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Isolation and Characterization of Human Heart Cytochrome *c* Oxidase

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Abstract

Cytochrome *c* oxidase was isolated from human hearts and separated by SDS gel electrophoresis. The identity of polypeptide bands with known subunits was demonstrated by immunoblotting with monospecific antisera to rat liver cytochrome *c* oxidase subunits. The polarographically determined kinetics of cytochrome *c* oxidation were similar to those reported for the bovine heart enzyme.

Key Words: Cytochrome *c* oxidase; human heart; subunit composition; monospecific antisera; immunoblotting.

Introduction

In previous studies human cytochrome *c* oxidase was isolated from heart (Jeffreys and Craig, 1977), placenta (Hare *et al.*, 1980; Kadenbach *et al.*, 1981), and HeLa cells (Hare *et al.*, 1980). The polypeptide composition of the isolated enzyme complex was analyzed by SDS-polyacrylamide gel electrophoresis and lead to the identification of four (Jeffreys and Craig, 1977), seven (Hare *et al.*, 1980), and ten (Kadenbach *et al.*, 1981) different polypeptide bands. The cytochrome *c* oxidase complex from various mammalian tissues could be separated into 13 different polypeptide components by high-resolution SDS-gel electrophoresis in the presence of urea (Kadenbach *et al.*, 1983a). The 13 polypeptides, denoted subunits (Kadenbach and Merle, 1981), were characterized by their N-terminal amino acid sequence (Buse *et al.*, 1982; Kadenbach *et al.*, 1983b) and by monospecific antisera (Kuhn-Nentwig and Kadenbach, 1984, 1985a). A definite identification of 13 subunits of the human cytochrome *c* oxidase complex, however, is still lacking.

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In the present study the presence of 13 different polypeptides in the isolated human heart cytochrome *c* oxidase complex was demonstrated by using monospecific antisera to nuclear-coded subunits of rat liver cytochrome *c* oxidase (Kuhn-Nentwig and Kadenbach, 1985b). The kinetics of cytochrome *c* oxidation were measured polarographically. An Eadie–Hofstee plot of the data revealed biphasic kinetics with similar K_m and V_{max} values to the isolated bovine heart enzyme.

Experimental

Nitrocellulose BA 85 (pore size 0.45 μm, 20 × 16 cm) was obtained from Schleicher and Schüll (Dassel), FITC-protein A from Pharmacia (Freiburg), swine anti-rabbit IgG conjugated with peroxidase from Dakopatts (Hamburg), and 3,3′-diaminobenzidine (DAB) and horse heart cytochrome *c*, Type VI, from Sigma (München).

The applied antisera to rat liver holocytochrome *c* oxidase and to specific subunits of the rat liver enzyme were previously described (Kuhn-Nentwig and Kadenbach, 1985a, b).

Human hearts were obtained from the Department of Pathology. Immediately after autopsy (6–30 h after death) mitochondria were isolated as described by Smith (1967) and were stored frozen at −70° in the presence of 1 mM tosyl lysyl chloromethane. Cytochrome *c* oxidase was isolated as previously described (Merle and Kadenbach, 1980) with the following modifications. The DEAE-Sephacel column was equilibrated with 30 mM KP_i, pH 7.2, since human cytochrome *c* oxidase is not bound to the column at the previously used concentration of 50 mM KP_i, pH 7.2. The gel filtration step was omitted. The precipitated enzyme was dialyzed either against 10 mM NaP_i, pH 7.0, in the presence of 1% lauryl-β-D-maltoside (for activity measurements) or against distilled water (for SDS-gel electrophoresis).

SDS-polyacrylamide gel electrophoresis was performed as described by Kadenbach *et al.* (1983a). Immunoblotting (Western blot) with an antiserum against rat liver holocytochrome *c* oxidase and with subunit-specific antisera (Kuhn-Nentwig and Kadenbach, 1985a, b) and immunodetection with FITC-protein A or with swine anti-rabbit IgG conjugated with peroxidase (Johnson *et al.*, 1982) were performed as previously described (Kuhn-Nentwig and Kadenbach, 1984).

Cytochrome *c* oxidase activity was measured polarographically (Ferguson-Miller *et al.*, 1976, 1978) at 25°C in 25 mM Tris acetate, pH 7.8, 7 mM Tris ascorbate, 0.014 mM EDTA, 1 mM laurylmaltoside, 0.7 mM tetramethyl-*p*-phenylenediamine (TMPD), 0.02–40 μM cytochrome *c*, and 139 pmole cytochrome *c* oxidase in a total volume of 1.7 ml.

Results

Human heart cytochrome *c* oxidase was isolated by use of the nonionic detergents Triton X-114 and Triton X-100 and of cholate (Merle and Kadenbach, 1980). The isolated enzyme complex was separated by high-performance SDS gel electrophoresis and compared with the rat liver enzyme as shown in Fig. 1. The human heart was obtained by autopsy 6 h after death. A similar band pattern is found although some polypeptides of the human

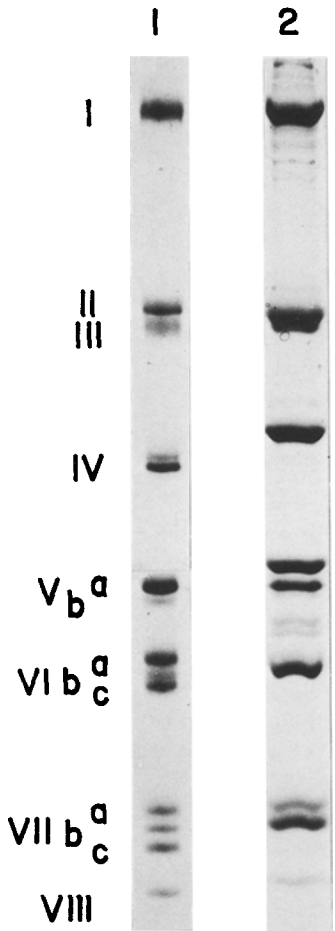

Fig. 1. Comparison of the polypeptide pattern of isolated human heart and rat liver cytochrome *c* oxidase. Isolated cytochrome *c* oxidase from rat liver (lane 1) and human heart (lane 2) were separated by SDS-gel electrophoresis. The human heart was obtained by autopsy 6 h after death.

enzyme show a different apparent molecular weight and partly run together. Subunit VIa appears as a faintly stained double band. Doubling of subunit VIa (unpublished results) and of subunit IV (Merle *et al.*, 1981) was frequently observed and has been explained by proteolytic degradation (Merle *et al.*, 1981). A weak staining by Coomassie blue was also found for subunit VIa from pig heart but not for subunit VIa from pig liver (Stroh and Kadenbach, 1986). Furthermore it appears that subunits VIb and c and VIIb and c of the human enzyme are not separated on the gel. In order to prove the presence of two different proteins in each of the two bands, monospecific antisera to subunits of rat liver cytochrome *c* oxidase were applied in a Western blot.

In Fig. 2A isolated human heart cytochrome *c* oxidase was separated by SDS gel electrophoresis, blotted onto nitrocellulose, cut into equal strips, and incubated separately with monospecific antisera to the indicated rat liver subunits. The exclusive reaction of the applied antisera with their corresponding subunit was demonstrated in a preceding publication (Kuhn-Nentwig and Kadenbach, 1985b). Antisera to subunits VIb and VIc and to subunits VIIb and VIIc were reactive with bands VIbc and VIIbc of the human heart enzyme, respectively, indicating the presence of four different proteins in the two bands. The antiserum to subunit VIIc reacts also with a faint Coomassie-blue stained band between subunits VIIc and VIII (Fig. 2A, lane 1). Obviously this band represents a proteolytic degradation product of subunit VIIc. In Fig. 2A an additional band IV* is visible below subunit IV which is suggested to originate from proteolytic degradation of subunit IV (Merle *et al.*, 1981). In contrast to the enzymes of Figs. 1 and 3, prepared from human hearts 6 h after death, the enzyme of Fig. 2 was prepared 16 h after death. The immunoreactive material on top of lanes 2–5 of Fig. 2A is suggested to represent aggregated protein.

Immunodetection in Fig. 2A was achieved with the more sensitive peroxidase reaction, because most nuclear-coded subunits of human heart cytochrome *c* oxidase react poorly with the corresponding antisera against rat liver subunits. This is demonstrated in Fig. 2B, where the immunoreaction of an antiserum to rat liver holocytochrome *c* oxidase with the rat liver and human heart enzyme is visualized by immunofluorescence of FITC protein A. A strong reaction was only found with subunits I, II, and IV (and IV*) of the human heart enzyme.

The oxidized and dithionite-reduced spectra of isolated human heart cytochrome *c* oxidase are identical to the corresponding spectra of the isolated enzyme from bovine heart (not shown). From the difference spectrum a heme *a* content of 9.2 nmol per mg protein was calculated, using $\varepsilon(605\text{–}630\,\text{nm}) = 12\,\text{mM}^{-1}\,\text{cm}^{-1}$ (von Jagow and Klingenberg, 1972).

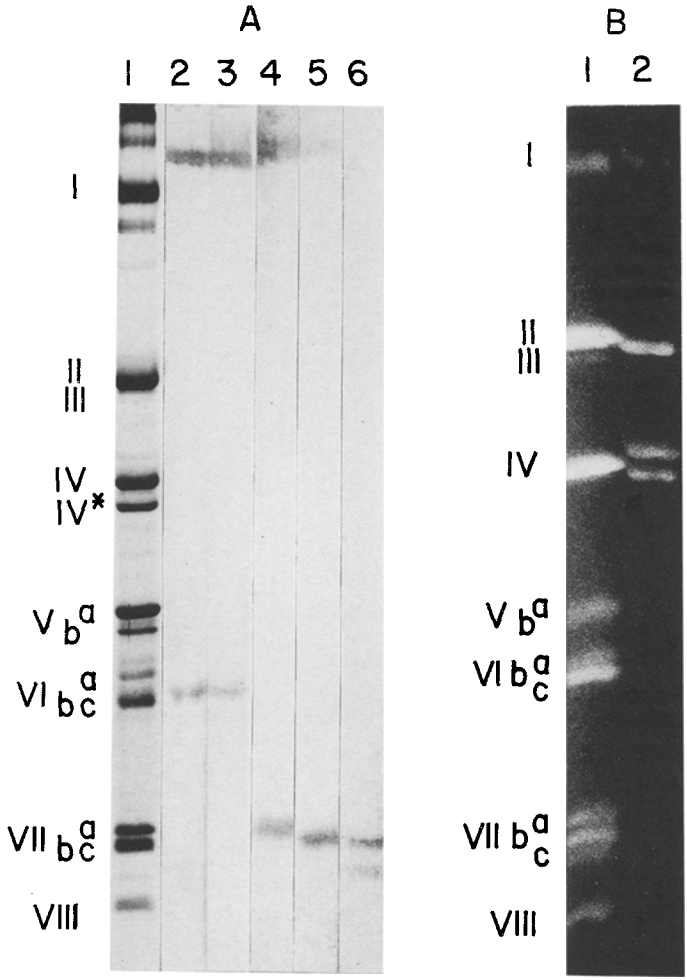

Fig. 2. Immunoblot analysis of human heart cytochrome *c* oxidase with antisera to the rat liver enzyme. (A) Human heart cytochrome *c* oxidase was separated by SDS gel electrophoresis and one lane was stained with Coomassie Blue R-250 (lane 1). The other part of the slab gel was transferred onto nitrocellulose, cut into equal strips, and separately incubated with monospecific antisera to rat liver cytochrome *c* oxidase subunit VIb (lane 2), VIc (lane 3), VIIa (lane 4), VIIb (lane 5), and VIIc (lane 6). Subsequent incubation with swine anti-rabbit IgG conjugated with peroxidase and with DAB and H_2O_2 for 10 min at room temperature was done as described in Experimental. (B) Equal amounts (52 μg protein) of isolated rat liver (lane 1) and human heart cytochrome *c* oxidase (lane 2) were separated by SDS-gel electrophoresis, transferred onto nitrocellulose, and incubated with an antiserum to rat liver holocytochrome *c* oxidase, followed by incubation with FITC-protein A as described by Kuhn-Nentwig and Kadenbach (1984). The human enzymes in lanes A, 1 and B, 2 were prepared from hearts, obtained by autopsy, 16 h and 20 h after death, respectively.

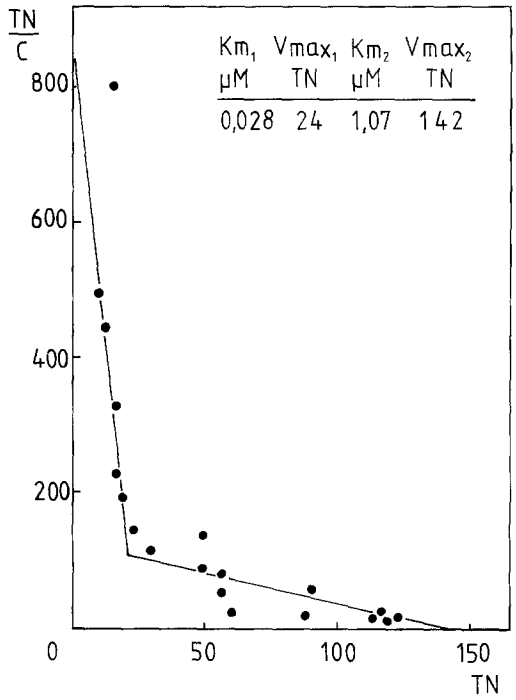

Fig. 3. Eadie–Hofstee plot of the kinetics of cytochrome *c* oxidation by isolated human heart cytochrome *c* oxidation. TN = turnover number, moles cytochrome *c* per mole cytochrome aa_3 per second. C = concentration of cytochrome *c*. For details see Experimental.

The activity of the enzyme was measured polarographically at various cytochrome *c* concentrations, and the data are presented in an Eadie–Hofstee plot in Fig. 3. Similar to the bovine heart enzyme (Ferguson-Miller *et al.*, 1976, 1978), a biphasic curve was obtained. The K_m and V_{max} values, measured for the high- and low-affinity phase of the reaction with cytochrome *c*, are very close to the values obtained with Keilin–Hartree particles for the bovine heart enzyme (Ferguson-Miller *et al.*, 1976, 1978). The high activity of the human heart cytochrome *c* oxidase found with our preparation is mainly due to the use of laurylmaltoside. This detergent was shown to activate cytochrome *c* oxidases from different sources (Rosevaer *et al.*, 1980).

Discussion

Characterization of the subunit composition of human cytochrome *c* oxidase is of actual interest, since several cases of human mitochondrial

myopathy have been described which are based on defective cytochrome *c* oxidase activity (Van Biervliet *et al.*, 1977; DiMauro *et al.*, 1980; Heiman-Patterson *et al.*, 1982; Müller-Höcker *et al.*, 1983; Johnson *et al.*, 1983; Bresolin *et al.*, 1985). It was suggested that these diseases could originate from genetic defects in nuclear-coded subunits of the enzyme (DiMauro *et al.*, 1985; Johnson *et al.*, 1984; Kuhn-Nentwig and Kadenbach, 1985a). The complete separation of nuclear-coded subunits is difficult due to the very similar molecular weight of these subunits (Buse *et al.*, 1982; Kadenbach *et al.*, 1983a).

In this study an immunological approach was applied to identify different proteins within single polypeptide bands. By this method, 13 different polypeptides could be identified in the purified cytochrome *c* oxidase complex from human heart.

In previous studies, isolated cytochrome *c* oxidase from human hearts showed very low activity (Jeffreys and Craig, 1977; Draijer *et al.*, 1982). We have found that the enzyme isolated from human hearts more than 6 h after death shows decreased activity which may be due to proteolytic degradation of subunits (see Fig. 1). The intact enzyme has a very high activity (Fig. 3), similar to the bovine heart enzyme. These results suggest that the basic structure and function of all mammalian cytochrome *c* oxidases is very similar.

On the other hand, a different distribution of SH groups was demonstrated in nuclear-coded subunits of cytochrome *c* oxidase from human heart as compared to those from the hearts of rat, pig, and bovine (Stroh and Kadenbach, 1986). In addition, a tissue-specific distribution of SH groups in subunits of the enzyme from the same organism was described. Recently a hypothesis on the function of multiple nuclear-coded subunits of mammalian cytochrome *c* oxidase was presented (Kadenbach, 1986). These subunits are assumed to represent receptors for intracellular effectors like substrates, ions, and nucleotides. The tissue-specific (Kadenbach *et al.*, 1982, 1983b) and developmental-specific (Kuhn-Nentwig and Kadenbach, 1985a) expression of nuclear-coded subunits is assumed to adapt the enzyme to the specific needs in different tissues. The different catalytic properties of the isozymes, however, can only be measured if suboptimal, "physiological" conditions are applied (Kadenbach *et al.*, 1986).

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