# Role of Nuclear-Encoded Subunits of Mitochondrial Cytochrome *c* Oxidase in Proton Pumping Revealed by Limited Enzymatic Proteolysis†

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ABSTRACT: The role of supernumerary subunits of bovine heart cytochrome *c* oxidase has been investigated by examining the effect on the enzymatic activity of limited proteolysis by chymotrypsin, thermolysin, and trypsin. All three proteases, when added to the soluble oxidase, digested subunits III, VIa, and VIb and caused inhibition of electron flow in the oxidase. In addition, trypsin and thermolysin also digested subunit IV. Trypsin cleaved off an N-terminal segment of seven residues; thermolysin cleaved only the first four residues at the N-terminus of subunit IV. Digestion of the soluble oxidase by trypsin but not by thermolysin caused decoupling of redox-linked proton pumping in the oxidase. It is concluded that the sequence V5-V6-K7 of the hydrophilic N-terminus of subunit IV, which protrudes out of the matrix side of the mitochondrial membrane, mediates the access of protons into the transmembrane proton translocating pathway in the oxidase.

Cytochrome *c* oxidase of mitochondria (cytochrome $a,a_3$, EC 1.9.3.1), the terminal enzyme of the respiratory chain, is made up of 3 core proteins (subunits I, II, and III), encoded by mitochondrial genes and highly conserved in the members of the heme–copper oxidase superfamily (Ludwig, 1987; Saraste, 1990; Gennis, 1991), and 10, nuclear-encoded supernumerary subunits (Kadenbach et al., 1983; Capaldi, 1990). Subunit I presents several putative transmembrane helices, which ligate by means of conserved histidines the heme $a_3$–$Cu_B$ binuclear center, the site of oxygen reduction, and heme *a*. Heme *a* and $Cu_A$, the latter ligated to subunit II, are located at the outer side of the membrane where they accept electrons from peripheral cytochrome *c*. The binuclear center, also located at the outer side of the membrane (Hosler et al., 1993), accepts protons, consumed in the reduction of dioxygen to water, from the inner aqueous space (Mitchell, 1966; Papa et al., 1974; Papa, 1976; Konstantinov et al., 1986).

Electron transfer in the oxidase results in the generation of transmembrane $\Delta\mu H^+$.[1] This derives first from the membrane asymmetry of reduction of dioxygen to water (Papa et al., 1974; Wikstrom, 1988). In addition, electron flow in the oxidase is associated with proton pumping from the inner to the aqueous space (Wikstrom, 1977; Wikstrom & Krab, 1979). This activity is, however, intrinsically variable, and there are conditions under which there is little proton pumping (Capuano et al., 1985; Papa et al., 1985, 1987). Papa et al. (Capitanio et al., 1991; Papa et al., 1991) have shown that the efficiency of proton pumping in cytochrome *c* oxidase, i.e., the $H^+/e^-$ ratios for proton ejection, varies under the influence of kinetic and thermodynamic factors [cf. Murphy (1989)].

Mutational analysis of *Paracoccus denitrificans* $aa_3$ oxidase, which has only subunits I, II, and III, indicates that subunits I and II in this prokaryotic enzyme are sufficient for both electron transfer activity and proton pumping [Haltia et al., 1991; see, however, Musser et al. (1993)]. The question is then raised of the role of subunit III and the supernumerary subunits in the eukaryotic enzymes. A role of these subunits in the assembly of the functional oxidase (Poyton et al., 1988), stabilization of the mature enzyme, and/or regulation of activity (Capaldi, 1990; Kadenbach et al., 1991) has been proposed from time to time. Some supernumerary subunits of eukaryotic $a,a_3$ oxidase present isoforms whose expression depends on the environmental conditions (Trueblood et al., 1988; Schiavo & Bisson, 1989), developmental stage (Kuhn-Nentwig & Kadenbach, 1985; Ewart et al., 1991), tissue (Kadenbach et al., 1986; Taanman et al., 1993), and age (Boffoli et al., 1994).

The present work deals with the role of supernumerary subunits and in particular subunit IV [nomenclature of Kadenbach et al. (1983)] in the bovine heart cytochrome *c* oxidase. The homologous counterpart of subunit IV in yeast cytochrome oxidase presents two isoforms, subunits Va and Vb (Trueblood et al., 1988; Capaldi et al., 1986), which are encoded by two nuclear genes, differently regulated by oxygen. Either of the two isoforms is required for functional oxidase, subunit Vb providing higher catalytic activity than Va (Trueblood et al., 1988). It is not clear whether there are also isoforms of mammalian subunit IV (Kuhn-Nentwig & Kadenbach, 1985; Romero et al., 1990; Van Kuilenburg et al., 1992).

Chemical labeling (Malatesta et al., 1983) and protease digestion (Zhang et al., 1984) indicate that subunit IV has the N terminus exposed at the inner matrix side and the C terminus at the outer C side, the two being connected by a single transmembrane helix (Capaldi et al., 1986; Zhang et al., 1984).

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[1] Abbreviations: COX, cytochrome *c* oxidase; COV, cytochrome *c* oxidase vesicle(s); ΔpH, transmembrane pH gradient; $\Delta\mu H^+$, transmembrane electrochemical potential; $H^+/e^-$, ratio of protons translocated per electron transferred by cytochrome *c* oxidase; $RCR_{\Delta\mu H^+}$, respiratory control ratio exerted by transmembrane electrochemical potential; $RCR_{\Delta pH}$, respiratory control ratio exerted by transmembrane pH gradient; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM, standard error of the mean; enzyme, ferrocytochrome *c*:oxygen oxidoreductase (cytochrome *c* oxidase) (EC 1.9.3.1).

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Binding of specific monoclonal antibodies to the N-terminal region of subunit IV affects the catalytic activity of the bovine heart enzyme (Gai et al., 1988). Chemical modification of lysine residues located in the outer part of subunits IV and II causes decoupling of proton pumping in isolated reconstituted bovine heart oxidase (Steverding et al., 1990). The results of the present study show that proteolytic digestion of the N-terminal segment of subunit IV, in particular removal of Val5-Val6-Lys7, produces decoupling of redox-linked proton translocation. It is proposed that this N-terminal segment of subunit IV mediates the access of protons from the inner space into the transmembrane proton translocating pathway of the oxidase.

## MATERIALS AND METHODS

*Enzyme Preparation.* Cytochrome *c* oxidase was purified from beef heart mitochondria as described in Errede et al. (1978); the nanomoles of heme $a+a_3$ per milligram of protein was 10–11. Reconstitution of cytochrome *c* oxidase in phospholipid vesicles was carried out by the cholate-dialysis method as described in Casey et al. (1979), and the percentage of right-side-out-oriented oxidases, measured as in Casey et al. (1982), was 80-85%. Cytochrome $a+a_3$, in the solubilized or liposomal state, was measured spectrophotometrically at room temperature using a $\Delta\epsilon_{605-630nm}$(reduced minus oxidized) = 14 $mM^{-1}\cdot cm^{-1}$.

*Protease Digestion.* Protease treatment of 5 mg of protein/mL soluble cytochrome *c* oxidase was performed in 0.1 mM K-Hepes, pH 8.0 at 25 °C. In some experiments, trypsin was preincubated with 2 mM TPCK; chymotrypsin and thermolysin were used without pretreatment. The protease:cytochrome oxidase ratio was 1:20 (mg/mg) for trypsin and chymotrypsin and 1:50 for thermolysin; the reaction was stopped with 2 mM PMSF for trypsin and chymotrypsin or with 2 mM EDTA for thermolysin (in the latter case, EDTA was present in all the media thereafter used). Cytochrome *c* oxidase vesicles (COV) were incubated, without dilution, with a protease:oxidase ratio of 1:10 (mg/mg) for trypsin and chymotrypsin. Samples for electrophoretic analysis were centrifuged in a sucrose gradient in the presence of Triton X-100 to remove the excess of phospholipids (Zhang et al., 1984). SDS–PAGE analysis was performed as in Kadenbach et al. (1983). Coomassie blue-stained gels were scanned at 590 nm with a Camag TLC scanner II densitometer. The areas corresponding to density bands were integrated with a D-2000 Chromato-integrator (Merck-Hitachi).

*Kinetic Assay.* Kinetic analysis was carried out with oxidase vesicles either polarographically or spectrophotometrically in 65 mM sodium phosphate, 0.1 mM EDTA, pH 7.5, and 2.0 $\mu$g of valinomycin/mL plus 3.0 $\mu$M CCCP. In the polarographic assay, oxygen consumption by COV (20 nM $aa_3$) was supported by 10 mM ascorbate plus 0.2 mM TMPD and 0.5–80 $\mu$M cytochrome *c* (correction for slow ascorbate oxidation in the absence of cytochrome *c* was made). In the spectrophotometric assay, the initial rate of oxidation of 0.5–50 $\mu$M ferrocytochrome *c* by COV (4 nM $aa_3$) was measured following the absorbance decrease at 550–540 nm; a $\Delta\epsilon$ = 19.1 $mM^{-1}\cdot cm^{-1}$ was used. The initial rates were estimated by semilogarithmic plot of the progress curve of ferrocytochrome *c* oxidation which followed first-order kinetics.

*Measurement of Proton Translocation, Respiratory Control Ratio, and Passive Proton Conductance in COV.* Redox-linked proton translocation was measured under level flow conditions from the initial rates of extravescicular pH changes and oxygen consumption obtained from measurements, by double-wavelength spectrophotometry, of absorbance changes of the membrane-impermeant pH indicator phenol red and those associated with hemoglobin deoxygenation, respectively (Papa et al., 1980, 1991). The respiratory control ratio was measured polarographically in 40 mM KCl, 10 mM Hepes (pH 7.4), 0.1 mM EDTA, 50 $\mu$M cytochrome *c*, and COV (40 nM $aa_3$) supplemented with 25 mM ascorbate plus 200 $\mu$M TMPD. Fully uncoupled respiration was achieved in the presence of 2 $\mu$g of valinomycin/mL plus 3 $\mu$M CCCP (Papa et al., 1987).

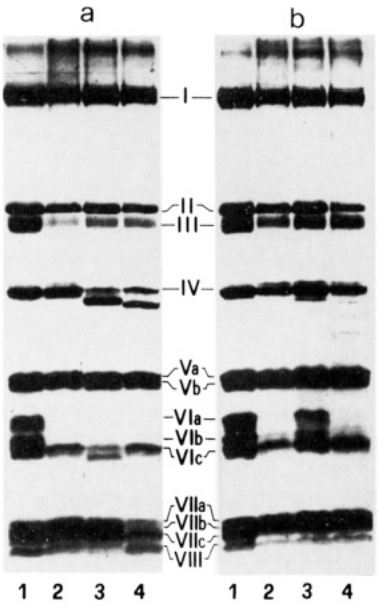


FIGURE 1: Effect of protease digestion on the subunit profiles of beef heart cytochrome *c* oxidase. The experimental conditions for the protease treatment of solubilized (a) and liposomal (b) cytochrome *c* oxidase are described under Materials and Methods. Lanes 1 show the controls without protease treatment; lanes 2 refer to treatment with chymotrypsin, lanes 3 with thermolysin, and lanes 4 with trypsin. Incubation time was 2 h, at 25 °C. Extraction of incorporated cytochrome *c* oxidase was performed by centrifugation in a sucrose gradient in the presence of Triton X-100 (Zhang et al., 1984); soluble cytochrome *c* oxidase was treated in the same way; the pelletted samples were resuspended in the lysis buffer, and 30 $\mu$g of protein was loaded for each lane. The SDS–PAGE and gel-staining conditions with Coomassie brilliant blue were as described in Kadenbach et al. (1983).

Passive proton conduction was measured electrometrically as in Papa et al. (1987), suspending 0.5 $\mu$M COV in 100 mM choline cloride, 5 mM NaCl, and 0.1 mM EDTA ($K^+$-free medium), pH 7.0. Addition of 2 $\mu$g of valinomycin/mL induced a $K^+$-diffusion potential which caused a compensating proton counterflow (uptake) whose initial rate was taken as a measure of passive membrane conduction.

*Sequencing.* Automatic sequencing by Edman degradation of polypeptides electroblotted on poly(vinylidene difluoride) membranes (Immobilon transfer) (Matsudaira, 1987) was performed by an Applied Biosystem Sequencer equipped with an on-line phenylthiohydantoin analyzer.

*Chemicals.* Horse cytochrome *c* (type VI), soybean phospholipids (type II), valinomycin, CCCP, TPCK, and PMSF were from Sigma Chemical Co.; trypsin, chymotrypsin, and thermolysin were from Boehringer Mannheim; TMPD was from BDH Chemical Ltd. All other reagents were of the highest purity grade commercially available.

## RESULTS

*Protease Digestion of Cytochrome c Oxidase Subunits.* Figure 1 shows SDS–PAGE analysis of digestion of soluble

Table 1: Effect of Protease Digestion on Kinetic Parameters of Cytochrome c Oxidase[a]

| | | control | | thermolysin | | chymotrypsin | | trypsin | |
|---|---|---|---|---|---|---|---|---|---|
| | assay conditions | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| soluble COX | (a) | 4.3 ± 0.2 | 105.1 ± 8.2 | 6.0 ± 1.5 | 68.3 ± 6.7 | 5.0 ± 1.4 | 50.9 ± 0.3 | 3.9 ± 0.2 | 61.9 ± 4.3 |
| | (b) | 3.5 ± 0.3 | 206.0 ± 9.7 | 4.5 ± 0.7 | 123.8 ± 6.9 | 4.5 ± 0.3 | 100.1 ± 7.3 | 3.9 ± 0.7 | 129.0 ± 5.0 |
| liposomal COX | (a) | 3.5 ± 0.2 | 128.3 ± 8.2 | 4.7 ± 1.5 | 125.1 ± 6.7 | 4.5 ± 1.4 | 106.0 ± 0.3 | 5.1 ± 0.2 | 110.0 ± 17.5 |
| | (b) | 5.6 ± 0.3 | 225.5 ± 13.8 | 6.0 ± 0.3 | 208.0 ± 21.0 | 5.8 ± 0.2 | 166.2 ± 26.3 | 5.6 ± 0.2 | 188.2 ± 11.2 |

[a] Protease treatment of soluble and liposomal cytochrome c oxidase was performed under the same conditions described in the legend of Figure 1. Spectrophotometric (a) and polarographic (b) kinetic assays were carried out as detailed under Materials and Methods. $K_m$ (in micromolar) refers to cytochrome c; $V_{max}$ is moles of cytochrome c oxidized per moles of cytochrome c oxidase per second. The effective concentration of cytochrome c oxidase was taken as that right-side-out-oriented in the liposomal membranes (85% of the total oxidase), measured as in Casey et al. (1982). The values reported, estimated from a Eadie–Hofstee plot of the experimental points best fitted by linear regression analysis, represent the average of 3–4 different experiments, ±SEM, and refer to the low-affinity phase of the oxidase activity which is measured under the prevailing experimental condition (Cooper, 1990).

and liposome-reconstituted beef heart cytochrome c oxidase by chymotrypsin, thermolysin, and trypsin. Each of the three proteases digested various subunits of the oxidase. There were, however, definite differences in the digestion of the oxidase subunits by the three proteases so that comparison of the relative patterns with the corresponding effects on the activities of the oxidase allowed for identification of specific roles of individual subunits.

When solubilized cytochrome c oxidase was treated with the proteases, chymotrypsin digested extensively subunit III [cf. Zhang et al. (1984, 1988)] whereas the other two proteases gave only some digestion of this subunit. Subunit IV, which was untouched by chymotrypsin, was extensively digested by both trypsin and thermolysin with the appearance of Coomassie blue detectable cleavage products. A difference in the position on the gel of the proteolytic products of subunit IV obtained with thermolysin and trypsin indicates more extensive digestion of this subunit by the latter protease. This was confirmed by direct sequencing of the proteolytic cleavage products of subunit IV. Cleavage occurred with both enzymes at the amino terminus, peptide bond 7–8 with trypsin and 4–5 with thermolysin, with removal of the sequences $H_2N$-Ala-His-Gly-Ser-Val-Val-Lys and $H_2N$-Ala-His-Gly-Ser, respectively [cf. subunit IV sequence reported in Sacher et al. (1979)]. Chymotrypsin, trypsin, and thermolysin all digested subunits VIa and VIb completely with no detectable proteolytic fragments. Thermolysin also digested subunit VIc, to a fragment detectable by Coomassie blue and subunit VIII. Trypsin also digested subunit VIIb.

No apparent digestion of the major subunits I and II (containing the redox centers) was observed in any case; moreover, absolute or difference (reduced minus oxidized) absorbance spectra and kinetics of cyanide binding of the protease-digested oxidase did not show any significant difference with respect to the undigested oxidase (data not shown). When cytochrome oxidase, incorporated in liposomes (COV) with 80–85% of the enzyme oriented "right-side-out" (the cytochrome c binding site exposed outside), was incubated with the proteases, digestion of subunit IV by both trypsin and thermolysin, and subunits VIIb and VIc by trypsin and thermolysin, respectively, was almost completely prevented [cf. Zhang et al. (1988)]. Subunit III was, although less extensively, still digested by chymotrypsin. Subunits VIa and VIb were still digested by each of the three proteases as they were in the solubilized oxidase.

*Effect of Digestion on Electron Flow.* Spectrophotometric and polarographic assay of aerobic oxidation of ferrocytochrome c by the oxidase showed that digestion of the enzyme in the soluble state by either chymotrypsin, thermolysin, or trypsin resulted in substantial depression of the $V_{max}$ of the reaction without any significant change of the $K_m$ for cytochrome c (Table 1). The depression of $V_{max}$ was largely prevented when the oxidase was incorporated in liposomes before its incubation with the proteases (Table 1). These results indicate that some of the supernumerary subunits, and hence the overall oligomeric structure of the oxidase, can influence the electron transfer activity. These include subunits IV, VIIb, and VIc which are digested by one protease or another in the soluble oxidase but not in COV. An effect of subunit VIa in electron transfer cannot be excluded. Although no proteolytic fragments of this subunit can be detected by Coomassie blue staining, immunoblot detection of fragments showed that while in the soluble enzyme both the amino- and carboxy-terminal segments are digested by proteases, in the reconstituted enzyme only the carboxy-terminal region is digested (Zhang et al., 1988). Direct involvement of subunit VIb in the oxidase activity can be excluded since it was shown that its removal from the complex after detergent treatment followed by chromatographic separation does not produce any significant functional effect (Planque et al., 1989).

*Proton Pumping Activity of Digested Cytochrome c Oxidase.* Figure 2 illustrates spectrophotometric measurement of respiration and proton ejection elicited by the addition of ferricytochrome to COV supplemented with ascorbate plus TMPD. It can be noted from the $H^+/e^-$ ratios for vectorial proton translocation calculated from initial rates of respiration and proton ejection that the efficiency of proton pumping at level flow (i.e., under conditions of negligible transmembrane $\Delta\mu H^+$), practically unaffected by digestion of the soluble oxidase with thermolysin and chymotrypsin, was severely depressed by digestion of the enzyme with trypsin.

Figure 3 presents results of a systematic analysis of the effect of proteolytic digestion of soluble cytochrome c oxidase on redox-linked proton translocation measured under the experimental conditions illustrated in Figure 2. It was previously shown (Capitanio et al., 1991; Papa et al., 1991) that the measured $H^+/e^-$ ratio for cytochrome c oxidase, under level flow as well as steady-state conditions, changes as a function of the respiratory rate. Since protease digestion results in depression of electron transfer by cytochrome c oxidase (see Table 1), measurements of the $H^+/e^-$ stoichiometries at various electron transfer rates were carried out. When $H^+/e^-$ ratios measured in the control and digested oxidase were plotted as a function of the rate of electron flow, it resulted that digestion with thermolysin or chymotrypsin did not alter significantly the rate dependence of the $H^+/e^-$ ratio with respect to that observed with undigested cytochrome c oxidase (Figure 3). In the control, as well as after digestion with these two proteases, a bell-shaped dependence of the $H^+/e^-$ ratio was found with values around 0.3 at relatively

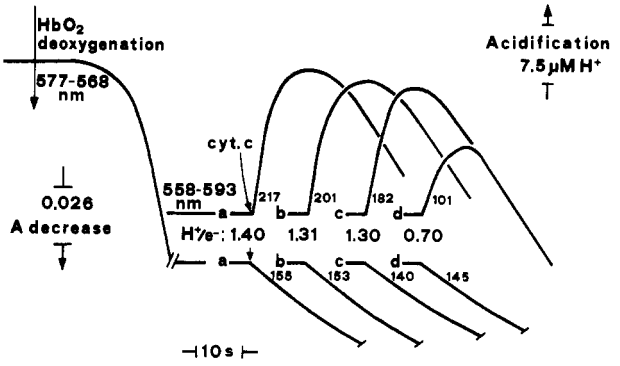


FIGURE 2: Measurement of level flow $H^+/e^-$ ratios for redox-linked $H^+$ translocation in COV: effect of protease digestion. Cytochrome oxidase vesicles (0.35 μM $a,a_3$) were suspended in 100 mM choline chloride, 5 mM KCl, and 0.1 mM EDTA, pH 7.5, supplemented with 6.5 mM ascorbate and 130–200 μM TMPD. The $O_2$ concentration was lowered by flowing argon onto the surface of the stirred suspension of COV in the spectrophotometric cuvette until recording of the absorbance change at 577–568 nm (lower traces) showed that 25 μM Hb was 50% deoxygenated. The cuvette was then sealed. The same experimental procedure was followed for measurement of pH changes on a separate sample of the same COV suspension where Hb was substituted with 50 μM phenol red and absorbance changes were monitored at 558–593 nm (upper traces). Respiration was started in both samples with the addition of 0.6 μM ferricytochrome *c*. The numbers on the traces refer to initial rates of electron transfer to $O_2$ and $H^+$ released (nequiv of $e^-\cdot min^{-1}\cdot mL^{-1}$ and nequiv of $H^+\cdot min^{-1}\cdot mL^{-1}$), respectively. For further details, see Papa et al. (1980, 1991). The $H^+/e^-$ ratios indicating the measured stoichiometries must be corrected for the scalar contribution due to the oxidation of ascorbate releasing 0.5 $H^+/e^-$. Protease treatment of soluble cytochrome *c* oxidase, successively reconstituted in liposomes, was carried out as detailed in the legend of Figure 1 and under Materials and Methods. (a) Control; (b) chymotrypsin; (c) thermolysin; (d) trypsin. The concentration of the redox mediator TMPD was higher in the protease-treated COV with respect to the control in order to achieve comparable electron transfer rates.

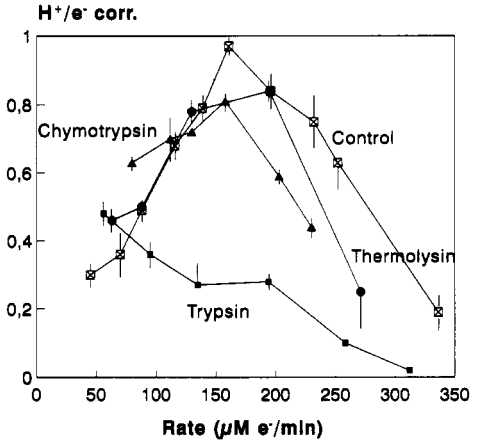


FIGURE 3: Effect of protease digestion of cytochrome *c* oxidase on level flow $H^+/e^-$ stoichiometry in COV. The experimental conditions were as described in the legend of Figure 2 with the TMPD concentration ranging from 5 to 400 μM, to vary the rate of electron transfer. The $H^+/e^-$ stoichiometries reported were corrected for the scalar contribution due to the oxidation of ascorbate releasing 0.5 $H^+/e^-$. Protease treatment of soluble cytochrome *c* oxidase successively reconstituted in liposomes was carried out as detailed in the legend of Figure 1 and under Material and Methods. (□) Control; (▲) chymotrypsin; (●) thermolysin; (■) trypsin. Means of 12 (control) and 4 (protease-treated oxidase) different experiments, ±SEM.

low and high respiratory rates and approaching 1.0 at intermediate rates [see also Capitanio et al. (1991)]. Trypsin digestion affected, on the other hand, the $H^+/e^-$ ratio which decreased progressively as the rate of electron transfer was enhanced, resulting in $H^+/e^-$ ratios which were much lower than those of the control enzyme.

Table 2: Effect of Protease Digestion on Respiratory Control Ratios and Passive Proton Conduction in Cytochrome *c* Oxidase Vesicles[a]

| conditions | $RCR_{\Delta\mu H^+}$ | $RCR_{\Delta pH}$ | passive $H^+$ conduction (μM $H^+$/min) |
|---|---|---|---|
| control | 11.7 ± 1.8 | 13.4 ± 0.6 | 63 |
| thermolysin | 15.0 ± 2.0 | 15.8 ± 1.5 | 61 |
| chymotrypsin | 12.9 ± 0.5 | 9.7 ± 0.6 | 61 |
| trypsin | 9.6 ± 0.2 | 7.0 ± 0.4 | 60 |

[a] Protease treatment of soluble cytochrome *c* oxidase before incorporation was carried out as described in the legend of Figure 1. Respiratory activity was measured polarographically in the medium as described under Materials and Methods. $RCR_{\Delta\mu H^+}$ and $RCR_{\Delta pH}$ were calculated by dividing the uncoupled respiratory rates by those measured in the absence of ionophores or in the presence of valinomycin alone, respectively. The uncoupled (valinomycin plus CCCP present) respiratory rates of the protease-treated oxidase were made comparable with those of the control oxidase (about 160 TN/s) by varying as opportune the concentration of TMPD, and the RCRs are relative to those conditions. Mean values of three different experiments, ±SEM. Passive $H^+$ conduction was measured electrometrically as detailed under Materials and Methods.

Digestion of cytochrome *c* oxidase with each of the three proteases did not alter passive proton conduction in the proteoliposomal membranes under conditions in which the $H^+/e^-$ ratio and the respiratory control index were lowered (Table 2).

No effect either on the redox-linked proton translocation or on the respiratory control ratio was observed when digestion was carried out on cytochrome *c* oxidase already incorporated in liposomal membranes (data not shown).

To further verify the impact of digestion of the various subunits on the protonmotive activity of cytochrome *c* oxidase, a time course of proteolytic digestion of the soluble enzyme was carried out, and the reconstituted enzyme, with a different extent of proteolysis, was tested for redox-linked $H^+$ translocation. The results presented in Figure 4 show a direct linear correlation between digestion of subunit IV by trypsin and depression of the $H^+/e^-$ ratio. Thermolysin digestion of subunit IV and that of subunit III by chymotrypsin were, on the other hand, completed without significant depression of the $H^+/e^-$ ratio.

A comparison (Figure 5) of the time course of digestion by trypsin of subunits IV, VIa, VIb, and VIIb with decoupling of proton pumping in oxidase vesicles shows that, at the various digestion times explored, the percentage of digestion of subunit IV was exactly equal to the percentage depression of the $H^+/e^-$ ratio. Digestion of subunits VIa and VIb was much faster than that of subunit IV and already extensive before significant depression of the $H^+/e^-$ ratio occurred. The rate of digestion of subunit VIIb was intermediate. Digestion of this subunit proceeded concomitantly with depression of the $H^+/e^-$ ratio. When it was completed, the $H^+/e^-$ ratio was, however, still 40% of the control.

## DISCUSSION

The present study shows that in bovine heart cytochrome *c* oxidase subunit IV, the largest of the nuclear-encoded polypeptides of the eukaryotic enzyme, is involved in proton pumping.

Of the three enzymes used for limited proteolysis of the oxidase, only trypsin caused, upon digestion of the enzyme in the soluble state, inhibition of redox-linked proton ejection by the reconstituted enzyme. Suppression of proton pumping did not result from enhancement of passive proton permeability of the vesicles, which was unaffected under conditions leading to a decrease of the $H^+/e^-$ ratio, nor from inhibition of electron flow. The $H^+/e^-$ ratio was, in fact, obtained from the initial

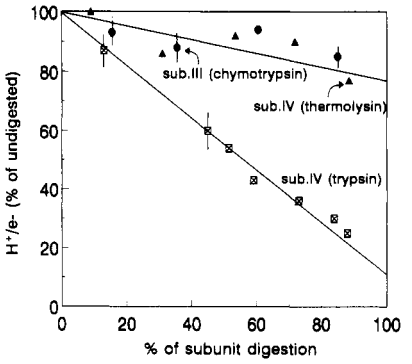


FIGURE 4: Correlation between protease digestion of subunits III and IV of cytochrome c oxidase and level flow $H^+/e^-$ stoichiometry. Protease treatment was carried out on soluble oxidase as described under Materials and Methods; the incubation time varied from 10 to 120 min, stopping the digestion with PMSF or EDTA for trypsin and chymotrypsin or for thermolysin, respectively. An aliquot of each sample was used for SDS–PAGE analysis; the rest was incorporated in phospholipid vesicles. The amounts of subunits III and IV remaining were calculated from the densitometric scans of the polyacrylamide gels and referred to the peak area of subunits in the undigested cytochrome c oxidase. Level flow $H^+/e^-$ ratios were measured as detailed in the legend of Figure 2, but the concentration of TMPD was such that the respiratory rates were between 130 and 200 $\mu$M $e^-$/min (intermediate rates); the $H^+/e^-$ ratio for the undigested oxidase was in this range 0.82 ● 0.04 on an average basis. (□) Trypsin, subunit IV; (▲) thermolysin, subunit IV; (●) chymotrypsin, subunit III. Mean values of 3–5 different experiments, ±SEM.

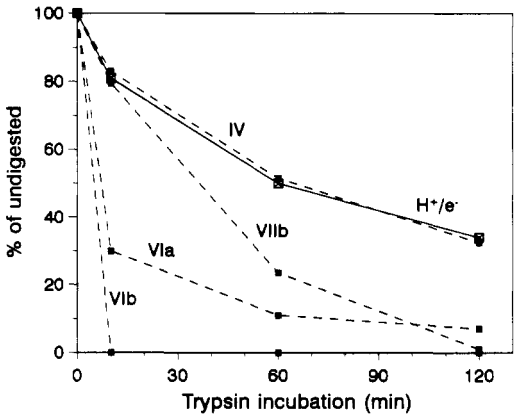


FIGURE 5: Correlation between the time course of digestion by trypsin of subunits of cytochrome c oxidase and decoupling of the redox-linked proton pump in COV. The experimental conditions were as described in the legend of Figure 4. (□) Level flow $H^+/e^-$ ratios; (■) subunits IV, VIa, VIb, and VIIb.

rates of electron flow and proton release measured upon activation of respiration by reductant addition. At equal intermediate respiratory rates, which give in the control an $H^+/e^-$ ratio around 1, proton pumping was almost completely suppressed in the trypsin-digested oxidase. The observed inhibition of proton release results, therefore, from direct decoupling of proton translocation from electron flow.

Trypsin digested subunits IV, VIa, VIb, and VIIb, and to some extent also subunit III. Chymotrypsin, which digested subunits III, VIa, and VIb [see also Zhang et al. (1984, 1988)], did not cause decoupling of proton pumping. Furthermore, suppression of subunit III digestion by trypsin treated with TPCK (inhibitor of chymotrypsin) did not prevent decoupling (results not shown). It thus seems possible to exclude that digestion of subunits III, VIa, and VIb is involved in the decoupling of proton pumping produced by trypsin.

Decrease of the $H^+/e^-$ ratio associated with proteolytic digestion of subunit III had been previously reported by others [for a review, see Brunori et al. (1987) and Prochaska and Fink (1987)]. In these experiments, the $H^+/e^-$ ratio was, however, obtained from the overall extent of $H^+$ ejection elicited by a reductant pulse. Under these conditions, the observed decrease of the $H^+/e^-$ ratio does not necessarily mean decoupling of the pump and could result from the observed inhibition of electon flow (see Table 1) [cf. Brunori et al. (1987)].

Comparison of the extent of digestion by trypsin of subunits IV, VIa, VI, and VIIb with the measured $H^+/e^-$ ratios showed the depression of proton pumping to be directly correlated with the extent of subunit IV digestion. Direct sequencing of subunit IV of cytochrome oxidase isolated from human heart (Van Kuilenburg et al., 1992) has shown the occurrence of a ragged N terminus, suggesting posttranslational proteolytic cleavage of the first 3, 7 or 11 residues. It would be interesting to verify whether this endogenous proteolysis of subunit IV is also associated with decoupling of proton pumping by the oxidase.

Subunits VIa and VIb were extensively digested before any depression of the $H^+/e^-$ ratio could be observed [see also Di Biase and Prochaska (1985)]. Digestion of subunit VIIb by trypsin was faster than the decoupling effected by this enzyme, and when it reached completion, the proton pumping was still substantial.

Thermolysin also caused partial digestion of subunit IV (together with digestion of subunits VIa, VIb, and VIc), but this protease did not result in significant depression of proton pumping. Direct sequencing of the proteolytic products of trypsin and thermolysin digestion showed that both proteases cleaved subunit IV at the N terminus. Trypsin cleaved off the N-terminal segment $H_2N$-Ala-His-Gly-Ser-Val-Val-Lys while thermolysin cleaved off only the segment $H_2N$-Ala-His-Gly-Ser.

The lack of inhibition of proton pumping by thermolysin and chymotrypsin seems to exclude the possibility that decoupling derives from perturbation of the oligomeric structure of the oxidase in the membrane and provides circumstantial evidence that it is specifically associated with cleavage of the Val5-Val6-Lys7 segment of subunit IV. These three residues belong to a sequence of protolytic residues which is apparently highly conserved in subunit IV of mammals and its homologues in lower eukaryotes (Kadenbach et al., 1987).

This segment of the N-terminal region of subunit IV of cytochrome c oxidase, protruding at the N surface of the mitochondrial membrane, is, thus, apparently involved in a critical step of proton pumping. The segment and in particular the lysine residue could mediate proton access in the transmembrane proton channel. A similar role of the N-terminal segment of subunit VIIb consisting of $NH_2$-Ile-Hys-Gln-Lys-Arg-, which is also cleaved off by trypsin in the soluble enzyme (Zhang, 1988), although less critical than that of the Val5-Val6-Lys7 segment of subunit IV cannot, however, be ruled out on the basis of the present results.

Site-directed mutagenesis of the *Escherichia coli* quinol oxidase has provided evidence that the conserved Asp135 of a peripheral cytoplasmatic loop of subunit I is possibly involved in the proton input channel in this oxidase (Thomas et al., 1993). Work from our laboratory shows that the hydrophilic N terminus of the 14 kDa subunit of cytochrome c reductase (Cocco et al., 1991) and the carboxyl terminus of the $F_o$I-PVP subunit of the ATPase complex (Zanotti et al., 1988) protrude from the matrix side of the membrane and facilitate transmembrane proton conduction by the membrane-spanning sector in these pumps. Hydrophilic extensions in the N space

of transmembrane helices, by means of polarizable hydrogen-bonded acidic and basic residues (Zundel & Brezinsky, 1992), might capture protons from the N phase at relatively high pH and mediate their selective access (filter function) into the proton input channel of proton pumps (mouth of the channel).

Subunit IV with its predictive membrane arrangement consisting of a single transmembrane $\alpha$-helix with hydrophilic N and C termini exposed at the matrix and cytosolic phase, respectively, could also serve as a transmembrane transmitter of signals brought by solutes from either aqueous phase (Gai et al., 1988; Anthony et al., 1993). These roles of subunit IV might represent peculiar features of the mitochondrial enzyme considering that this subunit is absent in bacterial oxidases (Ludwing, 1987). It is, however, possible that functional attributes which in prokaryotes are concentrated in a single protein (subunits I and II), in eukaryotes are distributed among more than one as is, indeed, the case for genetic information concentrated in prokaryotes in a single DNA molecule while distributed among separate DNA molecules in eukaryotes.

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