Transient kinetics of subunit-III-depleted cytochrome *c* oxidase

Francesco MALATESTA,* Giovanni ANTONINI,* Paolo SARTI† and Maurizio BRUNORI††

*Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', and †Institutes of Chemistry and Biochemistry and Centre of Molecular Biology (C.N.R.), University of Rome 'La Sapienza', Rome, Italy

Cytochrome *c* oxidase from ox heart was depleted of subunit III and its transient kinetic properties studied by stopped-flow and flash photolysis. It was found that the overall mechanism of electron transfer is very similar for subunit-III-depleted and native oxidase, although significant differences in some kinetic parameters have been detected. These include the second-order rate constant for cytochrome *c* oxidation and the rate-limiting step of the overall process. Moreover, at low cytochrome *c*/oxidase ratios (where the number of reducing equivalents is insufficient), the rate of reoxidation of cytochrome *a* was found to be very slow, even in air, and in fact for the subunit-III-depleted enzyme is even slower than for the native oxidase. The stability of reduced cytochrome *a* excludes the likelihood that removal of subunit III leads to a new O₂-binding site, and the result may be relevant to the lowered vectorial H⁺/e⁻ stoichiometry. The subunit-III-depleted oxidase can be pulsed under appropriate conditions and its combination with CO is unchanged, as shown by kinetic experiments and difference spectroscopy.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is an oligomeric intrinsic membrane protein composed of a relatively large number (7–13) (Azzi, 1980; Wikström *et al.*, 1981; Capaldi *et al.*, 1983; Buse *et al.*, 1985) of co-purifying polypeptides interacting via non-covalent forces. Isolation of individual constitutive polypeptides has so far been successful only under conditions so strongly denaturing that they eliminate the possibility of establishing structure–function relationships. Dissociation of subunits also leads to an irreversible modification of the classical spectroscopic properties of the oxidase, owing to the removal of its prosthetic groups (cytochrome *a*, *a*₃ and two copper ions called 'Cu_a' and 'Cu_{a3}').

A new emerging trend in the field of cytochrome *c* oxidase research is to establish the role of each subunit within the protein in order to account for the complex properties of the enzyme. Subunit III has been shown to be among the most weakly bound polypeptides, its removal being possible under various non-denaturing conditions (Penttilä, 1983). We have shown in previous papers (Sarti *et al.*, 1985; Püttner *et al.*, 1985; Brunori *et al.*, 1985) that the so-called 'vectorial' properties of cytochrome oxidase are only partially affected in the subunit-III-depleted enzyme ('III-less oxidase'). The data indicated that this modified enzyme is still capable of redox-linked proton translocation (H⁺/e⁻ ≈ 0.5) in synchrony with cytochrome *c* oxidation, leading to a possible role for subunit III in the regulation of energy transduction at site III, but excluding the likelihood that this subunit is the only, or even the main, proton channel.

In the present paper we have undertaken a kinetic survey on the pre-steady-state behaviour of III-less oxidase in the detergent-solubilized state in order to determine if the scalar kinetic properties of this enzyme are changed with respect to the unmodified native protein. The transient kinetics of oxidation of reduced

cytochrome *c* indicate that the overall mechanism of electron transfer is unmodified upon removal of subunit III, although some differences in the relevant parameters are clearly detected. The kinetics of combination with CO are identical in the native and III-less oxidase. The results are discussed with reference to the overall mechanism of reduction of the oxidase and also to the modified vectorial properties of this enzyme as observed in the reconstituted state.

MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared as described by Yonetani (1961), and the final pellet was dissolved in 0.1 M-phosphate buffer containing 0.2% sodium cholate, pH 7.4.

Subunit-III-depleted cytochrome oxidase (III-less) was prepared as described by Püttner *et al.* (1985) and used for the experiments within 1–5 h.

Cytochrome *c* (Type VI) was from Sigma. Reduced cytochrome *c* was prepared by adding solid dithionite and removing excess reductant by gel-permeation chromatography (Sephadex G-25, equilibrated with appropriate buffers). All other reagents were of analytical grade.

Stopped-flow experiments were carried out using a Durrum–Gibson thermostatically controlled apparatus with 2 cm-light-path observation chamber; transient kinetics were monitored at 550, 563 and 605 nm.

CO-binding experiments were performed by using either the stopped-flow or the flash-photolysis apparatus. For rapid-mixing experiments 1.25 or 2.5 μM (functional unit) dithionite-reduced oxidase, dissolved in 0.1 M-phosphate buffer containing 0.5% Triton X-100, pH 7.5, was mixed with 200 μM-CO in 0.1 M-phosphate buffer, pH 7.5, containing 50 mM-KCl, also in the presence of dithionite. Flash-photolysis measurements were carried out with the identical solutions used for the rapid-mixing

Abbreviations used: III-less oxidase, subunit-III-depleted cytochrome *c* oxidase; COV, cytochrome oxidase containing vesicles.

† To whom correspondence and reprint requests should be sent at the following address: Institute of Chemistry, Faculty of Medicine, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy.

experiments, the flash-photolysis apparatus previously described by Brunori & Giacometti (1981) being used. The functional unit is defined as containing four metal sites.

Cytochrome oxidase-containing vesicles (COV) were prepared as described by Sarti *et al.* (1985).

RESULTS AND DISCUSSION

Kinetics with reduced cytochrome *c*

The kinetics of oxidation of cytochrome *c* by III-less oxidase under steady-state conditions are somewhat dependent on the environment which the oxidase experiences. When III-less oxidase was reconstituted into artificial vesicles, it failed to show significant differences with respect to the control protein in the oxidation of ferrocycytochrome *c* in air, notwithstanding the decrease of the H^+/e^- stoichiometry to 0.5 (Sarti *et al.*, 1985). This is probably related to phospholipid activation of cytochrome oxidase, which could mask subtle differences in the rate constants. In the detergent-solubilized state, in Hepes buffer, pH 7.3, and at the same reactant concentrations as those described above, the initial electron-transfer phase (which will be referred to as the 'burst phase') is lost in the dead time of the stopped flow, as can be seen from absorbance recovery. The turnover phase, which corresponds to a simple exponential, is very similar for III-less and native oxidases, and the pseudo-first-order rate constant for cytochrome *c* oxidation can be lower for the III-less oxidase by (at most) a factor of 2, depending on the detergent used. It is generally observed, as previously reported (Antonini *et al.*, 1985), that the rate constants are smaller in Triton X-100 than in Tween 80. The turnover experiments are essentially in agreement with the extensive steady-state kinetics recently published which indicate that minor but significant differences are observed between native and III-less oxidase (Nalecz *et al.*, 1985).

Since it is known that native cytochrome oxidase can exist in two distinct functional conformations, called 'resting' and 'pulsed' (Antonini *et al.*, 1977), we also investigated the kinetic properties of the III-less enzyme in the pulsed state, obtained (as usual) by exposing the reduced protein to dioxygen. The III-less oxidase can indeed be pulsed, although under the conditions employed, the maximum activation was found to be 3 for both native and III-less oxidase (results not shown). In view of what has been reported elsewhere (Antonini *et al.*, 1985), this may be related, among other things, to the time after thawing the oxidase, since the preparation of the III-less enzyme takes a minimum of 5 h.

Transient kinetics with reduced cytochrome *c*

In order to observe conveniently the transient kinetics of cytochrome *c* oxidation and cytochrome *a* reduction, we carried out experiments in phosphate buffer where the initial electron-transfer phase can be monitored accurately from the beginning. All the experiments reported below have been conducted with the III-less oxidase in the resting state in phosphate buffer containing 0.5% Triton X-100, in air.

The reaction between ferrocycytochrome *c* and ferricytochrome *a*, under conditions in which cytochrome *c* is stoichiometric with the total amount of oxidizing equivalents of cytochrome oxidase, is clearly biphasic, as

Fig. 1. Time courses of ferrocycytochrome *c* oxidation and ferricytochrome *a* reduction for native and III-less enzymes, monitored at 550 nm (a) and 605 nm (b).

Ferrocycytochrome *c* (14 μM) in 0.1 M-sodium phosphate/50 mM-NaCl, pH 7.5, was mixed with 2.5 μM III-less (traces 1) and 3.5 μM native oxidase (traces 2) in the same buffer containing 0.5% Triton X-100. Closed arrows in (a) ($\rightarrow$, $\leftarrow$) indicate total absorbance recovery. For an explanation of the open arrows ($\nearrow$) see the text. Note the difference in the time scales (both expressed in s). Absorbance changes in (b) for the III-less oxidase are normalized to the concentration of the native enzyme. The temperature was 20 °C.

shown in Fig. 1, at 550 and 605 nm. The initial rapid phase, analysed at 605 nm, yields an apparent initial bimolecular rate constant of $3.7 \times 10^6 M^{-1} \cdot s^{-1}$ and $2.3 \times 10^6 M^{-1} \cdot s^{-1}$ for native and III-less oxidase respectively; this phase is best observed at low reactant concentrations in phosphate buffer of relatively high ionic strength (Wilson *et al.*, 1975). At 550 nm (Fig. 1a) the total absorbance recovery was close to 98% of the expected value, whereas it reached 93% for the control enzyme; this difference in absorbance recovery is consistent with an intrinsically lower bimolecular rate constant in the case of the III-less enzyme. The amplitude of the burst phase for cytochrome *c* oxidation corresponds closely to 2 mol of cytochrome *c* oxidized/mol of oxidase (expressed as functional units); therefore, within approx. 200 ms, close to two electrons have been transferred from the reduced substrate to the enzyme (see the open arrows in Fig. 1a), a result similar to that found consistently for the native enzyme under similar experimental conditions (Wilson *et al.*, 1975). The complete oxidation of cytochrome *c* proceeds with a slower velocity, which reflects the intramolecular electron transfer to the binuclear centre (Wilson *et al.*, 1981), and appears to be somewhat slower for the III-less enzyme. Over this same period of time, cytochrome *a* is reduced almost completely for both oxidases (Fig. 1b), and since more

than one electron is consumed in the burst, this demands that Cu_a is also (at least partially) reduced. These results imply that the reduction of Cu_a (the e.p.r. visible copper) in the III-less oxidase occurs with a rate similar to, or greater than, the rate of reduction of cytochrome *a*, as previously shown for native oxidase (Greenwood *et al.*, 1976). We can therefore conclude that the mechanism of the initial electron-transfer processes is quite similar in the native and III-less oxidase. It is noteworthy, however, that the fraction of cytochrome *a* reduced is always higher in the case of the III-less oxidase and, as an explanation, a possible perturbation in the redox equilibrium between cytochrome *a* and Cu_a may be postulated. Since the kinetic parameters are not dramatically changed (by a factor of 2 only), this observation may be accounted for by assuming that removal of subunit III is associated with a perturbation of the midpoint potential of either cytochrome *a* or Cu_a . Unfortunately, available redox estimates are, at the present time, not unequivocal, since the reported loss of pH-dependence of the midpoint potential of cytochrome *a* in the III-less oxidase (Penttilä, 1983) has been recently challenged (Thompson *et al.*, 1985).

Fig. 2 depicts the time course of cytochrome *a* reduction and reoxidation as monitored at 605 nm, under conditions in which cytochrome *c* was sub-stoichiometric by comparison with the total oxidizing equivalents on the oxidase. Under these conditions, previous results (Antonini *et al.*, 1970) have indicated that the available electrons from cytochrome c^{2+} distribute among different molecules of oxidase, leading to partially reduced intermediates. Assuming that the total contribution of cytochrome *a* to the absorbance change at 605 nm is 80%

Fig. 2. Time courses of cytochrome *a* reduction and reoxidation for native (Δ , $\blacktriangle$) and III-less ($\circ$, $\bullet$) cytochrome oxidase at 605 nm

Open and closed symbols refer to the lower and upper time scales respectively. All conditions were as for Fig. 1, except for cytochrome *c* concentration, which was $7 \mu\text{M}$ before mixing.

Fig. 3. (Reduced + CO)-minus-(reduced) kinetic difference spectrum of III-less (open symbols) and native (closed symbols) cytochrome oxidase as determined either by stopped-flow ($\circ$, $\bullet$) or flash photolysis (Δ , $\blacktriangle$)

For details, see the Materials and methods section. Absorbance changes are normalized as in Fig. 1.

of the total (Vanneste, 1966; Nicholls & Chance, 1974; Wikström *et al.*, 1981), the reduction of cytochrome *a* corresponds to 75% for the native enzyme and to almost 100% for the III-less oxidase. Under these conditions (sub-stoichiometric cytochrome c^{2+}), reduced cytochrome *a* is long-lived even in air ($270 \mu\text{M-O}_2$), and the reoxidation of cytochrome *a* is rate-limited by the redistribution of electrons between partially reduced oxidase molecules. This phenomenon was taken by Antonini *et al.* (1970) as an indication that the reduction of molecular O_2 by oxidase occurs via multi-electron steps. As shown in Fig. 2 (long time scale), the rate of reoxidation of cytochrome *a* is 2-fold slower for the III-less enzyme. If redistribution between oxidase molecules is taken to occur in a bimolecular mode, then the relevant second-order rate constants for electron exchange are $8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the III-less oxidase and $15 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the native enzyme. This finding may not be interpreted in terms of a changed aggregation state of the III-less oxidase because, although both dimeric and monomeric aggregation states have been reported for the III-less oxidase (Nalecz *et al.*, 1985; Thompson & Ferguson-Miller, 1983), the redistribution kinetics are slower, and not faster, than that of native oxidase. The lowered electron-exchange rate may also depend on the shuttling efficiency of cytochrome *c* between two distinct cytochrome *a* sites; as a matter of fact, since subunit III is part of the so-called 'high-affinity cytochrome *c*-binding site' (Capaldi *et al.*, 1983), removal of this subunit could affect the docking of cytochrome *c* and therefore reduce the electron-exchange rate.

The results shown in Fig. 2 also indicate that the species having cytochrome *a* reduced and cytochrome a_3 oxidized does not react directly with O_2 . In fact, if this

were the case, the decreased reoxidation rate of cytochrome *a* observed for the III-less enzyme may be difficult to account for. Therefore any possible mechanism which would account for the observed lower stoichiometry of proton translocation by the III-less oxidase cannot invoke the direct reaction of cytochrome *a* with dioxygen.

CO binding

CO binding to dithionite-reduced native and III-less oxidases was studied by using stopped-flow and flash photolysis in parallel. Both techniques, which measure directly CO binding to ferrocycytochrome *a*₃, yield the same bimolecular rate constant ($5.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) for both oxidases, a result in excellent agreement with published data (Gibson & Greenwood, 1963). The finding that the reactivity of cytochrome *a*₃ for CO is unchanged in the III-less oxidase suggests that also its reactivity towards O₂ may be unperturbed. CO binding was monitored as a function of wavelength in the Soret region, allowing the kinetic difference spectrum [(reduced + CO) - (reduced)] to be determined for the III-less and native oxidases. The kinetic difference spectrum, shown in Fig. 3, is the result of two independent experiments at two different oxidase concentrations. The normalized spectrum shows positive and negative peaks at 430 and 445 nm respectively, and an isosbestic point at 437 nm. The spectrum is overall symmetrical, identical (for the two oxidases) with the statically determined difference spectrum, and it shows no indication of asymmetry in the blue region, a result at variance with that obtained previously (Nalecz *et al.*, 1985).

Concluding remarks

The transient kinetics reported above indicate that the overall mechanism of the initial electron transfer from reduced cytochrome *c* to cytochrome *a* is unaffected by removal of subunit III, although quantitative differences have been detected. Moreover, we have also shown that, similarly to the native enzyme, the III-less oxidase also can exist in a more active pulsed state. CO combination rate constant and its kinetic difference spectrum are unchanged, excluding major perturbations of the reactivity of the binuclear centre and therefore suggesting that the oxygen reaction also will be, by and large, unaltered. We have definitely demonstrated that, in the presence of excess O₂, cytochrome *a* in the III-less enzyme is at least as stable as the control, excluding the presence of additional O₂-reducing sites created in partially reduced intermediates.

Given the mechanistic information presented here, the modified vectorial properties of III-less oxidase reconstituted into liposomes (Sarti *et al.*, 1985) cannot be accounted for on the basis of a wholly different mechanism for the redox reactions of this enzyme. Therefore we suggest that removal of subunit III results in a lowered H⁺/e⁻ stoichiometry because the

efficiency of coupling between the redox reaction and the proton pumping is under the control of this subunit (without excluding a specific role of the smaller polypeptides).

We thank Alfredo Colosimo and Michael T. Wilson for stimulating discussions and Emilio D'Itri for skilful technical assistance. This work has been partially supported by a grant from the 'Ministero della Pubblica Istruzione' of Italy to M.B. Thanks are extended to EMBO (European Molecular Biology Organization) for a short-term Fellowship to F.M.

REFERENCES

- Antonini, E., Brunori, M., Greenwood, C. & Malmström, B. G. (1970) *Nature* (London) **228**, 936-937
- Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. & Wilson, M. T. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3128-3132
- Antonini, G., Brunori, M., Colosimo, A., Malatesta, F. & Sarti, P. (1985) *J. Inorg. Biochem.* **23**, 289-293
- Azzi, A. (1980) *Biochim. Biophys. Acta* **594**, 231-252
- Brunori, M. & Giacometti, G. M. (1981) *Methods Enzymol.* **76**, 582-595
- Brunori, M., Antonini, G., Colosimo, A., Malatesta, F., Sarti, P., Jones, M. G. & Wilson, M. T. (1985) *J. Inorg. Biochem.* **23**, 373-379
- Buse, G., Meinecke, L. & Bruch, B. (1985) *J. Inorg. Biochem.* **23**, 149-153
- Capaldi, R. A., Malatesta, F. & Darley-Usmar, V. M. (1983) *Biochim. Biophys. Acta* **726**, 135-148
- Gibson, Q. H. & Greenwood, C. (1963) *Biochem. J.* **86**, 541-552
- Greenwood, C., Brittain, T., Wilson, M. T. & Brunori, M. (1976) *Biochem. J.* **157**, 591-598
- Nalecz, K. A., Bolli, R., Ludwig, B. & Azzi, A. (1985) *Biochim. Biophys. Acta* **808**, 259-272
- Nicholls, P. & Chance, B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 479-491, Academic Press, New York
- Penttilä, T. (1983) *Eur. J. Biochem.* **133**, 355-361
- Püttner, I., Carafoli, E. & Malatesta, F. (1985) *J. Biol. Chem.* **260**, 3719-3723
- Sarti, P., Jones, M. G., Antonini, G., Malatesta, F., Colosimo, A., Wilson, M. T. & Brunori, M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4876-4880
- Thompson, D. A. & Ferguson-Miller, S. (1983) *Biochemistry* **22**, 3178-3187
- Thompson, D. A., Gregory, L. & Ferguson-Miller, S. (1985) *J. Inorg. Biochem.* **23**, 357-364
- Vanneste, W. H. (1966) *Biochemistry* **5**, 838-848
- Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York
- Wilson, M. T., Greenwood, C., Brunori, M. & Antonini, E. (1975) *Biochem. J.* **147**, 145-153
- Wilson, M. T., Peterson, J., Antonini, E., Brunori, M., Colosimo, A. & Wyman, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7115-7118
- Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680-1688