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Demonstration of two isoforms of subunit VIIa of cytochrome *c* oxidase from human skeletal muscle

Implications for mitochondrial myopathies

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Two different isoforms of subunit VIIa have been found in cytochrome *c* oxidase isolated from human skeletal muscle. The first 22 residues of the N-terminal amino acid sequences showed 5 differences. Our results provide the first conclusive evidence for the existence of cytochrome *c* oxidase isoenzymes in man. Since the two cytochrome *c* oxidase isoforms were both present in skeletal muscle tissue, though not necessarily in the same cell type, this suggests that human cytochrome *c* oxidase isoforms are not strictly tissue-specific. These findings may have important implications for the elucidation of genetic diseases in man in which a deficiency of cytochrome *c* oxidase is restricted to certain tissues.

Human cytochrome *c* oxidase isoenzyme; Mitochondrial myopathy

1. INTRODUCTION

Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain, catalyzes the oxidation of ferrocytochrome *c* by molecular oxygen. The three largest subunits (I–III) of the mammalian enzyme are encoded by the mitochondrial genome [1], while the 10 smaller subunits are encoded by nuclear genes. Tissue-specific immunological and electrophoretic differences in these nuclearly coded subunits have been demonstrated in cytochrome *c* oxidase purified from several tissues of the same mammalian species [2,3]. The discovery of isoenzyme forms of bovine cytochrome *c* oxidase was confirmed by N-terminal amino acid sequence data, which revealed that different 'heart-type' and 'liver-type' isoforms of the subunits VIa, VIIa and VIII were present in cytochrome *c* oxidase purified from bovine heart and liver tissue, respectively [3]. In view of subsequent findings with cytochrome *c* oxidase from other mammalian species [4,5], we prefer to expand the definition of 'bovine heart-type' and 'bovine liver-type' isoforms to other species.

However, the situation in man with regard to cytochrome *c* oxidase was not clear. It is now well-established that in patients with mitochondrial diseases a mitochondrial enzyme may be deficient in some tissues but not in others (see [6–9] for reviews). This is often the case in patients with a deficiency of cytochrome *c* oxidase [8–15]. For instance, in one type of fatal infantile myopathy there is a deficiency of functional cytochrome *c* oxidase in skeletal muscle, while normal levels of the active enzyme are observed in other tissues. The existence of oxidase isoforms would provide an explanation for these observations.

Recently we determined the amino acid sequence of subunit VIII isolated from human heart cytochrome *c* oxidase, which proved to be a 'bovine liver-type' [4]. However, a 'bovine heart-type' subunit VIII has not yet been found in man. Furthermore, only 'bovine liver-type' cDNAs specifying the subunits VIa, VIIa and VIII of human cytochrome *c* oxidase have been published [16–18]. These findings led to doubt about the existence of tissue-specific isoenzymes of human cytochrome *c* oxidase. Our study proves the existence of human cytochrome *c* oxidase isoforms through the identification of two different isoforms of subunit VIIa in a single preparation of human muscle cytochrome *c* oxidase.

2. MATERIALS AND METHODS

2.1. *Purification of cytochrome c oxidase and fractionation of the small subunits*

Human skeletal muscle (quadriceps) was obtained at obduction. Cytochrome *c* oxidase was purified according to the same procedure as described before for human heart cytochrome *c* oxidase [4], essen-

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tially by differential extraction of submitochondrial particles with deoxycholate.

The nuclearly encoded subunits were separated from the mitochondrially encoded subunits using the method of Power et al. [19] with the following modifications: after preparing supernatant S1, the final pellet (P1) was resuspended in 1.25% triethylamine (TEA) and 1.25% trifluoroacetic acid (TFA). One volume of the mixed solvent acetonitrile/1-propanol (1:1, 0.05% TEA, 0.05% TFA) was added to the suspension and the mixture was stirred for 3 h on ice. After centrifugation, the supernatant was retained and the pellet extracted once more. The third extraction was carried out overnight and after centrifugation, the final extraction was carried out for 3 h. All four supernatants were pooled and designated supernatant S2. Both supernatants S1 and S2 and the final pellet P2 were lyophilized and saved for further analysis.

2.2. *Purification of subunits VIIa (1) and VIIa (2) with reversed-phase HPLC*

Subunits VIIa (1) and VIIa (2) (for a definition see section 3) were purified from supernatant S2. Aliquots of supernatant were dissolved in 8 M guanidine HCl/0.05% TFA and incubated for 1 h at room temperature. Dithiothreitol (10 mM) was added 15 min prior to injection into the HPLC system. HPLC was performed on a Brownlee (Santa Clara, CA) column (type RP-300, Aquapore octyl, 300 Å size, 7 μm spherical, 220 × 4.6 mm) and a guard column (type RP-8 Aquapore ODS, 7 μm spherical). The HPLC system consisted of two LKB 2150 pumps, a 2152 LC controller, a Rheodyne 7125 injector and an LKB Uvicord 2138 S detector operating at 280 nm. The solvents used for chromatography consisted of 0.05% TFA/0.05% TEA/5% acetonitrile/water (solvent A) and 0.05% TFA/0.05% TEA/50% acetonitrile/50% 1-propanol (solvent B).

2.3. *Polyacrylamide gel electrophoresis*

Tricine-SDS gel electrophoresis was performed according to Schägger and Von Jagow [20]. Gels (30 × 18 × 0.15 cm) were prepared with glycerol instead of urea. The samples were incubated in sample buffer and β-mercaptoethanol was added up to 2% (v/v) 1 h prior to the start of the gel electrophoresis run. Gels were stained overnight with Coomassie brilliant blue.

2.4. *Determination of the N-terminal amino acid sequences of subunits VIIa (1) and VIIa (2)*

The determination of the N-terminal sequence was carried out on a 477A pulsed-liquid sequencer (Applied Biosystems, USA) with online analysis of the phenyl thiohydantoin amino acids on a 120A PTH analyzer (Applied Biosystems).

3. RESULTS

From supernatant S2 we could purify two different subunits VIIa of human muscle cytochrome *c* oxidase which had different retention times on a reversed-phase column. Part of the elution profile of S2 is shown in Fig. 1. The two different subunits VIIa, designated by us as VIIa (1) and VIIa (2), eluted around 41% solvent B. Fig. 2 shows the analyses by gel electrophoresis of the two human muscle subunits VIIa, purified with reversed-phase HPLC. The two distinct bands in the subunit VII region of subunit VIIa (2) are probably due to different conformations of the protein, since subunits VIIb and VIIc have a free N-terminus (unpublished results) and analysis of the N-terminal amino acid sequence of both subunits VIIa revealed no significant contamination with other proteins. The additional minor bands of higher molecular mass are probably dimeric forms of the subunits VIIa (1) and VIIa (2).

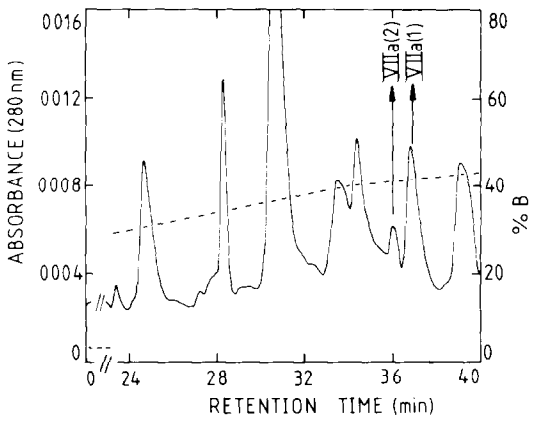

Fig. 1. Part of the HPLC-elution profile of supernatant S2 (for details see section 2). The 280 nm absorbance is given by the solid line, the dashed line represents the percentage of solvent B in the gradient.

This phenomenon was also observed for several other purified subunits (unpublished results).

In Table I the N-terminal amino acid sequences of subunits VIIa (1) and VIIa (2) from human muscle are presented together with the complete amino acid se-

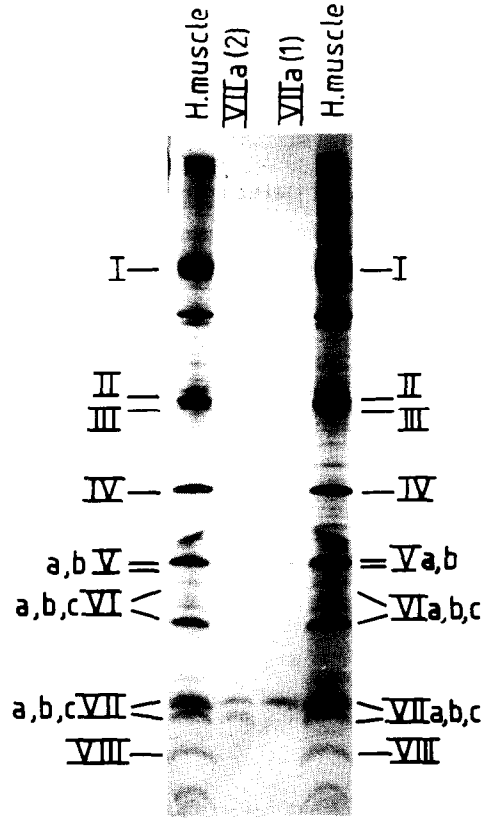

Fig. 2. The analysis by gel electrophoresis of 50 pmol of the human muscle cytochrome *c* oxidase subunits VIIa (1) and VIIa (2) (described under section 3), purified with reversed-phase HPLC. Purified human muscle cytochrome *c* oxidase (120 pmol) was used as a reference. The gel was stained with Coomassie blue.

Table I

The N-terminal amino acid sequences of the subunits VIIa (1) and VIIa (2) (see section 3) of human skeletal muscle cytochrome *c* oxidase as determined by us and comparison with the complete amino acid sequences of subunit VIIa of bovine heart [21], bovine liver subunit VIIa [22], and human endothelial cell subunit VIIa [16]

	1				5				10				15	
Bovine heart	Phe	Glu	Asn	Arg	Val	Ala	Glu	Lys	Gln	Lys	Leu	Phe	Gln	Glu	Asp
Human muscle (1)	Phe	Gln	Asn	Arg	Val	Arg	Glu	Lys	Gln	Lys	Leu	Phe	Gln	Glu	Asp
Human muscle (2)	Phe	Lys	Asn	Lys	Val	Pro	Glu	Lys	Gln	Lys	Leu	Phe	Gln	Glu	Asp
Human endothelial	Phe	Lys	Asn	Lys	Val	Pro	Glu	Lys	Gln	Lys	Leu	Phe	Gln	Glu	Asp
Bovine liver	Phe	Glu	Asn	Lys	Val	Pro	Glu	Lys	Gln	Lys	Leu	Phe	Gln	Glu	Asp

	16				20				25				30	
Bovine heart	Asn	Gly	Leu	Pro	Val	His	Leu	Lys	Gly	Gly	Ala	Thr	Asp	Asn	Ile
Human muscle (1)	Asn	Asp	Ile	Pro	Leu	Tyr	Leu	Lys	Gly	Gly	Ile	Val	Asp	Asn	Ile
Human muscle (2)	Asp	Glu	Ile	Pro	Leu	Tyr	Leu	...							
Human endothelial	Asp	Glu	Ile	Pro	Leu	Tyr	Leu	Lys	Gly	Gly	Val	Ala	Asp	Ala	Leu
Bovine liver	Asn	Gly	Ile	Pro	Val	His	Leu	Lys	Gly	Gly	Ile	Ala	Asp	Ala	Arg

	31				35				40				45	
Bovine heart	Leu	Tyr	Arg	Val	Thr	Met	Thr	Leu	Cys	Leu	Gly	Gly	Thr	Leu	Tyr
Human muscle (1)	Leu	Tyr	Arg	Val	Thr	Met	Thr	Leu	...						
Human muscle (2)	...														
Human endothelial	Leu	Tyr	Arg	Ala	Thr	Met	Ile	Leu	Thr	Val	Gly	Gly	Thr	Ala	Tyr
Bovine liver	Leu	Tyr	Arg	Ala	Thr	Leu	Ile	Leu	Thr	Val	Gly	Gly	Thr	Ala	Tyr

	46				50				55				60	
Bovine heart	Ser	Leu	Tyr	Cys	Leu	Gly	His	Ala	Ser	Lys	Lys			
Human muscle (1)	...													
Human muscle (2)	...													
Human endothelial	Ala	Ile	Tyr	Glu	Leu	Ala	Val	Ala	Ser	Phe	Pro	Lys	Lys	Gln	Glu
Bovine liver	Ala	Met	Tyr	Glu	Leu	Ala	Val	Ala	Ser	Phe	Pro	Lys	Lys	Gln	Asp

The sequences given for bovine liver and human endothelial cell subunit VIIa were derived from the cDNA sequence. Part of the sequences of the human muscle subunits VIIa (1) and VIIa (2) that have not yet been determined are indicated by dotted lines. Differences in sequence of subunit VIIa (1) and subunit VIIa (2) of human muscle cytochrome *c* oxidase have been boxed

quence of bovine heart [21], bovine liver [22] and human endothelial subunit VIIa [16]. The sequences given for bovine liver and human endothelial subunit VIIa were derived from the cDNA sequence. Amino acid sequence analysis allowed unambiguous identification of 38 and 22 residues, respectively, of the N-terminal amino acid sequences of subunits VIIa (1) and VIIa (2). The first 22 residues of the N-terminal amino acid sequence of subunits VIIa (1) and VIIa (2) found in human muscle cytochrome *c* oxidase showed 5 differences. Of the first 38 residues of the human skeletal muscle subunit VIIa (1), 30 residues proved to be identical to those of bovine heart, and 26 identical to those of bovine liver. On the other hand, of the first 22 residues of subunit VIIa (2), 14 residues were identical to those of bovine heart and 17 identical to those of bovine liver. Thus, the N-terminal sequence of the human muscle subunit VIIa (1) is more closely related to that of bovine heart than to its bovine liver counterpart, while that of the human muscle subunit VIIa (2) is more closely related to the sequence of bovine liver. This indicates that both a 'bovine heart-type' subunit VIIa (1) and a 'bovine liver-type' subunit VIIa (2) are present in human skeletal muscle.

Furthermore, the N-terminal sequence of the 'bovine liver-type' subunit VIIa (2) proved to be completely identical to the amino acid sequence deduced from the base sequence of a cDNA specifying subunit VIIa of human endothelial cells [16]. An important observation is that the concentration of the 'bovine liver-type' subunit VIIa (2) in our human skeletal muscle oxidase preparation was only about 10% of that of the 'bovine heart-type' subunit VIIa (1), as judged from the sequence yields.

4. DISCUSSION

Our results provide the first direct evidence at the level of gene-products for the occurrence of isoforms of human cytochrome *c* oxidase. It is as yet not clear whether these two isoforms occur in all skeletal muscle cells or fibers, or are restricted to certain cells or fibers. Since only a minor amount of subunit VIIa (2) was present, it cannot even be excluded that this 'bovine liver-type' subunit was derived from endothelial cells lining the blood vessels in skeletal muscle. Nonetheless, our results may have several implications in view of the molecular background related to the occurrence of mitochondrial myopathies in man. First, a mutation in a nuclear gene encoding an isoform of a subunit of cytochrome *c* oxidase expressed in skeletal muscle but not in other tissues or organs such as heart could provide a ready explanation for a tissue-specific deficiency of the enzyme. Of course, this need not always be the case: in many patients defects in the respiratory chain are accompanied by deletions in mitochondrial DNA [23–26] and the variability in the expression of the deficiency in different tissues has been attributed to heteroplasmy [24].

Second, since we have now shown that two isoforms of cytochrome *c* oxidase occur in skeletal muscle, this could provide an explanation for the fact that in certain mitochondrial myopathies there is only a partial deficiency of cytochrome *c* oxidase.

Third, there are several case reports of infants with a reversible form of infantile mitochondrial myopathy with cytochrome *c* oxidase deficiency [27–29]. A mutation affecting a fetal or neonatal isoenzyme form of a subunit of cytochrome *c* oxidase, but not the adult isoform, could explain the transient expression of cytochrome *c* oxidase deficiency in the neonatal period [8].

Our results offer also for the first time evidence at the protein level for the occurrence of two isoforms of a subunit of cytochrome *c* oxidase purified from one tissue. At the cDNA level two isoforms of subunit VIa have been identified in a rat-heart cDNA library [5]. Our findings might also imply that the human oxidase does not show the type of tissue specificity that has been reported for the oxidase in other mammals [3]. For instance, in bovine cytochrome *c* oxidase no evidence has been presented up to now for the occurrence of two different isoforms of subunit VIIa in one tissue. However, it cannot yet be excluded that bovine heart cytochrome *c* oxidase contains an additional minor amount of a 'bovine liver-type' subunit VIIa besides the published 'bovine heart-type' subunit VIIa. In that study [21], subunit VIIa of bovine heart cytochrome *c* oxidase was purified by gel filtration and the presence of a small amount of a second type VIIa polypeptide might well have been overlooked.

Reasoning along these lines, it will be most relevant to see whether cytochrome *c* oxidase purified from human heart also contains two different isoforms of subunit VIIa. Investigation of the amino acid sequences of several subunits of cytochrome *c* oxidase from human heart and skeletal muscle is in progress in our laboratories. This will yield more information about the tissue specificity of the human cytochrome *c* oxidase isoenzymes, and about the function of the different isoenzymes [30].

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