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Effect of Trypsin on the Kinetic Properties of Reconstituted Beef Heart Cytochrome *c* Oxidase

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Abstract

Isolated beef heart cytochrome *c* oxidase was reconstituted in liposomes by the cholate dialysis method with 85% of the binding site for cytochrome *c* oriented to the outside. Trypsin cleaved specifically subunit VIa and half of subunit IV from the reconstituted enzyme. The kinetic properties of the reconstituted enzyme were changed by trypsin treatment if measured by the spectrophotometric assay but not by the polarographic assay. It is concluded that subunit VIa and/or subunit IV participate in the electron transport activity of cytochrome *c* oxidase.

Key Words: Cytochrome *c* oxidase; kinetics; trypsin digestion; reconstitution; proteoliposomes.

Introduction

Cytochrome *c* oxidase from mammalian tissues is composed of 13 different polypeptide subunits. This was demonstrated by (1) separation of the isolated enzyme from different species and tissues into 13 polypeptide bands on high-resolution SDS[2] polyacrylamide gels (Kadenbach *et al.*, 1983a); (2) immunoprecipitation of 13 polypeptides from a total mitochondrial Triton X-100 extract with a monospecific antiserum against subunit IV (Merle *et al.*, 1981); (3) identification of 13 different N-terminal amino acid sequences (Kadenbach *et al.*, 1983b); and (4) exclusive reaction of monospecific antisera with their corresponding subunits (Kuhn-Nentwig and Kadenbach, 1984; Kuhn-Nentwig and Kadenbach, 1985a). Whereas the

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[2] Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; SDS, sodium dodecylsulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TLCK, L-1-chloro-3-tosylamido-7-amino-2-heptanone HCl; PMSF, phenylmethane sulfonylfluoride.

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catalytic function of the three mitochondrial coded subunits I–III is well established (for a review see Capaldi *et al.*, 1983, and Kadenbach, 1983), the functional role of the ten nuclear coded subunits is still unknown. It was suggested that they have a regulatory function (Kadenbach, 1983; Kadenbach and Merle, 1981). This view was supported by the discovery of tissue-specific (Kadenbach *et al.*, 1983b; Kadenbach *et al.*, 1982; Jarausch and Kadenbach, 1982; Merle and Kadenbach, 1980a) and developmental-specific isozymes (Kuhn-Nentwig and Kadenbach, 1985a), and by the demonstration of a tissue-specific participation of nuclear coded subunits in the binding domain for cytochrome *c* at subunit II of cytochrome *c* oxidase (Kadenbach and Stroh, 1984; Kadenbach *et al.*, 1984).

Recently the near neighborship of the 13 polypeptides in the enzyme complex was analyzed by using cleavable crosslinking reagents (Jarausch and Kadenbach, 1985a). The orientation of the subunits in the mitochondrial inner membrane was investigated by protease treatment of mitoplasts and subsequent immunoblotting (Jarausch and Kadenbach, 1985b) and by measuring the binding of monospecific antisera to mitoplasts by a quantitative immunoassay (Kuhn-Nentwig and Kadenbach, 1985b). In these studies the orientation to the cytosolic side was shown for all subunits of rat liver cytochrome *c* oxidase except subunits VIb and VIIc.

In the present study we investigated the effect of trypsin on specific cleavage of subunits and on the kinetic properties of isolated beef heart cytochrome *c* oxidase, reconstituted in liposomes by the cholate dialysis method (Casey *et al.*, 1979). With this method about 85% of the enzyme was shown to be oriented with its cytosolic side outside (Casey *et al.*, 1982). It will be shown that trypsin cleaves specifically subunit VIa and half of subunit IV of the enzyme with a concomitant change of the kinetics of its interaction with cytochrome *c*. This is the first report demonstrating directly the involvement of nuclear coded subunits in the catalytic activity of cytochrome *c* oxidase.

Materials and Methods

Asolectin (L-α-phosphatidylcholine, type II-s from soybean) was obtained from Sigma and was purified by the method of Kagawa and Racker (1971). Trypsin (type XI, DPCC treated) and cytochrome *c* (type VI, from horse heart) were purchased from Sigma. Valinomycin, TMPD, and FCCP were obtained from Fluka, Buchs.

Cytochrome *c* oxidase was prepared from bovine heart mitochondria as previously described (Merle and Kadenbach, 1980b; Kadenbach *et al.*, 1985). The final ammonium sulfate precipitate was dissolved in 0.25 M sucrose,

20 mM Tris-HCl, pH 7.6, and 2 mM EDTA, and stored in liquid nitrogen. The heme *a* content was 9.2 nmol/mg protein. Cytochrome *c* oxidase was reconstituted in liposomes by the cholate-dialysis procedure (Casey *et al.*, 1979) at a concentration of 40 mg/ml asolectin and 3 μM cytochrome aa_3. The orientation of cytochrome *c* oxidase within the membranes, determined by the method described in Casey *et al.* (1982), was 85 $\pm$ 2% accessible to exogenous cytochrome *c*. Respiratory control ratios were 6.5 $\pm$ 0.5.

Proteoliposomes (200 μl) were incubated at room temperature with the indicated amounts of trypsin in 1 mM K-Hepes, 30 mM KCl, 79 mM sucrose, and adjusted to pH 8.0 by addition of 50 mM Tris base. The reaction was stopped with TLCK and PMSF (1 mM final concentrations). To prepare samples for SDS-polyacrylamide gel electrophoresis, solid Na-cholate was added to a final concentration of 4% and diluted with an equal volume of water, and cytochrome *c* oxidase was precipitated by addition of saturated (°C) ammonium sulfate (50% final concentration). The precipitate was dissolved in sample buffer containing 8% SDS and 1% mercaptoethanol. SDS-polyacrylamide gel electrophoresis was performed as previously described (Kadenbach *et al.*, 1983a).

Cytochrome *c* oxidase activity was measured polarographically at 25°C according to Ferguson-Miller *et al.* (1976); see also Brautigan *et al.* (1977) and Ferguson-Miller *et al.* (1978). The assay buffer (1.75 ml final volume) contained 40 mM KCl, 10 mM K-Hepes, pH 7.4, 0.1 mM EDTA, 25 mM K-ascorbate, 0.7 mM TMPD, 1 μg/ml valinomycin, and 3 μM FCCP at various cytochrome *c* concentrations.

Cytochrome *c* oxidase activity was monitored spectrophotometrically (Yonetani, 1967) with a Kontron Uvikon 810 spectrophotometer. The initial oxidation rate of ferrocytochrome *c*, reduced with dithionite and separated by chromatography on a Sephadex G-25 column (1 $\times$ 10 cm), was recorded at 550 nm (25°C). The assay was performed in the presence of 0.8–80 μM ferrocytochrome *c* in three different buffers: 125 mM K-phosphate, pH 6.0; 40 mM KCl, 10 mM Hepes, pH 7.4; or 25 mM Tris-acetate, pH 7.8, 25 mM sucrose, always in the presence of 1 μg/ml valinomycin and 3 μM FCCP. The reaction was started by addition of 5 μl incubated proteoliposomes to 1 ml assay mixture. The activity is expressed as molecular turnover (TN) (mole cytochrome *c* per second per mole cytochrome aa_3^{-1}).

Results

Isolated beef heart cytochrome *c* oxidase was reconstituted in liposomes by the cholate dialysis method (Casey *et al.*, 1979), and the orientation was determined spectrophotometrically by the method of Casey *et al.* (1982).

We found that 85% of the enzyme was reducible by external ferrocytochrome *c* (not shown). The proteoliposomes were incubated for different times with trypsin, and the polypeptide pattern was analyzed on high-resolution SDS polyacrylamide gels (Kadenbach *et al.*, 1983a) after separation from excess lipids (see Materials and Methods). In Fig. 1, lanes 3 and 4, is shown the polypeptide pattern of control proteoliposomes incubated without trypsin. The 13 subunits of beef heart cytochrome *c* oxidase (Kadenbach *et al.*, 1983a)

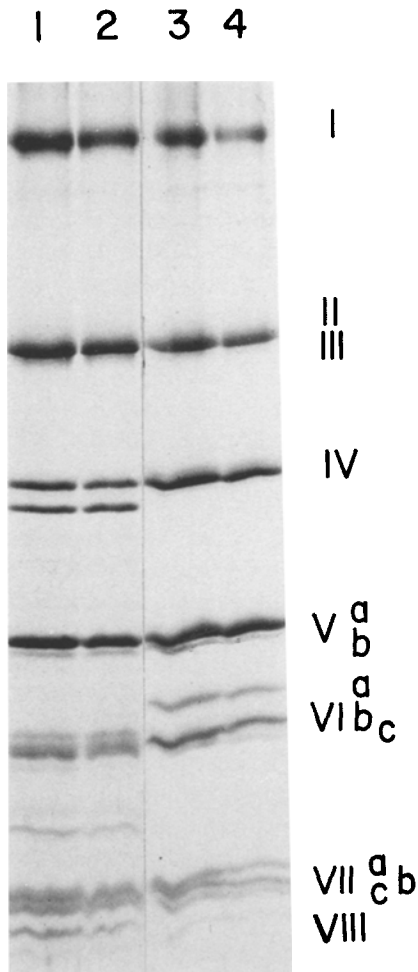

Fig. 1. Specific cleavage of subunit VIa and half of subunit IV of reconstituted beef heart cytochrome *c* oxidase by trypsin. Proteoliposomes were incubated with trypsin (1 mg per mg cytochrome *c* oxidase) for 30 min (lane 1), 60 min (lane 2), or without trypsin for 60 min (lanes 3 and 4) at room temperature and further treated as described under Materials and Methods.

are visible except subunit III, which easily tends to aggregate during SDS polyacrylamide gel-electrophoresis (Hundt and Kadenbach, 1977). On the left part of the gel (lanes 1 and 2) polypeptides VIb and c and VIIa and b run together. In lanes 1 and 2 of Fig. 1 are presented the polypeptide patterns of proteoliposomes incubated for 30 and 60 min with trypsin, respectively. Two main differences from the control proteoliposomes are visible: cleavage of about half of subunit IV into a new polypeptide band running below subunit IV and complete disappearance of subunit VIa. Two further new bands are visible, one above subunit VIb and another between subunits VIc and VIIa. A number of additional faint bands are detectable which can be explained by cleavage from the matrix side of the 15% cytochrome *c* oxidase with opposite orientation. The almost identical pattern of lanes 1 and 2 indicates the stability of the cleaved cytochrome *c* oxidase complex against further proteolytic attack by trypsin.

The enzymatic activity of cytochrome *c* oxidase in control and trypsin-treated proteoliposomes was measured spectrophotometrically at high ionic strength and high cytochrome *c* concentrations as shown in Fig. 2. A time-dependent inhibition of activity by trypsin to about 65% of the control is found. Incubation with trypsin for 2 h does not further decrease the partially inhibited activity (not shown).

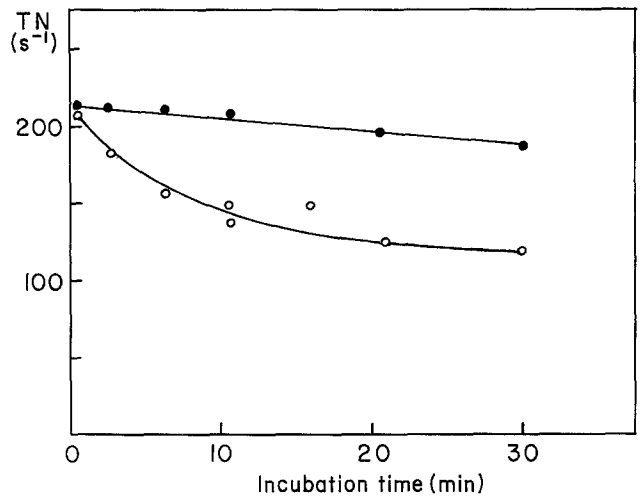

Fig. 2. Time-dependent decrease of reconstituted cytochrome *c* oxidase activity by trypsin. Proteoliposomes were incubated with trypsin (0.5 mg per mg cytochrome *c* oxidase) for the indicated times. The reaction was stopped by addition of TLCK (1 mM final concentration) and the activity was measured spectrophotometrically as described under Materials and Methods in 125 mM K P_i, pH 6.0, with 80 μM ferrocytochrome *c*. (●) Control proteoliposomes; (○) trypsin-treated proteoliposomes.

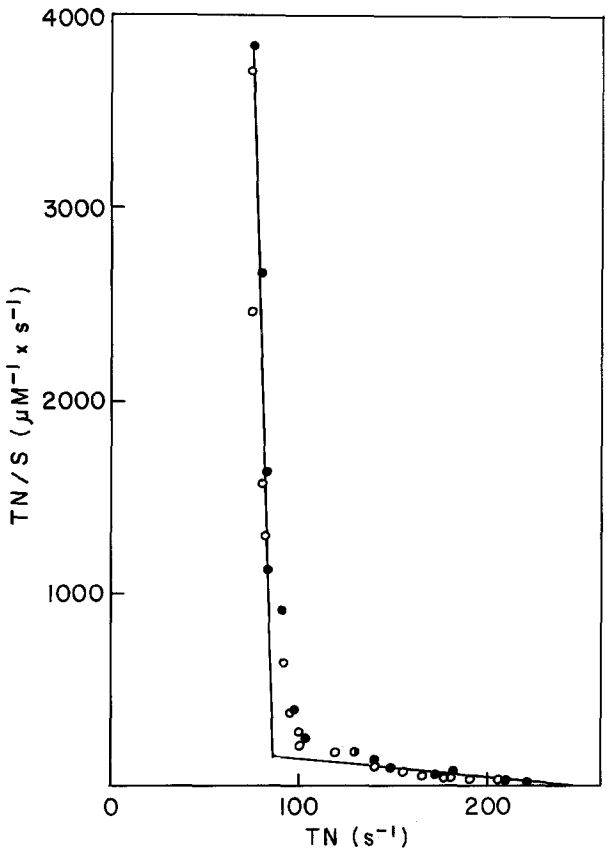

Fig. 3. Eadie–Hofstee plot of the kinetics of cytochrome *c* oxidation by reconstituted cytochrome *c* oxidase after incubation with or without trypsin, measured polarographically. Cytochrome *c* concentrations ranged from 0.02 to 40 μM and cytochrome aa_3 was 22 nM final concentration. (●) Control proteoliposomes; (○) proteoliposomes incubated for 60 min with 0.5 mg trypsin per mg cytochrome *c* oxidase.

The kinetics of the steady-state activity of reconstituted cytochrome *c* oxidase at various cytochrome *c* concentrations, measured polarographically, is demonstrated in an Eadie–Hofstee plot in Fig. 3. No difference between control and trypsin-treated proteoliposomes is found, which contrasts with the results of Fig. 2. Between the spectrophotometric and the polarographic assay applied for analysis of cytochrome *c* oxidase kinetics, large differences have been found (Ferguson-Miller *et al.*, 1978; Smith *et al.*, 1979). It was suggested that in the polarographic assay cytochrome *c*, bound to cytochrome *c* oxidase, can be repeatedly reduced by TMPD before it dissociates. This contrasts with the spectrophotometric assay, where each electron transfer involves binding of ferrocytochrome *c* and dissociation of

ferricytochrome *c* (Ferguson-Miller *et al.*, 1978; Smith *et al.*, 1979). Thus it appears that the spectrophotometric assay is more strongly influenced by the interaction between cytochrome *c* and the oxidase than the polarographic assay.

In Fig. 4 is demonstrated the spectrophotometrically measured kinetics of cytochrome *c* oxidation by reconstituted cytochrome *c* oxidase, incubated with or without trypsin. The assay was performed under three different conditions of ionic strength. With the buffer used for the polarographic assay, a higher K_m but the same V_{max} is found with the trypsin-treated proteoliposomes. This result corroborates previous data obtained with the detergent-dissolved enzyme, where an increased K_m but also an increased V_{max} was found (Kadenbach, 1983; Kadenbach *et al.*, 1981). At high ionic strength (125 mM potassium phosphate) a lower activity but an unchanged K_m is observed after trypsin treatment (Fig. 4A). Only at very low ionic strength are biphasic kinetics obtained. A slightly higher K_m and an unchanged V_{max} value are found at the low-affinity phase, and a slightly decreased K_m value at the high-affinity phase for the trypsin-treated proteoliposomes. From these data it can be concluded that cleavage of subunit VIa and half of subunit IV by trypsin changes the kinetic properties of beef heart cytochrome *c* oxidase which, however, can only be measured by the spectrophotometric assay.

Discussion

The effect of proteases on the kinetic properties of cytochrome *c* oxidase are preferentially measured with the reconstituted enzyme for two reasons: (i) the enzyme has a "physiological" boundary of phospholipids, and (ii) the proteases attack only those proteins protruding from the asymmetrically oriented enzyme complex into the medium. Trypsin appears to cleave only two subunits of reconstituted cytochrome *c* oxidase oriented with its cytochrome *c* binding site to the exterior: subunit VIa and half of subunit IV. "Removal" of subunit VIa by trypsin, corresponding to "impurity *b*" according to the nomenclature of Capaldi *et al.* (1983), has already been shown for the soluble enzyme by Ludwig *et al.* (1979). The appearance of new bands suggest, however, that part of the cleaved subunit remains attached to the enzyme complex (see Fig. 1). Separation of proteoliposomes from the medium did not remove the additional bands (not shown).

Only half of subunit IV appears to be cleaved by trypsin. A half-site reactivity of cytochrome *c* oxidase has already been shown by Bisson *et al.* (1980), who found complete inhibition of activity when 1 mol Lys-13 modified arylazidocytochrome *c* was covalently bound per cytochrome *c* oxidase dimer. We suggest that the reconstituted enzyme forms dimers in the lipid

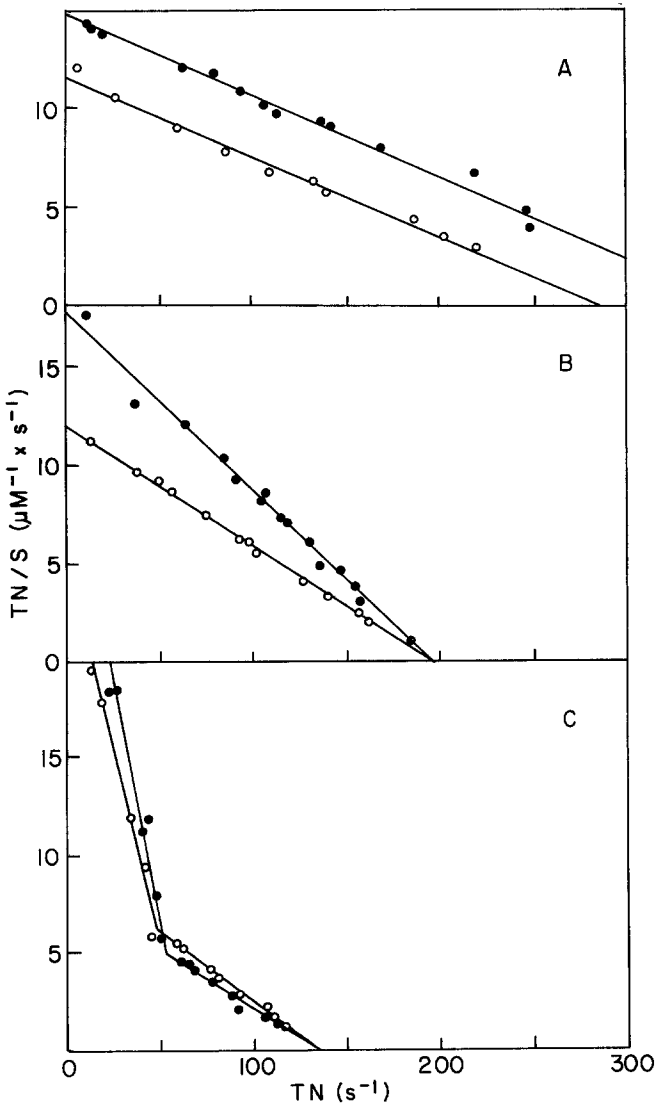

Fig. 4. Eadie–Hofstee plot of the kinetics of cytochrome *c* oxidation by reconstituted cytochrome *c* oxidase after incubation with or without trypsin, measured spectrophotometrically with different buffers. A: 125 mM K P_i, pH 6.0; B: 40 mM KCl, 10 mM Hepes, pH 7.4; C: 25 mM Tris-acetate, pH 7.8, 25 mM sucrose. The final concentration of cytochrome *c* oxidase, applied as liposomal suspension, was 5.4 nM. Cytochrome *c* concentrations ranged from 0.8 to 80 μM. (●) Control proteoliposomes; (○) proteoliposomes incubated for 60 min with 0.5 mg trypsin per mg cytochrome *c* oxidase.

bilayer with different accessibility of corresponding subunits of the two monomers. Cleavage of half of subunit IV was also previously shown with pronase in rat liver mitoplasts (Jarausch and Kadenbach, 1985b). These data contrast with other results, where an exclusive orientation to the matrix was found for subunit IV (Ludwig *et al.*, 1979; Eytan and Broza, 1978; Malatesta *et al.*, 1983; Zhang *et al.*, 1984), but they are supported by reaction of a monospecific antiserum against subunit IV of rat liver cytochrome *c* oxidase with rat liver mitoplasts (Kuhn-Nentwig and Kadenbach, 1985b).

Trypsin changed the kinetic properties of reconstituted cytochrome *c* oxidase if measured spectrophotometrically but not polarographically. The rate-determining step in the polarographic assay was suggested to be the oxidation of bound cytochrome *c* by the oxidase, and in the spectrophotometric assay, the dissociation of bound cytochrome *c* (Ferguson-Miller *et al.*, 1978). Therefore we suggest that trypsin changes the binding and/or dissociation of cytochrome *c* rather than the electron transfer within the oxidase. The changed kinetic properties may be due to cleavage of subunit VIa because it was shown to be arranged in the isolated rat liver enzyme complex close to subunit II (Jarausch and Kadenbach, 1985a), the binding site for cytochrome *c* (Bisson *et al.*, 1980), and to be oriented to the cytosolic side in rat liver mitoplasts (Jarausch and Kadenbach, 1985b; Kuhn-Nentwig and Kadenbach, 1985b). Furthermore, a carboxylic group of subunit VIa was shown to participate in the binding domain for cytochrome *c* in pig liver, but not in pig heart cytochrome *c* oxidase (Kadenbach and Stroh, 1984; Kadenbach *et al.*, 1984). The difference can be explained by different N-terminal amino acid sequences (Kadenbach *et al.*, 1983b). Subtilisin was also found to cleave subunit VIa, but not subunit IV in rat liver mitoplasts (Jarausch and Kadenbach, 1985b), and to change the kinetic properties of reconstituted beef heart cytochrome *c* oxidase similarly to trypsin if measured spectrophotometrically but not polarographically (Büge and Kadenbach, unpublished results). On the other hand, two further possibilities cannot be excluded which can also explain the changed kinetic properties of the trypsin-treated enzyme: (i) cleavage of half of subunit IV, and (ii) cleavage of a few amino acids from larger subunits (e.g., subunit I–III) which may not be detected from the apparent molecular weight. The different changes by trypsin treatment of the spectrophotometrically measured kinetic parameters at different pH and ionic strength (Fig. 4) is difficult to explain. The complex binding domain for cytochrome *c* (Kadenbach and Stroh, 1984) and the electrostatic nature of the interaction between cytochrome *c* and the oxidase (Koppenol and Margoliash, 1982), however, cannot be easily explained. Further work is required to understand the influence of the multiple subunits on the kinetic properties of cytochrome *c* oxidase.

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