'PULSED' CYTOCHROME OXIDASE MAY BE PRODUCED WITHOUT THE ADVENT OF DIOXYGEN

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Received 25 February 1981

1. Introduction

We have reported [1–3] that the product of the reaction of fully reduced cytochrome oxidase with oxygen (either in excess of, or stoichiometric with the functional unit aa_3) corresponds to a more active form of the enzyme, which we have termed 'pulsed' oxidase. The spectral and kinetic properties of 'pulsed' oxidase have been characterized [2–6], and particular attention has been paid to the observation that this state of the enzyme catalyzes the oxidation of reduced cytochrome *c* more rapidly than the 'resting' form [2,3].

The spectral properties of 'pulsed' oxidase, as determined in stopped-flow experiments [3,4], are similar to those of the classical 'oxygenated' compound(s), originally described by Okunuki and coworkers [7] and subsequently investigated in [8–11]. In addition its kinetic and spectral properties are independent of the nature of the reductant employed to achieve reduction of the enzyme (whether ascorbate and cytochrome *c*, or dithionite, or NADH and PMS [2–5]).

Although it was shown [3] that 'pulsed' oxidase, similarly to 'resting' [12–14], contains 4 oxidizing equivalents, it is not yet known whether dioxygen in any of its redox states is still bound to the enzyme or, more importantly, whether it is the only oxidant capable of yielding 'pulsed' oxidase. We now report that anaerobic oxidation of reduced cytochrome oxidase with an excess ferricyanide, in the presence or in the absence of cytochrome *c*, yields a form of the enzyme which is catalytically more active, like 'pulsed', and which undergoes a slow transition back to the 'resting' state, similar to that reported for 'oxygenated' oxidase [7–10]. Therefore our experiments show that dioxygen is not unique in producing 'pulsed' oxidase.

2. Materials and methods

Beef heart cytochrome oxidase was prepared as in [15]: its concentration was determined spectrophotometrically [16] and is expressed in terms of total heme *a*.

All reagents were analytical grade, used without further purification. Absorption spectra were recorded using a Cary 219 instrument. Stopped-flow experiments were carried out with a Gibson-Durrum Instrument. The flash-photolysis apparatus is similar to that in [17,18].

3. Results and discussion

To test the proposition that molecular oxygen is not a unique oxidant, we performed an experiment to determine whether reduced oxidase exposed to excess ferricyanide, under strictly anaerobic conditions, is 'pulsed' or 'resting'.

A 1 ml sample of oxidase (140 μM total heme), reduced with dithionite in the presence of cytochrome *c* (67 μM), was mixed anaerobically with a 10 μl solution of ferricyanide (final conc. 4 mM). After 1 min, the solution was cooled in ice, and rapidly transferred to a Sephadex G-25 column equilibrated with 50 mM phosphate buffer (pH 7) containing 1% Emasol (at 4°C). The enzyme solution, passed through the column under pressure to separate the excess ferricyanide, was collected within 3 min after the addition of the oxidant and divided into 2 aliquots, one of which was transferred to the spectrophotometer, and the other to the stopped-flow apparatus, where its activity was determined. Spectra (from 450–380 nm) and activity were recorded as a function of time, starting ~4 min after the anaerobic addition of ferricyanide (half of

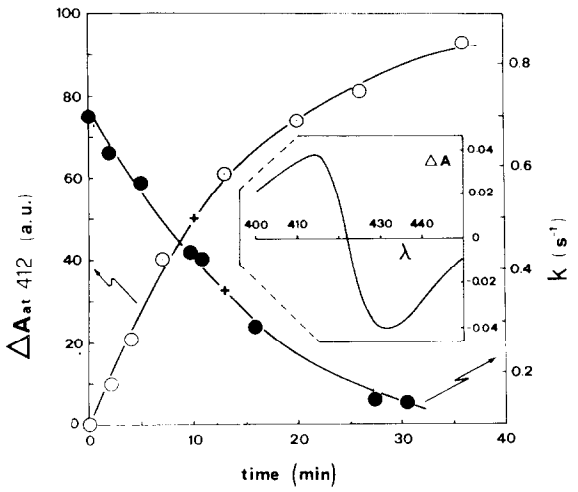

Fig.1. Time dependence of: (•) the first-order rate constant for the aerobic oxidation of 12 μM reduced cytochrome *c* by a ferricyanide oxidized oxidase solution (3.6 μM) and (⊙) the absorbance of the same sample at 412 nm (in arbitrary units); (+) indicates the half-time for the two processes (i.e., $t_{1/2}$ = 10 min for the spectral decay and $t_{1/2}$ = 13 min for the functional decay). Insert: the difference spectrum between $t \rightarrow \infty$ (i.e., 'resting' oxidase) and first observation (i.e., 5 min and 20 s after anaerobic addition of ferricyanide). Conditions detailed in the text, t = 15°C.

this time span was at 0–4°C). The first order rate constant for the aerobic oxidation of reduced cytochrome *c* is shown in fig.1. This rate, which was initially high, decayed to that characteristic of the 'resting' form with $t_{1/2} \sim 13$ min (at 15°C). The initial spectrum, with a maximum at 428 nm, also changed as a function of time yielding a final state with a maximum at 415 nm (isosbestic at 422 nm) (see insert to fig.1). This spectral decay, with $t_{1/2} \sim 10$ min, parallels the functional decay, indicating that the 2 phenomena reflect the same process. The time-dependent spectral change corresponds closely to that described for the transition between 'oxygenated' oxidase and the 'resting' enzyme [7–11].

To interpret this experiment, it was essential to establish that reduced oxidase exposed to 4 mM ferricyanide was fully oxidized before being applied to the G-25 column which was air equilibrated, i.e., within ~1 min. Since cytochrome *a* is rapidly oxidized by ferricyanide, both in the presence and absence of cytochrome *c* [19], we carried out control experiments to ensure that cytochrome a_3 was also fully oxidized within this time. The following controls were therefore performed:

(i) Cytochrome oxidase at 40 μM total heme, in 50 mM phosphate buffer (pH 7) plus 1% Emasol 4130, was anaerobically reduced with an excess (2–3-fold) of dithionite dissolved in the same buffer. After reduction of both cytochrome *a* and a_3, monitored spectrophotometrically, 10 μl of a carefully degassed solution of ferricyanide was added to give 4 mM final conc. After 40 s, pure CO gas was admitted to the sample, which was thereafter rapidly equilibrated with the gas phase and then transferred into the flash photolysis apparatus, where the presence of reduced cytochrome a_3, if any, could be unequivocally estimated from the appearance of photosensitivity [20]. As shown in fig.2, flash experiments carried out at different time intervals (starting ~90 s after the addition of ferricyanide) indicate that the photosensitive material (i.e., the amount of reduced cytochrome a_3–CO complex in solution) is initially very small and increases with time reaching a maximum which approaches the value obtained after complete reduction with excess dithionite (100% of signal). The $t_{1/2}$ for the appearance of photosensitivity was found to be 1–2 min after admission of CO gas and in agreement with [21–25] shows that mM CO levels stabilize cytochrome a_3 in the reduced state even in the presence of an excess ferricyanide. It may be added that if (dithionite) reduced cytochrome oxidase was exposed to oxygen before being exposed to CO, there was no recovery of photosensitivity even after several minutes.

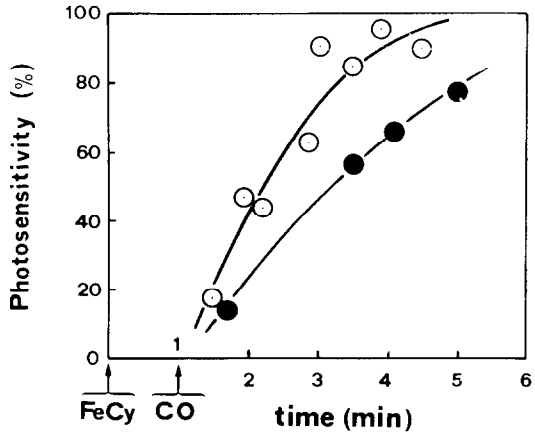

Fig.2. Time course of appearance of photosensitivity (%) after the addition of CO gas (at t = 1 min) to a solution of reduced cytochrome oxidase pre-exposed to ferricyanide (4 mM final conc., at t = 0). 100% corresponds to the value obtained after addition of excess dithionite. Conditions detailed in the text; $t \simeq 20$°C; (•,⊙) indicate two separate experiments.

(ii) Considering the statement [19] that addition of 4 mM ferricyanide to reduced oxidase did not completely restore the 655 nm band even after 1 min, we carried out additional experiments. Cytochrome oxidase (20–40 μM total heme under the above conditions was reduced anaerobically to completion with an excess (2–3-fold) of dithionite. After addition of ferricyanide (4 mM final) under strictly anaerobic conditions (see above), the spectrum of the sample was recorded as a function of time over 700–550 nm. These experiments were performed both in the absence and in the presence of stoichiometric cytochrome *c*, to ensure complete and rapid oxidation of cytochrome *a* at all times. It was found in 4 independent experiments that, while the absorbance in the α-band region changed with $t_{1/2} \sim 5$–7 min (corresponding to a decay process yielding finally the spectrum of 'resting' oxidase), the absorbance at 655 nm did not change significantly after the first 1–2 min following the addition of ferricyanide.

These control experiments show that ~1 min after the addition of 4 mM ferricyanide to fully reduced cytochrome oxidase, under strictly anaerobic conditions, cytochrome a_3 (as well as cytochrome *a*) is fully oxidized, and the 655 nm band is restored. The apparent inconsistency with the statement in [19] (see fig.4, p. 347 in [19]) may possibly be attributed to minor differences in the experimental conditions (e.g., temperature, which in our experiments was $\geqslant 20^{\circ}$C).

4. Conclusion

These experiments show that 'pulsed' cytochrome oxidase, the active state produced by mixing the fully reduced enzyme with molecular oxygen [2,3], may also be obtained by anaerobic oxidation with ferricyanide (and presumably with other oxidants). The experiment is possible because the decay from 'pulsed' to 'resting' in the fully oxidized state is slow under the conditions described, as monitored by both functional and spectral assay (fig.1), and EPR spectroscopy [5].

The enhancement in the turnover number of cytochrome oxidation typical of 'pulsed' oxidase is essentially the same whether the fully reduced enzyme is oxidized with dioxygen or with ferricyanide. It is proposed in [26] that the activation process, whereby the rate of the internal electron transfer leading to reduction of cytochrome a_3 is enhanced [2], involves a transition in the fully reduced cytochrome oxidase, and subsequent oxidation leading to fully oxidized 'pulsed' oxidase is independent of the nature of the oxidant.

These experiments are also relevant in the assessment of a possible structure for 'pulsed' oxidase [2,3,5,14], since they show that this active form of the enzyme contains no bound dioxygen, being obtainable even under complete anaerobiosis. They are also consistent with the hypothesis [26] that the difference between 'pulsed' and 'resting' oxidase may be conformational in nature, as suggested also by crosslinking experiments [27].

The similarities in the spectral properties (both in the Soret and α-band region) and the spectral decay of the ferricyanide 'pulsed' enzyme with those of the so-called 'oxygenated' oxidase [7–11] indicate that we may in fact be dealing with the same species. This being so, 'oxygenated' oxidase may be otained without the advent of dioxygen, supporting the hypothesis that this form of the enzyme is a conformational variant of oxidized oxidase [9,11]. It seems reasonable to abandon the term 'oxygenated' to define a state of the enzyme which can be produced without oxygen, especially since a primary oxygen-adduct has been identified by low-temperature kinetic spectrophotometry [28].

Acknowledgement

Partially supported by NATO exchange grant 1767 to M. T. W.

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