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Human cytochrome *c* oxidase isoenzymes from heart and skeletal muscle; purification and properties

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(1) Human cytochrome *c* oxidase was isolated in an active form from heart and from skeletal muscle by a fast, small-scale isolation method. The procedure involves differential solubilisation of the oxidase from mitochondrial fragments by laurylmaltoside and KCl, followed by size-exclusion high-performance liquid chromatography. (2) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate showed differences between the subunit VI region of cytochrome *c* oxidases from human heart and skeletal muscle, suggesting different isoenzyme forms in the two organs. This finding might be of importance in explaining mitochondrial myopathy which shows a deficiency of cytochrome *c* oxidase in skeletal muscle only. (3) In SDS polyacrylamide gel electrophoresis most human cytochrome *c* oxidase subunits migrated differently from their bovine counterparts. However, the position of subunits III and IV was the same in the human and in the bovine enzymes. The much higher mobility of human cytochrome *c* oxidase subunit II is explained by a greater hydrophobicity of this polypeptide than of that of the subunit II of the bovine enzyme.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is a large membrane protein that catalyses the oxidation of ferrocytochrome *c* by molecular oxygen, thus forming the terminal step in mitochondrial respiration.

Since the oxidase is a multi-subunit enzyme, the origin, assembly and function of the different polypeptides has been the subject of many investigations. The enzyme isolated from prokaryotes was found to contain two to three different polypeptide chains [1–5]. In the oxidase of *Neurospora crassa* and of yeast, eight and nine different subunits, respectively, have been identified [6,7], and mammalian cytochrome *c* oxidase has been resolved into 13 different polypeptides on SDS-polyacrylamide gel electrophoresis [8].

In eukaryotes, the three largest subunits are coded for by the mitochondrial genome [9]. The primary functions of the oxidase have been assigned to these polypeptides. Subunits I and II are considered to contain all four prosthetic groups [10–13]. The cytochrome *c* binding domain has been located on subunit II [14–17], and subunit III is supposed to be involved in the proton-translocating function of the oxidase [18,19].

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The other subunits are synthesized in the cytoplasm and transported into the mitochondrion. In studies of mammalian cytochrome *c* oxidase, Kadenbach and coworkers [20] demonstrated that tissue-specific immunological and electrophoretic differences can be observed in these subunits. As a result of this discovery of isoenzyme forms of cytochrome *c* oxidase, they proposed that the nuclearly encoded subunits function as organ-specific modulators of enzyme activity [21].

A species of mammalian cytochrome *c* oxidase that has as yet hardly been studied is that of human origin. This gap in cytochrome *c* oxidase research is mainly caused by the fact that up to now it has not been possible to isolate a native cytochrome *c* oxidase preparation from human material. The enzyme was found to lose activity upon purification [22,23].

Recently, the necessity of studies of the human oxidase was stressed by medical reports on organ-specific deficiencies of the enzyme. A disease often described in this connection is fatal infantile myopathy, in which patients lack a functional cytochrome *c* oxidase in skeletal muscle, while normal levels of the enzyme are observed in other organs [24–26]. A deficiency of one or more of the isoenzyme forms of cytochrome *c* oxidase suggests a defect in an organ-specific, nuclearly encoded subunit.

In order to be able to study cytochrome *c* oxidase from human material, little of which is generally available, we developed a fast, small-scale isolation procedure of the enzyme. As described in this paper, with this method cytochrome *c* oxidase activity was fully retained in the purified preparation.

The differences in subunit pattern between bovine heart cytochrome *c* oxidase and that of human heart and skeletal muscle isoenzymes were studied by gel electrophoresis.

Materials and Methods

Cytochrome c oxidase isolation procedure. Human hearts and skeletal muscle (quadriceps) were obtained at obduction. Cytochrome *c* oxidase was purified from different hearts that had all become available within 24 h post mortem. The sample of skeletal muscle used, was obtained 12 h after death.

Cytochrome *c*-depleted Keilin–Hartree submitochondrial particles were prepared from human heart and human skeletal muscle by the method of King [27], adapted as described before [28]. The particles were suspended to a protein concentration of 30 $mg \cdot ml^{-1}$ in 0.66 M sucrose/50 mM Tris-sulphate (pH 8.0). Cytochrome *c* oxidase was selectively solubilised from these particles with laurylmaltoside plus 1 M KCl. Complex III was extracted at approx. 2% of the detergent. After centrifugation (100000 × *g* for 15 min), cytochrome *c* oxidase was solubilised from the pellet with approx. 3% laurylmaltoside. The precise detergent concentrations required in each particular case were determined in pilot experiments. The solubilised cytochrome *c* oxidase was concentrated to 30 mg protein $\cdot ml^{-1}$ on an Amicon PM-30 filter and stored in liquid nitrogen.

Finally, cytochrome *c* oxidase was purified in one HPLC size-exclusion chromatography step. The HPLC system developed in our laboratory [29] was used, and 100-µl samples of the crude enzyme were applied to a Dupont GF-250 column. Elutions were performed at 0.5 ml/min in 50 mM Tris-acetate, in 1 mM EDTA/100 mM Na_2SO_4/0.1% laurylmaltoside (pH 7.5, 20°C). Protein and haem were detected at 280 nm and 405 nm, respectively. The fractions containing cytochrome *c* oxidase (always the main peak in the elution profile) from 3 to 6 runs were pooled and stored in liquid nitrogen.

Large-scale isolations of bovine and human heart cytochrome *c* oxidase were performed according to the method of Fowler [30], as modified in our laboratory [31]. For bovine heart this procedure led to an active and pure preparation; when applied to human heart, the final oxidase preparation was pure but inactive.

Spectrophotometric measurement of cytochrome c oxidase activity. Human heart cytochrome *c* was prepared by the method of Margoliash and Walasek [32]. Ferrocytochrome *c* was obtained by incubating cytochrome *c* with ascorbate, followed by gel filtration on Sephadex G-50 superfine (Pharmacia) in 25 mM Tris-Mops (pH 7.8)/1 mM EDTA.

The rate of oxidation of ferrocytochrome *c* was measured in 25 mM Tris-Mops (pH 7.8)/1 mM EDTA/0.1% laurylmaltoside (25°C) with a spec-

trophotometer built in our laboratory using the optics of a Cary-14 spectrophotometer, or with a Durrum stopped-flow apparatus, as described before [33]. K_m and TN_{max} values of high-affinity and low-affinity reactions were determined by computer analysis of the data [28].

The absorbance coefficients (reduced-minus-oxidised) used for cytochrome aa_3 and cytochrome c were 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm and 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm, respectively [34,35].

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to the method of Kadenbach et al. [8]. The gels, which contained 4 M urea, were prepared 16 h before use. The samples were incubated for 20 h at room temperature in a sample buffer containing 0.4% β-mercaptoethanol and 3% SDS. We added up to 0.8% β-mercaptoethanol 15 min prior to the start of the gel electrophoretic run. Electrophoresis was run for 1–2 h at 80 V and for 28–30 h at 210 V. Gels were stained with Coomassie brilliant blue followed by silver staining as in Ref. 8.

Gradient gel electrophoresis was performed using a running gel from 12–20% acrylamide and 0.39–0.65% *N,N'*-methylenebisacrylamide. The stacking gel contained 4% acrylamide and 0.13% *N,N'*-methylenebisacrylamide.

Separation of polypeptides by gel permeation chromatography. Cytochrome *c* oxidase (300 nmol) was incubated for 4 h at 20°C with a 10-fold excess of SDS in 50 mM sodium phosphate at pH 6.5, and 1 mM EDTA in a total volume of 4 ml. Chromatography was performed on AcA 54 (LKB) in a column of 2.5 × 150 cm (Glenco, glass-Teflon system 3500) with 50 mM Tris-sulphate, 1 mM EDTA, 3% SDS (pH 8.0). Migration velocity was 2.5 $cm \cdot h^{-1}$. Column eluates were monitored at 280 nm with a Zeiss PMQ-II spectrophotometer.

Results

The limited availability of human material prompted us to develop a method suitable for small-scale purification of cytochrome *c* oxidase. The observation of Hare et al. [22] that the use of bile acids as detergents leads to irreversible inactivation of the human oxidase, was confirmed in our laboratory. Therefore, we used the mild detergent laurylmaltoside to solubilise the enzyme. To minimise the risk of denaturation, the crude enzyme preparation was purified in a single gel permeation HPLC step lasting less than 30 min, as described in the Materials and Methods section. Fig. 1A shows the elution profile of the purification of crude human heart cytochrome *c* oxidase on a Dupont GF-250 column as described in Materials and Methods. The cytochrome *c* oxidase purified in this way elutes as a sharp peak on the same column (Fig. 1B). The combined yield of six HPLC runs was generally about 20 nmol purified cytochrome *c* oxidase.

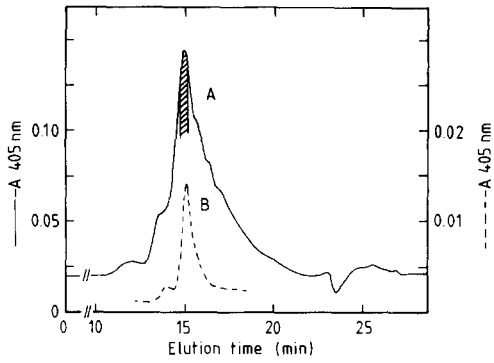

Fig. 1. Elution profile of human heart cytochrome *c* in 50 mM Tris-acetate, 1 mM EDTA, 100 mM Na_2SO_4, 0.1% laurylmaltoside on a Dupont GF-250 column. (A) The crude enzyme; (B) rechromatography of a sample of the purified oxidase (the hatched fraction in (A)).

Fig. 2 shows the absorbance spectra of human heart cytochrome *c* oxidase isolated in this way. The Soret peak of the oxidised enzyme lay at 419 nm, in the reduced form the Soret peak and α band were at 443 and 604 nm, respectively. The absorbance ratios ($A_{443\ nm}$(red)/$A_{419\ nm}$(ox) = 1.3

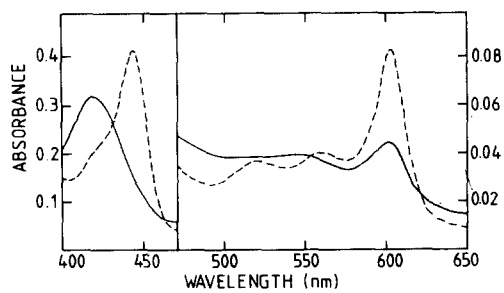

Fig. 2. Absorbance spectra of oxidised (———) and dithionite-reduced (— — —) human heart cytochrome *c* oxidase (1.6 μM) in 50 mM Tris-acetate (pH 7.5)/1 mM EDTA/100 mM Na_2SO_4/0.1% laurylmaltoside.

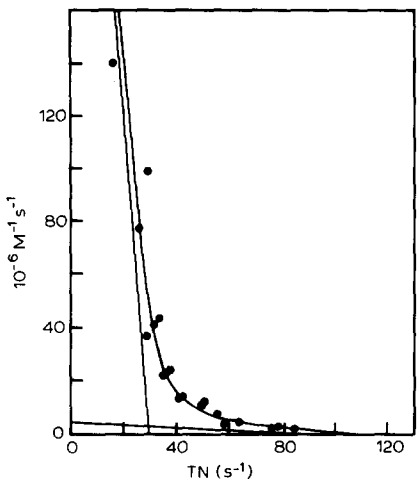

Fig. 3. Eadie–Hofstee plot of steady-state oxidation of human heart ferrocytochrome *c* (0.085–63 μM) by purified human heart cytochrome *c* oxidase, measured spectrophotometrically in 25 mM Tris-Mops/1 mM EDTA/0.1% laurylmaltoside (pH 7.8). The curve was simulated by computer as described in Materials and Methods.

and $A_{443\ nm}(red)/A_{419\ nm}(red) = 2.2$ demonstrate that the preparation is fully reducible [36].

Steady-state activity of purified human heart cytochrome c oxidase

Spectrophotometric measurement of the steady-state activity of human heart cytochrome *c* oxidase with human cytochrome *c* was carried out in a medium of low ionic strength ($I = 25$ mM). The Eadie–Hofstee plot depicted in Fig. 3 shows that biphasic kinetics were observed, well-known for the bovine enzyme under the same conditions [37,38]. Fitting the data with the sum of two first-degree functions [28] resulted in a high-affinity reaction with $K_m = 5 \cdot 10^{-8}$ M and $TN_{max} = 30\ s^{-1}$ and a low-affinity reaction with $K_m = 2 \cdot 10^{-6}$ M and $TN_{max} = 80\ s^{-1}$.

The maximal turnover numbers are comparable to those found for bovine cytochrome *c* oxidase under similar conditions [28]. This result demonstrates, just as the absorbance spectra did, that with the method described we isolated a native cytochrome *c* oxidase from human heart.

Subunit composition of human heart and skeletal muscle isoenzymes

As a first step in our search for isoenzymes of human cytochrome *c* oxidase we purified the

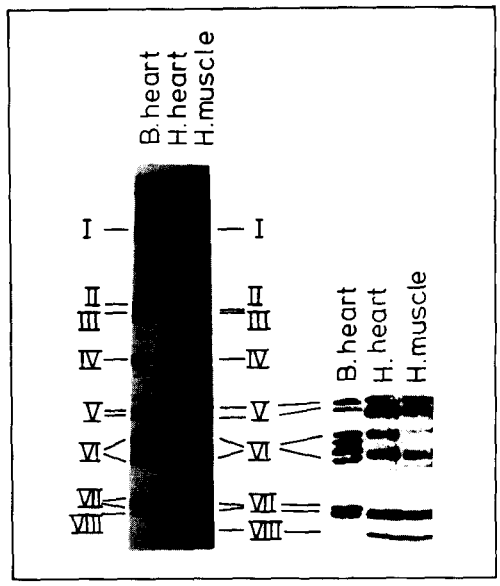

Fig. 4. Comparison of the subunit composition of bovine heart, human heart and human skeletal muscle cytochrome *c* oxidase. Gel electrophoresis was performed according to the method of Kadenbach et al. [8]. Left-hand part: Coomassie staining; right-hand part: silver staining.

oxidase from human skeletal muscle. The subunits of the enzyme were compared with those of human heart cytochrome *c* oxidase on an SDS polyacrylamide gel (Fig. 4). Bovine heart cytochrome *c* oxidase purified by using the Fowler method was used as a reference. The bands were numbered according to the system of Kadenbach [39].

The left-hand panel of Fig. 4 (Coomassie staining) shows that the resolution of the gel was adequate to separate all bovine subunits. It is remarkable that for this cytochrome *c* oxidase we observed four bands in the subunit VI region instead of three as has been published by Jarausch and Kadenbach [40]. An extra subunit VI has also been reported for pig liver cytochrome *c* oxidase [41]. All our bovine heart preparations showed the four subunits VI and none of them comigrated with one of the bands of the cytochrome bc_1 complex (not shown). Thus, we conclude that the additional band we observed in the subunit VI region may represent a polypeptide of cytochrome *c* oxidase which is only visible at high gel electrophoretic resolution.

A comparison of bovine and human heart cytochrome *c* oxidase shows differences in mobility

for most corresponding subunits. The human subunits II, V_b and VIII clearly had a higher mobility, and subunit V_a had a lower mobility than their probable bovine counterparts. The position of the human subunit I (in this gel just above bovine I) was sometimes found to be equal to that of bovine I and is still under investigation. The lack of complete subunit separation, compared to the separation in the bovine lane, in the human subunits VI and VII regions demonstrates additional differences in these polypeptides, but prevents exact naming of them.

From a comparison of the human heart and skeletal muscle oxidases (lanes 2 and 3) it is clear that the mitochondrially encoded subunits I, II and III had identical electrophoretic mobility, as expected. For a precise study of the differences in the human isoenzymes, silver staining of the gel was performed (right-hand panel of Fig. 4). Here, differences in the subunit VI region can be observed. The upper band in the skeletal muscle preparation showed slightly lower mobility and less staining than the corresponding heart polypeptide. Furthermore, in the human muscle preparation we observed an additional weakly staining band which is absent in the heart enzyme.

The anomalous electrophoretic migration of human oxidase subunit II

The hydrophobic cytochrome *c* oxidase subunits I and III always migrate faster in SDS gel electrophoresis than expected from their known molecular mass, whereas subunit II behaves normally [42]. As a result, on most SDS gels the band of subunit III is situated below the much smaller subunit II.

We observed (Fig. 4) a clear difference in mobility between human and bovine cytochrome *c* oxidase subunit II, even though they have equal molecular weights [43,44]. In order to investigate this phenomenon we isolated a large batch of the human heart enzyme using the method of Fowler et al. [30] and submitted the oxidase to SDS gel permeation chromatography on an LKB AcA 54 column [45]. The elution profile is shown in Fig. 5. In contrast to the gel electrophoretic pattern, the elution profile of the four largest subunits (I–IV) of the human oxidase is similar to that of bovine oxidase [9].

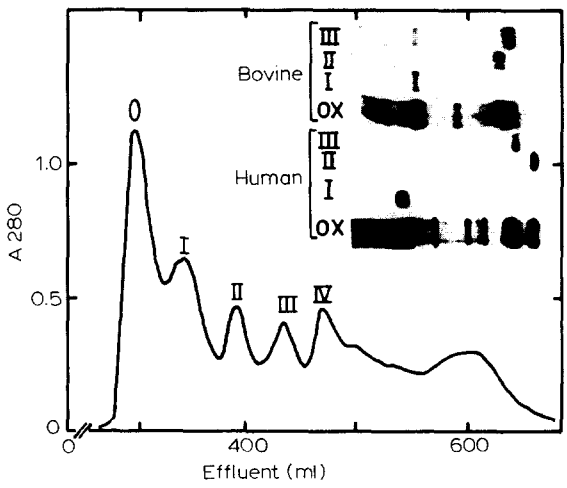

Fig. 5. Separation of the subunits of human cytochrome *c* oxidase on a column of Ultrogel AcA 54 in the presence of SDS. For conditions see methods. The subunits are marked by Roman numerals, peak 0 represents aggregated material. The lower part of the inset shows analysis by gradient gel electrophoresis of the subunit fractions indicated (I–III; OX, total oxidase). The upper part of the inset shows the result of the control experiment with bovine cytochrome *c* oxidase.

The lower part of the insert to Fig. 5 gives the electrophoretic mobility (migration from left to right) of the peak fractions from the gel permeation column. In the gradient gel electrophoretic system used here, the human oxidase subunit II moved even faster than subunit III.

The fact that the subunits II and III of the bovine oxidase showed the usual electrophoretic behaviour (III moving faster than II) was demonstrated in a parallel experiment shown in the upper part of the inset to Fig. 5.

Discussion

In this paper we demonstrated that, using the mild detergent laurylmaltoside and HPLC size-exclusion chromatography, we isolated human cytochrome *c* oxidase in the native state. The enzyme showed normal reducibility and steady-state kinetics. The amount of enzyme that could be obtained in this way is limited due to the HPLC procedure, but one needs only small amounts of the protein to perform steady-state kinetics, gel electrophoresis or spectral studies. This method is useful particularly for comparative studies of human isoenzymes that do not require large amounts of en-

zyme. In our studies we found that human heart cytochrome *c* oxidase showed a subunit pattern on SDS gels that was very different from that of the bovine heart enzyme. Only subunits III and IV showed equal mobility for both oxidase species. In this paper we paid special attention to the electrophoretic behaviour of the human subunit II. We demonstrated that, in contrast to subunit II in bovine and yeast cytochrome *c* oxidase, human subunit II showed the same anomalous electrophoretic behaviour as subunit I and III. A possible explanation for anomalous behaviour on SDS-polyacrylamide gel electrophoresis could be the hydrophobic nature of polypeptides, resulting in increased SDS-binding. To test this hypothesis, we calculated the hydrophobicity index of the three largest subunits of both species from the primary structure inferred from the base sequence of their mtDNA [43,44].

Table I shows that human subunits I and III are of a hydrophobicity comparable to the corresponding bovine polypeptides. The human subunit II, on the other hand, has a much higher content of hydrophobic residues than its bovine counterpart. Therefore, we conclude that the observation that human subunit II has a higher mobility than bovine II, in spite of equal molecular weights, can be explained by the more hydrophobic nature of the human polypeptide.

Apart from the human heart oxidase we also purified the human skeletal muscle isoenzyme. Reports on patients suffering from cytochrome *c* oxidase deficiency in skeletal muscle [24–26] suggest a defect in one or more of the subunits that are specific for this isoenzyme. In order to gain more insight into this class of diseases it is necessary to determine which cytochrome *c* oxidase subunits are different in the human isoenzymes. In this paper we compared cytochrome *c* oxidase from human skeletal muscle to human heart cytochrome *c* oxidase on SDS-polyacrylamide gel electrophoresis and found differences in the subunit VI region consisting of one extra band and of one band showing less silver staining and slightly lower mobility in the muscle preparation. It is possible that the extra polypeptide observed in skeletal muscle cytochrome *c* oxidase is a digestion product of the upper subunit VI band, resulting in low intensity of the two bands. But it is known that cytochrome *c* oxidase subunits which are present in full stoichiometric amounts can show low staining intensities. This is true for both Coomassie staining (subunit III) and silver staining (subunit VIII).

Since the same subunit pattern was observed in all preparations of human skeletal muscle cytochrome *c* oxidase, and since we did not observe digestion of any of the other polypeptides, we attribute the observed differences in subunit pattern to the presence of organ-specific isoenzymes of human cytochrome *c* oxidase.

The fact that all other nuclearly encoded subunits in both isoenzymes show the same mobility during gel electrophoresis does not indicate that they are identical. This is demonstrated by the fact that DiMauro et al. [25] raised a monoclonal antibody against human heart subunit IV that did not cross-react with the corresponding muscle subunit. Therefore, we conclude that there is evidence that the human heart and skeletal muscle isoenzymes differ in at least three of the small subunits. Further investigation of the nature and function of cytochrome *c* oxidase isoenzymes in general, and of human isoenzymes in particular, might reveal information about mitochondrial myopathies.

TABLE I

HYDROPHOBICITY INDEX OF HUMAN AND BOVINE CYTOCHROME *c* OXIDASE SUBUNITS I–III

The hydrophobicity index is the sum of the mol percentages of Asx, Glx, Lys, His, Arg, Ser and Thr. The values were calculated using the mtDNA sequences [43,44].

Subunit	Hydrophobicity index	
	Human	Bovine
I	29.0%	29.6%
II	36.6%	41.4%
III	36.8%	35.6%

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