Volume 258, number 2, 285–288 FEB 07893 December 1989

Role of supernumerary subunits in mitochondrial cytochrome *c* oxidase

Y. Planques, N. Capitanio, G. Capitanio, E. De Nitto, G. Villani and S. Papa

Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

Received 17 October 1989

Role of supernumerary subunits of bovine heart cytochrome *c* oxidase has been investigated by examining the influence on the enzymatic activity of their removal by chromatographic procedures or controlled digestion by trypsin Is has been shown that partial proteolytic cleavage of subunit IV results in depression of respiratory activity and of redox-linked proton translocation Selective removal by gel-filtration of subunit VIb has no significant influence on the redox and protonmotive activity of the oxidase

Cytochrome *c* oxidase, Supernumerary subunit, Proton translocation

1. INTRODUCTION

Cytochrome *c* oxidase of mitochondria and bacteria (EC 1.9.3.1) (COX) catalyzes the aerobic oxidation of cytochrome *c* with reduction of dioxygen to H_2O and formation of transmembrane proton-motive force (PMF). PMF is, first of all, generated as a direct consequence of the anisotropic organization in the membrane of the reduction of O_2 to H_2O, whereby electrons are delivered by cytochrome *c* at the outer surface and H^+ derived from the inner aqueous phase [1–3]. It is generally accepted that, in addition, electron flow in eukaryotic and certain prokaryotic cytochrome *c* oxidases results in proton pumping from the inner to the outer space [3, 4]. The characteristics, mechanism and function of proton pumping in cytochrome *c* oxidase have, however, to be established (see [5, 6]).

COX of bovine heart consists of 13 subunits [7]. The first two, for which analogous subunits are present in prokaryotic oxidases [6], hold the 4 redox prosthetic centers and apparently perform the basic events of proton-motive activity of the oxidase. The function of the other subunits is still unknown [6].

In this paper information is presented on the role of two supernumerary subunits, IV and VIb (according to the nomenclature in [7]), as deduced from the influence on the enzymatic activity of their removal by chromatographic procedures or controlled digestion by trypsin.

Correspondence address Y Planques, Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

Abbreviations CCP, carbonylcyamide *p*-trifluoremethoxyphenyl hydrazone, $Ph(NMe_2)_2$, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

2. MATERIALS AND METHODS

2 1 *Enzyme preparations*

Cytochrome *c* oxidase, 10–12 nmol heme $a+a_3$/mg protein was prepared from beef-heart mitochrondria as in [8] Subunit VIb-less oxidase was prepared as follows 2–4 mg of purified oxidase were incubated for 1 h at 4 or 20°C in 1 ml of 0.1 M NaCl, 25 mM Tris-HCl pH 7.4, 4% Na-cholate The oxidase suspension was then applied on a column (7 cm long, 1 cm diameter) of Sephadex G-150 (Pharmacia) suspended in 0.1 M NaCl, 25 mM Tris-HCl pH 7 4, 2% Na-cholate and eluted at 4 or 20°C with 2% Na-cholate

Reconstitution of the cytochrome oxidase vesicles (COV) was performed by the cholate dialysis method as described in [9]. The percentage of cytochrome *c* oxidase incorporated 'right-side out' was calculated as in [10] or after reduction by impermeant and permeant reducing agents in anaerobiosis in the absense of cyanide [11] With both methods more than 80% of the oxidase molecules were found to be oriented 'right-side out' as in intact mitochondria [10, 11]

2 2 *Cytochrome assay*

Cytochrome *a* and a_3, in the soluble enzyme or in reconstituted vesicles, were measured spectrophotometrically at room temperature using a $\Delta\epsilon_{605-630} = 14\ mM^{-1}\ cm^{-1}$ Ferrocytochrome *c*, prepared as in [10], was measured using a $\Delta\epsilon_{550}$ (reduced-oxidized) = 21 $mM^{-1}\ cm^{-1}$ Ferrocytochrome *c* was oxidized by adding a few grains of ferricyanide (for other details see [11])

2 3 *Trypsin digestion*

COX, dissolved in 0 1% Triton X-100, 0 1 M K-Hepes, pH 8 0, at a final concentration of 4 0 mg protein/ml, was incubated with trypsin at a ratio of 1 20 (w/w) at 25°C, and the reaction stopped by adding phenylmethylsulfonylfluoride (Sigma) at final concentration of 1 mM Aliquots of trypsinized oxidase (20–50 μg protein) were analyzed by SDS-PAGE (as in [7])

Cytochrome oxidase reconstituted in vesicles was directly incubated with trypsin at the same protease/protein ratio reported above and the cleaved enzyme separated from phospholipids by sucrose gradient centrifugation prior to SDS-PAGE, as described in [12]

Cleavage of individual subunits of cytochrome *c* oxidase was calculated from the densitometric scans (Camag TLC densitometer, Switzerland) of the SDS-PAGE gels as peak-area of the remaining subunits

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/89/$3 50

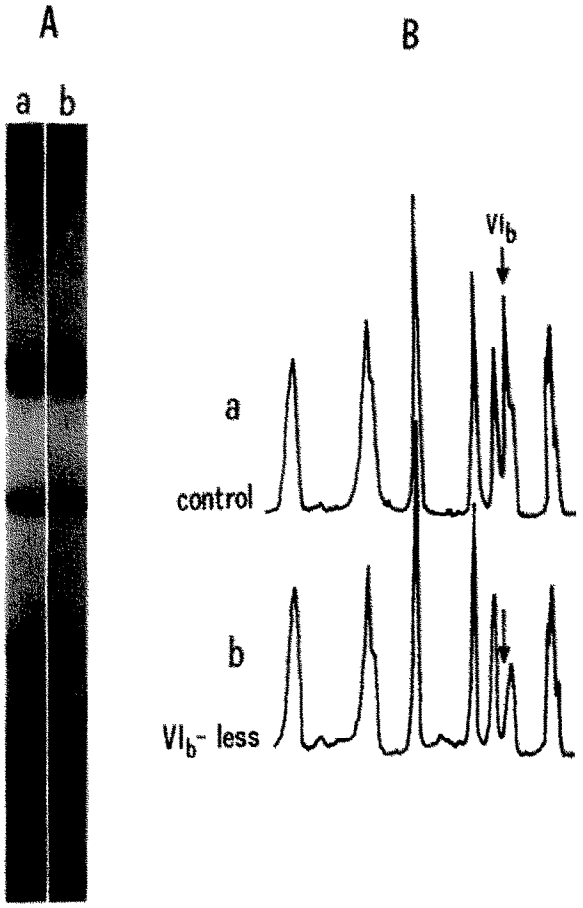

Fig.1. SDS-PAGE and densitometric scan of isolated bovine heart cytochrome *c* oxidase before and after gel-filtration on Sephadex G-150. For experimental details see section 2 and [7]. (A) SDS-PAGE of: (a) 20 μg control oxidase; and (b) 20 μg of oxidase passed through Sephadex G-150. (B) Densitometric analysis of gel-slots of cytochrome *c* oxidase performed at 590 nm.

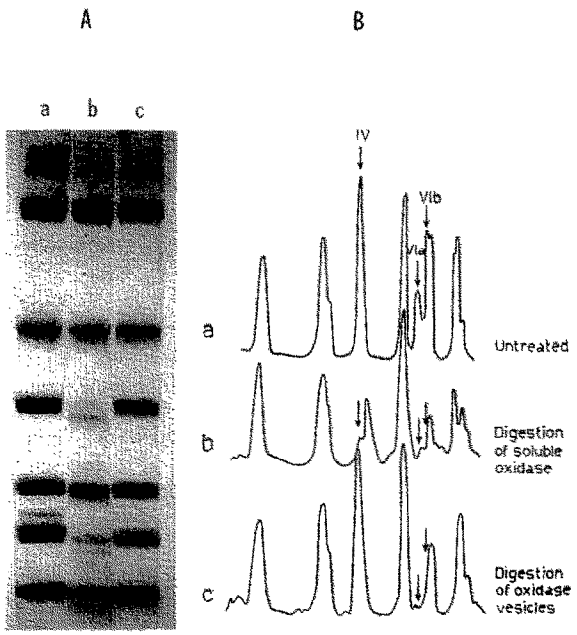

Fig.2. SDS-PAGE and densitometric scan of isolated bovine heart cytochrome *c* oxidase after digestion with trypsin. For experimental details see section 2 and [12]. (A) SDS-PAGE of: (a) 20 μg of control oxidase supplemented simultaneously with trypsin (1 : 20, w/w) and 1 mM (protease inhibitor) phenylmethylsulfonylfluoride; and (b) 20 μg of soluble oxidase incubated with trypsin for 120 min prior addition of the protease inhibitor. (B) Densitometric analysis of gel-slots of cytochrome *c* oxidase. (a) control oxidase; (b) soluble oxidase digested 120 min with trypsin; (c) cytochrome *c* oxidase reconstituted in vesicles was incubated with trypsin as in (b). After digestion, the oxidase was separated from phospholipids as in [12].

2.4. *Measurements of proton translocation and oxygen uptake*

Proton translocation in COV was measured with a pH combination electrode connected to a rapidly responding electrometer amplifier [11]. Oxygen consumption was measured potentiometrically with a Clark-type electrode coated with a highly sensitive membrane.

Valinomycin (2 μg/ml) plus CCP (5 μM) stimulated the oxygen uptake of COV around 10 times in 40 mM KCl, 10 mM Hepes (pH. 7.4), 0.1 mM EDTA, 25 mM ascorbate, 50 μM cytochrome *c* and 20 μl vesicles (0.08 nmol heme $a+a_3$) in 1.5 ml. For proton translocation measurements, 0.25 ml vesicles (0.5 μM oxidase) were suspended in

Table 1

Effect of removal by gel-filtration of subunit VIb from isolated bovine heart cytochrome *c* oxidase on redox kinetics of the soluble enzyme and respiratory activity and proton translocation in COV

Oxidase	Soluble oxidase		COV				
	K_m (μM)	V_{max} (nmol · s^{-1} · $nmol^{-1}$)	T.N. (uncoupled state)	R.C.R.	H^+/e^-		
					Reductant pulses	Oxidant pulses	H^+ conductivity μM H^+/min
Control oxidase	7.5 ± 0.05	254 ± 15	210 ± 12	9.9 ± 0.3	0.85 ± 0.02	0.17 ± 0.13	40.0 ± 5.2
Subunit VIb-less oxidase	5.5 ± 0.03	200 ± 14	183 ± 20	8.7 ± 1.0	0.74 ± 0.05	0.15 ± 0.06	31.4 ± 4.1

Kinetics assay of the soluble oxidase was carried out as in [11]. The values represent the mean of 7 different experiments. K_m are concentrations of cytochrome *c*; V_{max} values are nmol cytochrome *c* oxidized · s^{-1} (nmol cytochrome aa_3)$^{-1}$. For the measurement of the respiratory activity T.N. nmol cytochrome *c* oxidized · s^{-1} (nmol cytochrome aa_3)$^{-1}$, respiratory control ratio (R.C.R.) and passive proton conductivity, see section 2 and in [11]. H^+/e^- ratio for redox-linked proton ejection in COV was measured in reductant pulses of 4 nequiv. O_2 to anaerobic, reduced COV supplemented with 4 μM ferrocytochrome *c*, 10 mM ascorbate and 0.2 mM $Ph(NMe_2)_2$. The H^+/e^- ratio in oxidant pulses was corrected for scalar acidification deriving from oxidation of ascorbate. The values are the mean of 7 different experiments

100 mM choline/Cl 0.2 mM choline/Hepes, 0 1 mM choline/EDTA, 5 mM KCl, 2 μg valinomycin/ml, final vol. 1.5 ml. The redox-linked pH changes, elicited by pulses of 4–10 μM ferrocytochrome *c* were quantified with small aliquots of a standard solution of 10 mM HCl

2 5 *Chemicals*

$Ph(NMe_2)_2$ was from BDH Chemicals Ltd, valinomycin, CCP, horse-heart cytochrome *c* (type VI) and soybean phospholipids were from Sigma All other reagents were of the highest purity grade commercially available

3. RESULTS

Gel filtration on Sephadex G-150 of purified cytochrome *c* oxidase suspended in 4% cholate and eluted with 2% cholate, pH 7.4 at 20°C or 4°C, resulted in the selective removal of the enzyme of subunit VIb [7] (subunit AED of the first N-terminal residues [13]). No other structural alteration was apparent judging from the SDS-PAGE pattern (fig.1) and the optical spectra of the oxidized and reduced enzyme (not shown). Polarographic assay of the activity of the soluble enzyme did not reveal any substantial change in the K_m for cytochrome *c* and turnover number (table 1). The subunit VIb-less enzyme reconstituted in liposomes with the cholate dialysis method, resulted in being incorporated 'right-side out' by around 80% as the control enzyme and exhibited practically unchanged

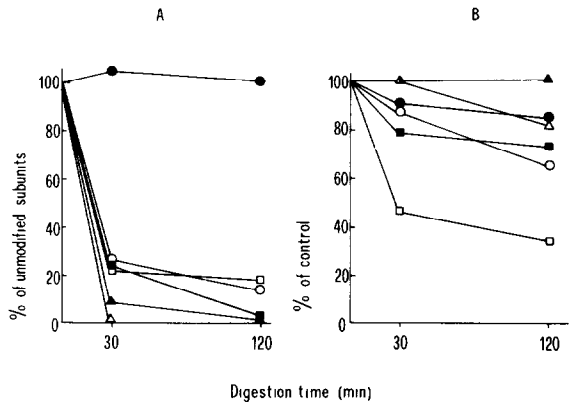

Fig 3 Time course of trypsin digestion of subunits IV, VIa and VIb in soluble and reconstituted cytochrome *c* oxidase and the effect on respiratory activity and redox-linked proton ejection in oxidase vesicles For experimental details see section 2 and [7,11,12] (A) Peak-areas of cytochrome oxidase subunits calculated from the densitometric scans of gel-slots of 20 μg samples of the oxidase after various intervals of trypsin digestion, expressed as % of the areas in the control enzyme. (○---○,●---●) areas of subunits IV after trypsin digestion of soluble (open symbols) and reconstituted (closed symbols) oxidase, (□---□,■---■) subunit VIa, (△---△,(▲---▲) subunit VIb (B) Time course of the effect of trypsin digestion on respiratory activity (○---○,●---●) (control value 230 mol of cytochrome *c* oxidized per mol of oxidase per s), (△---△,▲---▲) respiratory control index (control value 8.2); (□---□,■---■) H^+/e^- ratio for proton ejection induced by the addition of 4 μM ferrocytochrome *c* to the aerobic suspension of COV (control value 0 75) All the activities were measured in COV. Open symbols, oxidase incubated with trypsin prior to reconstitution; closed symbols, COV were incubated directly with trypsin The values presented are means of 3 different experiments

respiratory activity, respiratory control index, and H^+/e^- ratio for redox-linked proton translocation (table 1). Passive H^+ conductance of COV reconstituted with the subunit VIb-less enzyme was the same as that exhibited by control COV (table 1).

Incubation of the soluble oxidase with trypsin resulted in a partial digestion of subunit IV with production of a fragment which exhibited from its migration on the gel an apparent decrease in the molecular mass of some 3 kDa (cf. [14]). Subunits VIb and VIa were also digested but no product could be identified under the prevailing conditions used (fig.2). The trypsin-digested oxidase, when reconstituted in vesicles, exhibited a significant decrease in the respiratory activity and respiratory control index and even more marked decrease of the H^+/e^- ratio of redox-linked proton translocation (fig.3).

When the oxidase was incubated with trypsin after reconstitution in liposomes, digestion of subunit IV was fully prevented; subunits VIb and VIa were, on the other hand, digested to the same extent as observed with the soluble enzyme (fig.3). Under these conditions the respiratory activity and the respiratory control index were practically unaffected by the trypsin treatment and the decrease of the H^+/e^- ratio for redox-linked proton ejection was much smaller than that obtained when the oxidase was digested by trypsin prior to its reconstruction (fig.3).

4. DISCUSSION

The role of the supernumerary subunits of mitochondrial cytochrome *c* oxidase is as yet unclear. They can be involved in biogenesis and membrane assembly of the holoenzyme, proton-motive activity and regulatory properties of the mature enzyme (see [6]). Subunit III, which is mitochondrial encoded, is apparently involved in the correct assembly of the holoenzyme in the membrane [15] and in the proton-motive activity of the mature enzyme [16, 17]. It has been suggested that subunit VIb is somehow involved in stabilizing the dimeric form of the oxidase [18] which would be essential for the proton pumping activity of the oxidase [3]. The present observations show, however, that selective removal of subunit VIb does not impair either redox or proton-motive activity of the reconstituted oxidase.

The data on the tryptic digestion of subunit IV indicate that this subunit plays a role in the proton-motive activity of cytochrome *c* oxidase. Subunit IV apparently consists of a single hydrophobic stretch traversing the membrane and presents the N-terminal region extending in the inner aqueous space [14]. This is in fact, the segment cleaved off by trypsin in the soluble enzyme and protected towards digestion when the oxidase is reconstituted in phospholipid vesicles (fig. 3) (see [14]). The C-terminal region of subunit IV extends out of the C-side of the membrane. Lysine residues in

this region [19] are modified by the impermeant reagent, succinic acid anhydride, to the same extent in the soluble and membrane reconstituted oxidase [17]. It is conceivable that basic residues in the N-terminal region of subunit IV pick up H^+ from the matrix space, where the pH is relatively higher than in the cytosol, and promote their translocation in the membrane. Subunit IV is reported to bind ATP [20]. ATP binding, possibly involving the lysine residues in the peripheral C-terminal region protruding in the intermembrane space, may induce conformational changes affecting the protonmotive activity of the oxidase.

Acknowledgements The skillful technical assistance of M Minuto is gratefully acknowledged. This work was supported by CNR Grants 88 00794 and 88 00376 11

REFERENCES

[1] Mitchell, P (1977) in. Chemiosmotic Coupling in Oxidative Phosphorylation, Glynn Research Ltd, Bodmin
[2] Papa, S (1976) Biochim Biophys Acta 456, 39–84
[3] Wikstrom, M (1981) in· Cytochrome Oxidase. a Synthesis, Academic Press, London
[4] Wikstrom, M (1976) Nature 271, 271–273.
[5] Papa, S. (1988) in: Oxidases and Related Redox Systems (King, T E , Morrison, H.S , eds) pp 707–730, Alan R Liss, New York
[6] Papa, S , Chance, B and Ernster, L (1987) in Cytochrome Systems Molecular Biology and Bioenergetics, Plenum Press, New York
[7] Kadenbach, B , Jarausch, J., Hartmann, R and Merle, P (1983) Anal Biochem 129, 517–521.
[8] Errede, B , Kamen, M O and Hatefi, Y (1978) Methods Enzymol 53, 40–47
[9] Casey, R P , Chappell, J B and Azzi, A (1979) Biochem J 182, 149–156
[10] Casey, R P , Ariano, B H and Azzi, A (1982) Eur J Biochem 122, 313–318
[11] Papa, S , Capitanio, N and De Nitto, E (1987) Eur J Biochem., 164, 507–516
[12] Zhang, Y.Z , Georgevich, G and Capaldi, R.A (1984) Biochemistry 23, 5615–5621
[13] Takamiya, S , Lindofer, M.A and Capaldi, R A (1987) FEBS Lett 218, 277–282.
[14] Zhang, Y Z , Lindofer, M A and Capaldi, R A (1988) Biochemistry 27, 1389–1394
[15] Schon, E.A , Rizzuto, R., Moraes, C T , Nakase, H , Zeviani, M. and Di Mauro, E (1989), Science 244, 346–349
[16] Wikstrom, M (1988) FEBS Lett 231, 247–252
[17] Capitanio, N , Planques, Y , Steverding, D , Capitanio, G , De Nitto, E., Villani, G and Papa, S (1989) in Structure, Function and Biogenesis of Energy Transfer Systems (Quagliariello, E et al eds) Elsevier, Amsterdam, in press
[18] Finel, M, (1987) Biochim. Biophys Acta 894, 174–179.
[19] Buse, G , Steffens, G C M., Biewald, R , Bruch, B , Hensel, S (1987) in. Cytochrome Systems. Molecular Biology and Bioenergetics (Papa, S , Chance, B. and Ernster, L eds) pp 791–793, Plenum Press, New York
[20] Montecucco, C , Schiavo, G and Bisson, R (1986) Biochem J 234, 241–243