibility.

The N-terminal amino-acid sequence of the rat heart subunit VIII is largely different from the liver subunit VIII, showing only 41% homology in a sequence of 38 amino-acid residues. The brown fat cytochrome *c*

COX subunit VIII

| | 10 | 20 | 30 | 40 |
|---|---|---|---|---|
| Bovine liver | IHSKPPREQL | GTMEIAIGLT | SCFLD..... | .....MENY KKRE |
| Human liver, cDNA | IHSLPPEGKL | GIMELAVGLT | SCFVTFLLPA | GWILSHLETY RRPE |
| Human heart* | IHSLPPEGKL | GIMELAVGLT | SC.... | |
| Rat liver, cDNA | VHSKPPREQL | GVLDITIGLT | SCFVCCLLPA | GWVLSHLESY KKRE |
| Rat liver* | VHSKPPRE.. | | | |
| Rat brown fat* | ISSKPAKSPT | SAMDQAV... | | |
| Rat heart* | ISSKPAKSPT | SAMDQAVGMS | VIIAGFMVPA | GWVLSHLE.. |
| Bovine heart | ITAKPAKTPT | SPKEQAIGLS | VTFLSFLLPA | GWVLYHLDNY KKSSAA |

Fig. 4. Comparison of the N-terminal amino-acid sequences of cytochrome *c* oxidase subunits VIII from rat liver, brown fat tissue and heart, and from human heart as determined from gel bands after blotting onto glass-fiber sheets (*) with the deduced amino-acid sequence of cDNAs, isolated from human liver [29], and rat liver [20] cDNA libraries, and of protein sequences of isolated subunit VIII from bovine heart [27] and liver [19]. The initial yields obtained with the gas-phase amino-acid sequencer were: human heart, 30 pmol; rat liver, 250 pmol; rat brown fat, 25 pmol; rat heart, 400 pmol.

oxidase contains the heart-type subunit VIII, as revealed from the sequence of 17 N-terminal amino-acid residues. Thus, a third cytochrome *c* oxidase isozyme occurs in rat brown adipose tissue, which corresponds to one-third of the heart enzyme containing the liver-type subunit VIa. This isozyme differs from the liver isozyme and from the other two thirds of heart cytochrome *c* oxidase containing the heart-type subunit VIa. A general principle of cytochrome *c* oxidase isozyme structure can be concluded. In mammals the cytochrome *c* oxidase appears to be assembled always from 13 subunits, where 55% of the protein mass consists of three invariable subunits encoded on mitochondrial DNA which carry the catalytic activity, and of ten different but variable nuclear-encoded subunits. Assuming that only two different functional genes occur in the mammalian genome for each of the ten nuclear-encoded subunits, $2^{10} = 1024$ different isozymes could in theory be assembled.

The different isozyme structure of cytochrome *c* oxidase from brown fat tissue, could indicate a different regulatory property. In fact, the role of brown fat tissue in nonshivering thermogenesis requires a rapid and several-fold increase of respiration upon the extracellular signal noradrenaline [31,32].

The occurrence of the liver-type subunit VIII in cytochrome *c* oxidase from human heart contrasts with the subunit structure of cytochrome *c* oxidase from other mammals and from birds. In chicken [33], rat (Fig. 4), pig [23] and bull [19], the liver-type subunit occurs exclusively in liver and the heart-type in heart cytochrome *c* oxidase, as concluded from their different apparent molecular weights (chicken, pig, bovine) or amino-acid sequences (rat, bull). From the variable expression of the two isoforms for subunits VIa and VIII in liver and heart in the different species we must conclude that the two isoforms of both subunits are expressed not only under the control of tissue-specific signals, but also under the control of additional as yet unknown signals which could be elicited by age, nutritional status, season, etc.

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