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Human heart cytochrome *c* oxidase subunit VIII

Purification and determination of the complete amino acid sequence

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Subunit VIII was purified from a preparation of the human heart cytochrome *c* oxidase and its complete amino acid sequence was determined. The sequence proved to be much more related to that of the bovine liver oxidase subunit VIII than to that found in bovine heart. Our finding of a 'liver-type' subunit VIII in the human heart enzyme suggests that either there are no isoforms of human subunit VIII or the human oxidase does not show the type of tissue specificity that has been reported for the oxidase in other mammals.

Cytochrome oxidase; Subunit VIII; Amino acid sequence; (Human)

1. INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is located in the mitochondrial inner membrane, where it catalyses the oxidation of ferrocytochrome *c* by molecular oxygen and contributes to the establishment of a proton gradient across the membrane. The subunit composition depends on the source of the enzyme: 3 dissimilar subunits have been identified in the oxidase from *Paracoccus denitrificans* [1], at least 9 in that from *Saccharomyces cerevisiae* [2], and 13 in mammalian cytochrome *c* oxidase [3].

In eukaryotes the three largest subunits, I–III, are encoded by the mitochondrial genome [4]. Subunits I and II contain the haem *a* groups and copper atoms [5–7], subunit II contains the cytochrome *c*-binding site [8,9], and subunit III is probably involved in the proton-translocating function of the enzyme [10,11]. The question of the function of the 10 smaller, nuclear encoded subunits of the mammalian oxidase (IV, Va,b, VIa–c, VIIa–c, VIII) remains unsettled.

Kadenbach and co-workers [12] compared the enzyme purified from several tissues of the same mammalian species and reported tissue-specific immunological and electrophoretic differences in some of the corresponding subunits. The presence of cytochrome *c* oxidase isoenzymes was later confirmed by extensive N-terminal amino acid sequence data [13,14]. However, no sound evidence has been presented for the existence of isoenzyme forms for the human cytochrome *c* oxidase. Recently, many cases of cytochrome *c* oxidase deficiency have been reported [15–17]. The patients usually showed decreased cytochrome *c* oxidase activity in skeletal muscle but normal activity in some other tissues investigated. The existence of tissue-specific oxidase isoforms would provide an explanation for these observations, although other explanations are possible.

Despite its small size of about 45 residues,

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Abbreviations: TEA, triethylamine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography

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subunit VIII seems to be quite variable: the homology between bovine heart and liver oxidase subunits VIII is weak [13,14]. Therefore, subunit VIII was an obvious starting point for our investigation of the possible existence of human cytochrome *c* oxidase isoenzyme forms. Surprisingly, our study revealed that the amino acid sequence of oxidase subunit VIII from human heart is closely related to that from bovine liver and strikingly different from that of bovine heart.

2. MATERIALS AND METHODS

2.1. *Isolation procedure for cytochrome* c *oxidase*

Human hearts were obtained at obduction. Cytochrome *c* oxidase, from hearts that had become available within 24 h post-mortem, was purified essentially according to the method of Fowler et al. [18] modified in our laboratory [19] in the following ways: after decreasing the concentrations of deoxycholate and KCl by dialysis, pure cytochrome *c* oxidase was precipitated between 13 and 20% ammonium sulphate and the final ammonium sulphate fractionation in the presence of 3% cholate was omitted. For determination of the concentration of cytochrome *c* oxidase an absorption coefficient (reduced minus oxidised) of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm was used [20].

Bovine heart cytochrome *c* oxidase was isolated as described [18,19].

2.2. *Fractionation of the subunits of cytochrome* c *oxidase*

The nuclear encoded subunits were separated from the mitochondrially encoded subunits using the method of Power et al. [21] with the following modifications: supernatant S1 was prepared by incubating the enzyme with 42% acetonitrile for 1 h on ice, and the pellet was extracted twice more with 42% acetonitrile. All supernatants were pooled and designated S1. The final pellet (P1) was resuspended in 1.25% triethylamine (TEA) and 1.25% trifluoroacetic acid (TFA). 1 vol. 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 twice more. The final extraction was carried out overnight. All three supernatants were pooled and designated supernatant S2. Both supernatants S1 and S2 and the final pellet P2 were lyophilized and saved for further analysis.

2.3. *Purification of subunit VIII with reversed-phase HPLC*

Subunit VIII was isolated from supernatant S1. An aliquot of supernatant S1 was treated with acetone to remove most of the cholate. After centrifugation, the pellet was dissolved in 6 M guanidine HCl/0.1% TFA and incubated for 30 min 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 pore size, 7 μm spherical, 220 × 4.6 mm) and a guard column (type RP-18 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 in acetonitrile (solvent B).

2.4. *Polyacrylamide gel electrophoresis*

SDS-urea gel electrophoresis was performed according to Kadenbach et al. [3]. Gels (16 × 18 × 0.15 cm) were prepared 16 h before use. The samples were incubated for 20 h at room temperature in sample buffer containing 0.4% β-mercaptoethanol and 8% SDS. We added up to 0.8% β-mercaptoethanol 15 min prior to the start of the gel electrophoresis run. Electrophoresis was carried out first for 1.5 h at 16 mA and subsequently for 7–8 h at 26 mA. Gels were stained with Coomassie brilliant blue. Silver staining was performed as in [22].

2.5. *Determination of the amino acid sequence of subunit VIII*

Automatic sequence analysis was carried out on a 477A pulsed-liquid sequencer (Applied Biosystems, USA) with on-line analysis of the phenylthiohydantoin amino acids on a 120A PTH analyser (Applied Biosystems). The subunit VIII preparation was loaded on a polybrene-containing GFC filter, washed by carrying out two Edman cycles. In order to obtain the C-terminal peptide, digestion of the remainder of the material with *Staphylococcus aureus* V8 protease (Miles, England) was carried out in 0.1 M ammonium acetate (pH 4.0) for 12 h at 37°C at an enzyme/substrate ratio of 1:40. The peptides were separated by reversed-phase HPLC on a Vydac C_4 column 214 TP 54 (4.6 × 250 mm), using the following DuPont equipment: an 870 three-piston chromatographic pump, an 8800 gradient controller and a UV spectrophotometer set at 220 nm.

3. RESULTS

In contrast to the bovine oxidase, sufficiently pure human cytochrome *c* oxidase precipitated between 13 and 20% ammonium sulphate saturation. Therefore, the final fractionation with ammonium sulphate in the presence of cholate was omitted. The purity of the isolated enzyme preparation was confirmed by its subunit pattern on SDS-polyacrylamide gel electrophoresis (fig.1). The bands were numbered according to Kadenbach et al. [3] using the bovine heart cytochrome *c* oxidase as a reference. The left-hand panel of fig.1 (Coomassie staining) shows the subunit patterns of bovine heart and human heart cytochrome *c* oxidase. In order to visualise subunit VIII, which stains weakly with Coomassie blue, an additional silver staining of the gel was performed (right-hand panel of fig.1). A comparison of the electrophoretic mobility of subunit VIII of bovine and of human heart cytochrome *c* oxidase showed that the human subunit VIII clearly had a higher mobility than its bovine counterpart. This is in

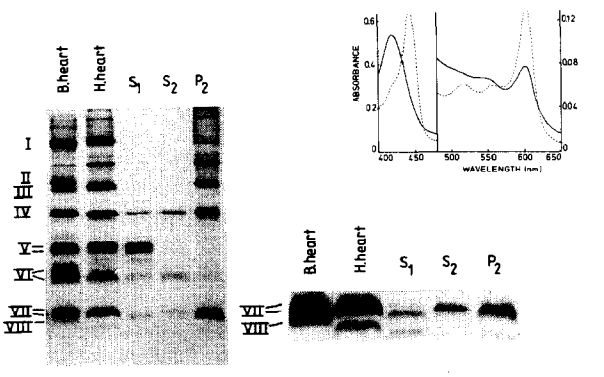

Fig.1. Subunit composition of human heart cytochrome *c* oxidase and of supernatants S_1 and S_2 and pellet P_2. Bands numbered according to Kadenbach et al. [30]. Bovine heart cytochrome *c* oxidase was used as a reference. Left-hand panel: Coomassie staining; right-hand panel: silver staining. The inset shows the absorbance spectra of oxidised (——) and dithionite-reduced (– – –) human heart cytochrome *c* oxidase (2.3 μM) in 50 mM Tris-sulphate (pH 8.0).

agreement with earlier data of Sinjorgo et al. [23].

The absorbance spectrum of human heart cytochrome *c* oxidase is shown in the inset to fig.1. The Soret peak of the oxidised enzyme was at 419 nm, in the reduced form the Soret peak and the α-band being at 443 and 604 nm, respectively. The absorbance ratios $A_{443\,nm}(red)/A_{419\,nm}(ox)$ = 1.2 and $A_{443\,nm}(red)/A_{419\,nm}(red)$ = 2.2 demonstrate that the preparation was fully reducible. The data are in good agreement with the values reported for the small-scale HPLC isolation method of human cytochrome *c* oxidase described by Sinjorgo et al. [23].

In order to purify subunit VIII of human heart cytochrome *c* oxidase with reversed-phase HPLC, we separated the nuclear encoded subunits from those encoded mitochondrially by first incubating the holoenzyme with acetonitrile at neutral pH, followed by extraction with an acetonitrile/1-propanol mixture at acidic pH (see section 2). The three large hydrophobic subunits remained in the pellet, eliminating the risk of irreversible binding to the stationary phase during reversed-phase column chromatography.

The subunit composition, determined by SDS gel electrophoresis, of supernatants S_1 and S_2 and pellet P_2 is shown in fig.1. The silver staining of the gel (right-hand panel of fig.1) showed that almost all of subunit VIII could be extracted via this method. Subunit VIII was predominantly present in supernatant S_1, although a small part appeared in S_2 and in the final pellet P_2.

Human heart subunit VIII was further purified from supernatant S_1 with reversed-phase HPLC. Part of the elution profile of supernatant S_1 is shown in fig.2. Subunit VIII eluted as a small peak at approx. 56% solvent B. The purity of the polypeptide was tested by SDS gel electrophoresis. The inset to fig.2 shows that isolated subunit VIII was not contaminated with other polypeptides.

In table 1 the amino acid sequence of subunit VIII from human heart oxidase is presented, together with that from bovine heart [24] and the major part of the sequence from bovine liver [14] and rat liver subunit VIII [31]. The automatic sequence analysis of approx. 1.5 nm of the human polypeptide allowed unambiguous identification up to residue His-36. From cycle 37 onwards, a substantial carry-over phenomenon occurred. It was established that the polypeptide consisted of 44 residues, but the nature of residues 41 and 44 was not clear. The exact sequence of the C-terminal part of the protein was obtained from sequence analysis of a peptide that eluted at 5.6% acetonitrile during HPLC separation of an *S. aureus* protease V8 digest of the protein. The yield of the phenylthiohydantoin amino acids in each cycle was (in pmol): Thr-1, 105.6; Tyr-2, 117.2; Arg-3, 21.6; Arg-4, 19.0; Pro-5, 108.8; Glu-6, 19.1. Subsequent cycles gave no further residues. This confirmed and established the sequence of the terminal residues 38–44. Obviously, subunit VIII

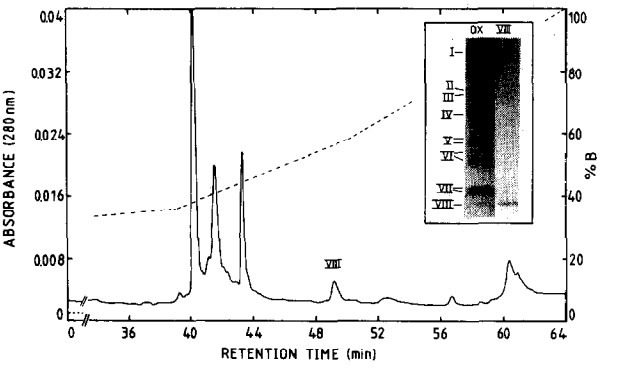

Fig.2. Part of the HPLC-elution profile of supernatant S_1. The inset shows the analysis by gel electrophoresis of the human heart subunit VIII purified with reversed-phase HPLC (OX, total human heart oxidase).

Table 1

Primary structure of human heart cytochrome *c* oxidase subunit VIII

	1	5	10	15
Human heart	Ile-His-Ser-Leu-Pro-Pro-Glu-Gly-Lys-Leu-Gly-Ile-Met-Glu-Leu-			
Bovine liver	Ile-His-Ser-Lys-Pro-Pro-Arg-Glu-Gln-Leu-Gly-Thr-Met-Glu-Ile-			
Bovine heart	Ile-Thr-Ala-Lys-Pro-Ala-Lys-Thr-Pro-Thr-Ser-Pro-Lys-Glu-Gln-			
Rat liver	.....-Gly-Gly-Val-Leu-Asp-Ile-			
	16	20	25	30
Human heart	Ala-Val-Gly-Leu-Thr-Ser-Cys-Phe-Val-Thr-Phe-Leu-Leu-Pro-Ala-			
Bovine liver	Ala-Ile-Gly-Leu-Thr-Ser-Cys-Phe-Leu-Asp-.....			
Bovine heart	Ala-Ile-Gly-Leu-Ser-Val-Thr-Phe-Leu-Ser-Phe-Leu-Leu-Pro-Ala-			
Rat liver	Thr-Ile-Gly-Leu-Thr-Ser-Cys-Phe-Val-Cys-Cys-Leu-Leu-Pro-Ala-			
	31	35	40	45
Human heart	Gly-Trp-Ile-Leu-Ser-His-Leu-Glu-Thr-Tyr-Arg-Arg-Pro-Glu			
Bovine liver	.....-Met-Glu-Asn-Tyr-Lys-Lys-Arg-Glu			
Bovine heart	Gly-Trp-Val-Leu-Tyr-His-Leu-Asp-Asn-Tyr-Lys-Lys-Ser-Ser-Ala-Ala			
Rat liver	Gly-Trp-Val-Leu-Ser-His-Leu-Glu-Ser-Tyr-Lys-Lys-Arg-Glu			

Comparison of the complete amino acid sequence of subunit VIII of human heart cytochrome *c* oxidase as determined by us, with that of subunit VIII of bovine heart [24] and with the partial sequence of bovine liver subunit VIII [14]. The partial sequence given for rat liver oxidase subunit VIII was derived from cDNA [31]. Parts of sequences that have not yet been determined are indicated by the dotted lines

of human heart oxidase is two residues shorter than that of bovine heart and has the same size as subunit VIII of bovine liver.

4. DISCUSSION

In this paper we present the complete amino acid sequence of human heart cytochrome *c* oxidase subunit VIII that had been purified from the enzyme by solvent extractions and reversed-phase HPLC. To our knowledge this is the first amino acid sequence of a subunit of human cytochrome *c* oxidase that has been directly determined by protein sequencing. From a human liver cDNA bank the group of Schon and DiMauro recently obtained the nucleotide sequence and the corresponding amino acid sequence of the liver form of human oxidase subunit VIII (Schon, E.A., personal communication). The sequence is completely identical to that determined by us for the heart protein. This is a very unexpected finding because subunit VIII is one of the most variable polypeptides in other mammals [25–27]. This is illustrated by the fact that for the first 20 amino-terminal residues, subunits VIII from bovine heart and bovine liver have only 8 residues in common (table 1).

Jarausch and Kadenbach [28] proposed that for the tissue-dependent subunits VIa, VIIa and VIII, tissue specificity overrides species specificity. Reasoning along these lines, one would have expected that the sequence of the human heart subunit VIII should show more homology to that of its bovine heart counterpart than to subunit VIII from human liver cytochrome *c* oxidase. From the sequence for human heart subunit VIII we found that for the first 20 amino-terminal residues, there were only 6 residues identical to those of bovine heart, but 13 residues of the human heart subunit were identical to those of bovine liver subunit VIII. Thus, the sequence of human heart subunit VIII is much more related to that of bovine liver than to its bovine heart counterpart. This indicates that in the human cytochrome *c* oxidase a 'liver form' of subunit VIII is present not only in liver (as deduced from the liver cDNA sequence) but also in the heart. A comparison of the human heart sequence with that derived from cDNA for rat liver [31] also supports the notion that a 'liver type' subunit VIII is present in human heart oxidase. A conspicuous difference between the human heart subunit VIII and the three other subunits VIII is found near the carboxy-terminus, where at positions 41–42 the human subunit VIII has two arginine residues, while all other subunits VIII have two lysines (see table 1).

Up to now, our results with subunit VIII of human heart cytochrome *c* oxidase have not supported the idea that isoenzyme forms of cytochrome *c* oxidase occur in man, in contrast to the situation in other mammals. However, the fact that the human heart subunit VIII sequence was identical to that deduced from human liver cDNA does not prove that there are no isoenzyme forms of this subunit present in man. If two isoforms of a subunit are strongly dissimilar, a DNA probe based on the sequence of one of the isoforms may fail to detect the presence of the other. Secondly, we have shown the presence of a liver type subunit VIII in human heart, but it has not been proved that a second, 'heart type', subunit VIII is absent in our enzyme preparation. It might be relevant that a second fraction of subunit VIII was extracted with a less polar organic solvent mixture (S_2) from the holoenzyme and that part of subunit VIII was still present in pellet P_2 (see fig.1). This issue will be investigated further.

The occurrence of tissue-specific cytochrome *c* oxidase deficiencies in man has stimulated research on the possible existence of tissue-specific isoforms of the oxidase. These could either arise from separate multiple genes for one or more of the subunits or by alternative splicing of a primary transcript. Even the presence of two different genes coding for the 'same' subunit would not necessarily lead to the occurrence of isoenzymes, if the differences reside only in the presequences. This has been found to be the case for the ATP synthase proteolipid [29]. Such a difference will only be detectable by nucleotide sequencing of the gene. Of course, tissue-specific oxidase deficiency could also be caused indirectly by a defect in tissue-specific proteins or other gene products involved in subunit biosynthesis or import, or by a defect in the assembly of the oxidase holoenzyme from its polypeptides and prosthetic groups.

Most relevant with respect to cytochrome *c* oxidase deficiencies is the oxidase that is present in skeletal muscle. A disease often described in this connection is the form of fatal infantile myopathy in which the patient lacks a functional cytochrome *c* oxidase in skeletal muscle while normal levels of the enzyme are found in other tissues [13,15–17]. Investigations of the amino acid sequences of several subunits of cytochrome *c* oxidase from human heart and skeletal muscle are in progress in our laboratories. This will yield more information about the possible existence of human cytochrome *c* oxidase isoenzymes.

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